An oligonucleotide microarray for the detection of vaccinia virus

M. WENLI, W. YAN, W. HONGMIN and Z. WENLING*
*Institute of Molecular Biology, First Uniform Medical University, Guangzhou 510515; and *Medical Research Laboratory, Guangzhou Liu Hua Qiao Hospital 510010, P. R. China*

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ABSTRACT

Vaccinia virus is a member of the orthopoxvirus group, to which also belongs variola virus, one of the most hazardous pathogens known to man. To establish a model system to detect orthopoxviruses, a vaccinia oligonucleotide microarray is designed, produced and tested. Vaccinia virus is used to test the prepared microarrays. The virus DNA samples in different propagation phases are extracted and hybridised with the oligonucleotide microarray. The results showed that the oligonucleotide microarray can detect vaccinia virus with high specificity and sensitivity.

KEY WORDS: Microarrays.
Oligonucleotides.
Orthopoxvirus.
Vaccinia virus.

Introduction

Vaccinia virus, which has the potential to infect humans, is the largest virus in nature. The vaccinia and variola genomes show high homology in their sequence alignment. Variola virus, the causative agent of smallpox, is the most dangerous member of the orthopoxvirus family, and can cause severe epidemics in humans.[1]

The massive vaccination programme with vaccinia virus resulted in the eradication of smallpox in 1980.[2] However, recent active bioterrorism has rekindled concern about the use of smallpox, anthrax and other pathogens as bioweapons.[3] Furthermore, reports of the high frequency of intra- and intermolecular recombinations[4-6] indicate that vaccinia virus can undergo further evolution, and a particular danger is the potential increase in its virulence for humans, as even the attenuated strains of vaccinia virus that are widely used in genetic engineering and biotechnology can cause severe disease in immunocompromised people.[7] Thus, there is an urgent need to establish reliable, inexpensive and rapid methods to detect vaccinia virus, which also can be expanded to cover variola virus.

The use of DNA microarrays to detect pathogens has the advantage of high-throughput as massive probes can hybridise with DNA specimens under the same conditions, ensuring the reliability of detection. An oligonucleotide microarray, comprising a large number of probes of controlled length, is currently available and shows high specificity and sensitivity in gene detection.

In this study, a vaccinia oligonucleotide microarray is designed, produced and tested, and vaccinia virus is used to test the prepared microarrays.

Materials and methods

Viruses and DNAs

The Tian-Tan vaccinia virus strain was used in this study (Medical Research Laboratory, Guangzhou Liu Hua Qiao Hospital). The viruses were titrated and propagated by growth on a human tk-negative osteosarcoma cell line (10 pfu/cell in 75 cm^2 dishes). After overnight incubation, the cells were removed and centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 200 µL extraction buffer containing 0.14 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 8.6), 0.5% NP-40 and 1 mmol/L 2-mercaptoethanol.

The solution was incubated at 0°C for 10 min, shaken occasionally then centrifuged at 13,000 rpm for 3 min. The supernatant was transferred to a fresh Eppendorf tube, to which 20 µL 10 mg/mL RNase A was added, and the mixture was incubated at 37°C for 1 h. Then, 200 µg proteinase K and 20 µL 10% sodium dodecyl sulphate (SDS) were added and the mixture was incubated at 55°C for 3 h. The mixture was then extracted (x2) with phenol/chloroform (1:1), avoiding vortex-mixing, and then purified by ethanol precipitation. The pellet was air-dried and dissolved in 10 µL TE (pH 8.0).

Design of oligonucleotide probes

The vaccinia gene sequences were aligned using the Viral Genome Organiser (VOG) and Poxvirus Orthologous Clusters (POCs) softwares,[8] in order to find specific regions suitable for oligonucleotide (oligo) probe design. Criteria for oligo probe design were as follows: length of probes were 60 nucleotides, with calculated melting temperature of 85.2±5°C; no more than seven continuous nucleotides in a probe; the stem of the hairpin no longer than 6 bp; dimers formed in the probe no more than 6 bp; and mismatch between the probes and non-specific sequences no longer than 20 continuous nucleotides.

Microarray production

The oligo probes (60 mer) were synthesised using an ABI3900 DNA synthesiser, then purified using 12% denatured SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purified probes were dissolved in a 50% dimethyl sulphoxide

Correspondence to: Dr Wenli Ma
Email: wenli@fimmu.com

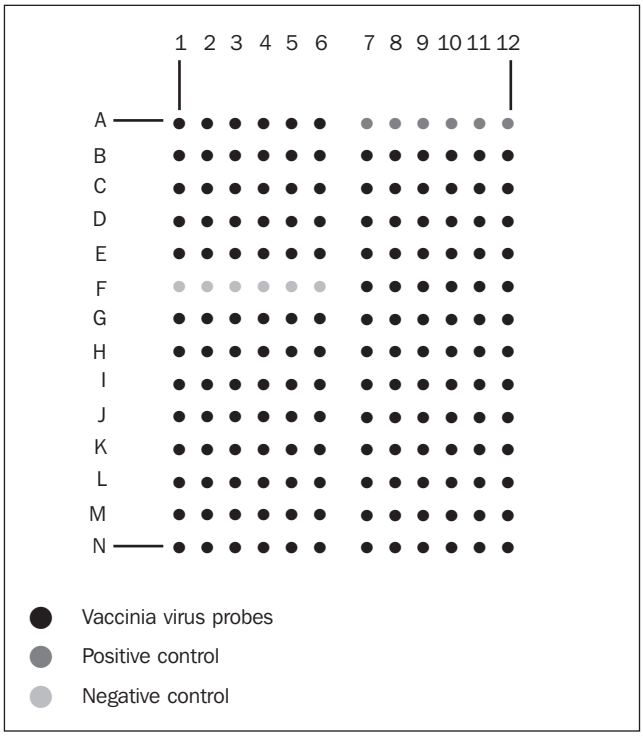

Fig. 1. Layout of the vaccinia virus oligo chip. Probe 1 located in row A, column 1-6 (A1-A6); probe 2 located in A7-A12; probe 3 located in B1-B6, and so on.

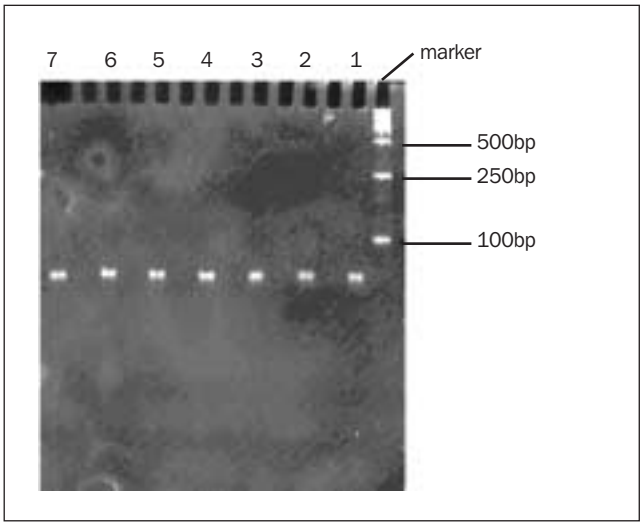

Fig. 2. Oligonucleotide probes (60 mer) purified by PAGE, 12% acrylamide/bis-acrylamide (19:1) and 7 mol/L urea.

(DMSO) solution, the final concentration was adjusted to 1 μg/μL and stored in a 384-well plate. Oligonucleotide microarrays were printed using a contact micro-spotting robotic system (PixSys 5500) equipped with a single micro-spotting pin. The slides (DakoCytomation) were covered with poly-L-lysine beforehand. After printing, the slides were rehydrated by passing them through 90°C water vapour and then fixed using ultraviolet (UV) irradiation (65 mJ). The oligo probes for the microarray were spotted on to the slides as depicted in Figure 1.

Labelling of DNA samples

The labelled DNA samples of vaccinia virus for microarray analysis were prepared by a restriction display polymerase chain reaction (RD-PCR) technique,[9,10] in the presence of the Cy3 universal primer (Tri-link Co). Briefly, 1 μg vaccinia virus DNA was added to 1 μL Sau3AI (10 units/μL), 2μL 10Xbuffer and double-distilled water up to a final volume of 20 μL. This was digested at 37° for 1 h and then heated at 75°C for 5 min to stop the reaction.

The completely digested products with a stick end of GATC were annealed with the adapter, which was synthesised by two oligonucleotides, SIP (5′-pGATCmCACACCAGCCAAACCCA-3′) and SIR (5′-GGTTTG GCTGGTGTG-3′). The digested DNA sample (10 μL) was mixed with 3 μL adapter (50 mmol/L), 1 μL T4-lingase (350 U/μL), 2 μL buffer and double distilled water up to 20 μL total volume, and allowed to link at 16°C for 4 h.

The linked products (3 μL) were mixed with 1 μL Cy3 universal primer and 10 μL 2XPCR buffer containing 2 units *Thermus aquaticus (Taq)* DNA polymerase (TaKaRa), 1.5 mmol/L $MgCl_2$ and 0.2 mmol/L dNTP mixture. The PCR was performed in an ABI3700 thermocycler for 5 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C and a final incubation of 7 min at 72°C. The products were purified using a PCR purification kit (TaKaRa) and collected in 30 μL TE, stored at -20°C in the dark.

Microarray hybridisation

The slides were pretreated by immersing in prehybridisation buffer (25% formamide, 5XSSC, 0.1% SDS, 1% BSA) prewarmed to 42°C to block the non-specific areas. Hybridisation of Cy3-labelled DNA samples with oligo probes on the microchip was carried out using the following protocol: the final hybridisation mixture containing 2 μL Cy3-labelled DNA and 2 μL hybridisation buffer (50% formamide, 10XSSC, 0.2% SDS) was denatured at 95°C for 5 min, centrifuged at 13,000 rpm for 1 min and then added to the microarray on the slide. The slide was loaded into a hybridisation chamber (Corning) and placed in a water bath at 42°C for 3 h. After hybridisation, the slides were washed once in 2XSSC containing 0.2% SDS for 5 min at 42°C, once with 0.1XSSC containing 0.2% SDS, four times with 0.1XSSC and finally dried in air to remove any remaining solution.

Microarray scanning

Fluorescence microarray images were obtained by scanning the slides with an Agilent scanner (570 nm). Images were analysed using the Array-Pro analyser software. Background fluorescence readings obtained from the region surrounding each spot were subtracted.

Results

Design of oligonucleotide probes

The genome of the Tian-Tan vaccinia strain was analysed with POC and VOG softwares via internet programmes (www.poxvirus.org) and compared with other species using Cluster W. Finally, 26 genes specific to vaccinia virus were selected for probe preparation. Each DNA fragment was analysed using oligo 6.0 and primer 5.0 softwares. The probes designed are listed in Table 1.

Table1. Nucleotide sequence, gene, T_m and GC% of oligo probes.

No	Sequence	Gene	T_m value	GC content
1	5'-GACGGTATGTATTGTAGATGCTCTCATGGTTATACAGGCATTAGATGTCAGCATGTAGTA-3'	TC18R	85.8	37.1%
2	5'-GGCTAATCAATTTCGGGCTTGGAAAAAACGTATCGCTGGAAGGGACTATATGACTAACTT-3'	TC16R	86.3	38.6%
3	5'-GTTTGCAAATACGGTTAATCCTTTGACCTCGTCGATTTCCGACCAATCTGGGCGTATAAT-3'	TC7L	87.5	41.4%
4	5'-GACGCATTATCTGGATTAAACATACTAGGAGCCATCATTTCGGCTATCGACTTAATATCC-3'	TK3L	85.8	37.1%
5	5'-CACAGGCATAAAATGTAGGAGAGTTACTAGGCCCCACTGATTCAATACGAAAAGACCAAT-3'	TE3L	86.9	40.0%
6	5'-GGGTTAAATTTGAACAAGGTGACTATAAAGTGGAAGAGTATTGTACAGGACTATGCATCG-3'	TB8R	85.2	35.7%
7	5'-CGATGTACACATTCCCAAGTTTAAGGTAACAGGTTCGTATAATCTGGTGGATACTCTAGT-3'	TB13R	85.2	35.7%
8	5'-TCGAAAATCAACGTAATTATAGGAGAACCTGCCAATATAACATGCACTGCTGTATCAACG-3'	TB19R	85.2	35.7%
9	5'-GCTATCAAGTCTTTATACAATGACATGGAAATATCTGTGAACACTTTATACTTCTCCAAC-3'	TA11L	84.6	34.6%
10	5'-CATCGACTGTACGATACATAATGTTACCGTGTTGCGTACATTGCTCGTAAAAGACTTTCG-3'	TA55L	85.8	37.1%
11	5'-CTTTTTAGCCAGAGATATCATAGCCGCTCTTAGAGTTTCAGCGTGATTTTCCAACCTAAA-3'	TA36L	85.8	37.1%
12	5'-GGTAAAATTAATTCCTTATTGACTAGTCCACTGCCCATTAATACTCGCATGACAGTTGTT-3'	TB14R	85.2	35.7%
13	5'-GAAATAGAATCGTTAGAAGCAACTTATCATATAATCATAGTAGCGTTGACAATTATGGGC-3'	TB5R	84.6	34.3%
14	5'-CTAGAGTACTTAAACCATCCGTTATCGAAGAATGGAAAAAATCTCACCATATAAGCCACG-3'	TB1R	85.2	35.7%
15	5'-CTGCTAGAAAACCTCATCGAAGAAGATACCATATTTTTTGCAGGAAGTATATCTGAGTAT-3'	TL4R	85.2	35.7%
16	5'-AATCTTAATATCTTCGTCACACGTTAGCTCTTTGAACTGTTTAAGAGATGCATCAGTTGG-3'	TH5L	85.2	35.7%
17	5'-CGATGATATTTTCCCGGAAGATGTAATAATTCCATCTACTAAGCCCAAAACCAAACGAGC-3'	TH6R	86.9	40.0%
18	5'-CTTCCACTATTGCCATCGAATGCCATAGAATAAATATCCTTGGAATTGATAGAAATCGGAC-3'	TA3L	85.8	37.1%
19	5'-CTGGCATAACGATGACCCTACCTGATGAATCAGACAATGTACTGGGCCATGTAGAATAAA3'	TA16L	85.8	37.1%
20	5'-GGGAATCGATGTTATTATCAAAGTCACAAAGCAAGACCAAACACCGACCAATGATAAGAT-3'	TB23R	85.2	35.7%
21	5'-CGTTTCCATCGCTTTAAAGACGTTTCCGATAGATGGTCTCATTTCATCAGTCATACTAAG-3'	TC6L	86.3	38.6%
22	5'-GCGAGAACTCATACCGCACTTATATTTTTGATGGGTAAGCCAACAACATCTAGACGTGAT-3'	TA53R	86.3	38.6%
23	5'-CGGTTTTATACTGTTCGAGATTCTCATTGATTATATTCTCATCTATCATCTCCACACAGT-3'	TF6L	85.2	35.7%
24	5'-CGTCTTAGGCGTCGGAAAGGATGATTCATCAAACGAATAAACAATTTCACAAATGGATGT-3'	TI6L	85.2	35.7%
25	5'-GTACGATGTAATACCAGCGTGAACAACTTACAGATGGATAAAACTTCCTCATTAAGATTG-3'	TG10R	85.2	35.7%
26	5'-TATAGGTGTCCCGTCAGCGTGCAGACAAAATGAAGATCCAAGATTTGTAGAAGCATTTAA-3'	TD5R	85.7	40.0%
27	5'-GTTTGGCTGGTGTGGATCGTTTGGCTGGTGTGGATCGTTTGGCTGGTGTGGATCGTTTGG-3'	Ctrl (+)	88.7	44.6%
28	5'- TAACGTTAAGAGACTACCATTGCACATGCCCTAAGAACAGGTACAATAGAGTAGGTACA-3'	Ctrl (-)	86.5	42.9%

Note: Ctrl (+) indicates the positive control and Ctrl (-) the negative control.

Synthesis of oligonuleotide probes

The oligo probes synthesised were purified by PAGE and isopropyl alcohol precipitation. Figure 2 shows seven samples of the purified oligo probes. The bands were clear and uniform.

Intensity of hybridisation signal

Vaccinia virus DNA was extracted during different infection phases (i.e., 12-h, 16-h and 24-h post-infection). Figures 3a, b and c represent virus DNA extracted from samples at 12-h, 16-h, and 24-h post-infection, respectively, while Figure 3d is human genomic DNA. The array colours reflected the intensity of the signal from weak to strong. The scanned images showed that all the virus samples displayed positive signals, especially probes 10, 12, 15, 22, 23 and 28. However, negative samples, such as the human genomic DNA, showed no evidence of hybridisation.

The mean intensity of the group of six dots from a single probe was calculated using the formula: (signal of probe)-(signal of negative probe)/(signal of positive probe)-(signal of negative probe). The signal intensities of six probes in Figures 3a, b and c (12-h, 16-h and 24-h post-infection) were compared (Fig. 4) and mean values for signal intensities were calculated. In different infection phases, the oligo microarray signals showed little diversity, which indicated that this oligo microarray was a uniform and reliable means to detect different phases of vaccinia virus infection.

Discussion

Orthopoxviruses are among the most dangerous microorganisms that might be used in a bioterrorist attack; thus, a rapid and accurate detection method is of great importance. Several approaches to virus detection, such as immunoenzyme analysis of viral antigens,[5] immunoblotting[6] and PCR, have been proposed for virus detection in recent years. These methods could help to initiate a rapid defensive response and prevent the possible spread of smallpox.

Several PCR-based methods to detect and discriminate orthopoxviruses are available; however, a PCR technique that is based on single or several special fragments can produce both false-positive and false-negative results. Thus, better sequence-specific approaches that can detect more genes may provide more accurate results.

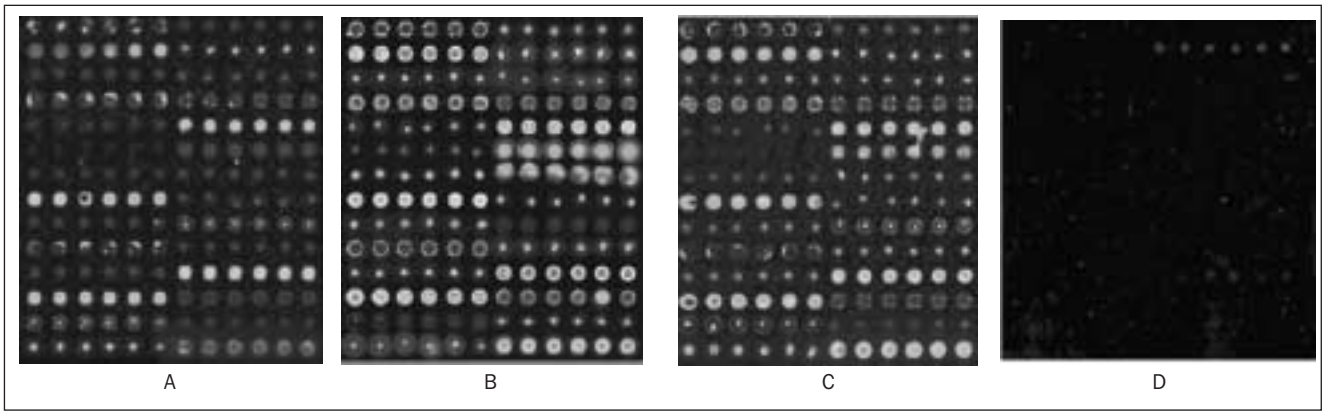

Fig. 3. Hybridisation with different vaccinia DNA samples and controls: a) 12-h, b) 16-h and c) 24-h post-infection; d) human genome DNA sample.

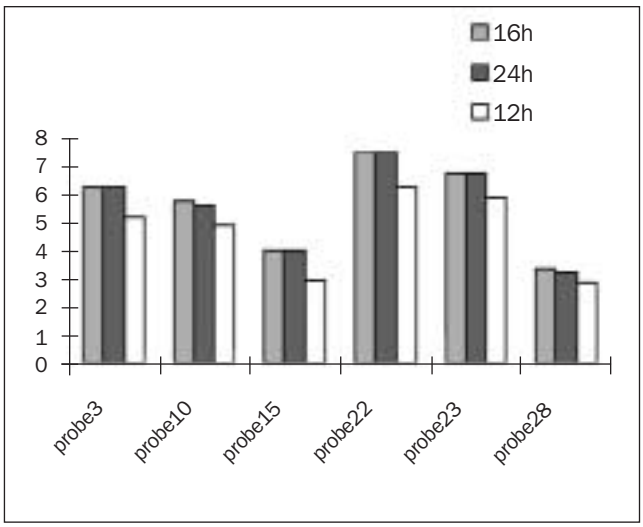

Fig. 4. Comparison of the signal intensities of six probes in Figures 3a, b and c (12-h, 16-h and 24-h post-infection).

In the present study, vacccina virus was selected as a detection model to assess the oligonucleotide microarray technique. The oligonucleotide microarray in this study is different from the Affymetrix genechip, which is protected by patents. It was produced from presynthesised probes, which were coherent in T_m value and GC content, to permit uniform hybridisation conditions. Oligo probes show high specificity for short lengths and do not allow hybridisation with non-target genes, even if some sections are homologous.

In the present study, the positive control was designed from several tandem repeats of a universal primer sequence, which was able to hybridise with the labelled DNA sample by adaptors complementary to the universal primer. A sequence of rice genome was used as a negative control, in order to confirm the specificity of the hybridisation.

The intensity of dots in the hybridisation image correlated with the concentration of the probe present: the greater the intensity, the higher the concentration. However, a relatively high concentration was needed to achieve perfect hybridisation quality.

From a practical point of view, the use of oligo probes with 200-400 gene microarrays means that the slides can be treated more cheaply. This will help to reduce the cost associated with the clinical application of the oligo microarray technique, which is a reliable method for the detection of pathogens. □

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