Microarray Multiplex Assay for the Simultaneous Detection and Discrimination of Influenza A and Influenza B Viruses

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Abstract In this study, we present a microarray approach for the typing of influenza A and B viruses, and the subtyping of H1 and H3 subtypes. We designed four pairs of specific multiplex RT-PCR primers and eight specific oligonucleotide probes and prepared microarrays to identify the specific subtype of influenza virus. Through amplification and fluorescent marking of the multiplex RT-PCR products on the M gene of influenza A and B viruses and the HA gene of subtypes H1 and H3, the PCR products were hybridized with the microarray, and the results were analyzed using a microarray scanner. The results demonstrate that the chip developed by our research institute can detect influenza A and B viruses specifically and identify the subtypes H1 and H3 at a minimum concentration of 1×10^2 copies/ μ L of viral RNA. We tested 35 clinical samples and our results were identical to other fluorescent methods. The microarray approach developed in this study provides a reliable method for the monitoring and testing of seasonal influenza.

Keywords Influenza A virus · Influenza B virus · Typing · DNA microarray

Introduction

Influenza (flu) is a contagious respiratory illness caused by influenza viruses. Some demographic populations, such as the elderly and young children, are at a high risk of serious complications from the flu, but some subtypes can severely affect healthy adults. In the United States, 350,000 people are hospitalized with seasonal flu each year, and 50,000 die of the illness [1].

The influenza virus is a segmented, minus-strand RNA virus belonging to the family *Orthomyxoviridae*. The influenza viruses are classified into influenza A, B and C according to the antigenic differences in the nucleocapsid protein (NP) and matrix protein (M) [2]. Studies have shown that the influenza C virus causes only mild respiratory disease and typically does not cause flu epidemics. The seasonal epidemics are primarily caused by influenza A and B viruses. Existing methods to test for the presence of influenza include virus isolation in cell culture, serological diagnostics and immunological and molecular biology techniques. There are many shortcomings in the traditional virus isolation in cell culture, as they are time-consuming and have low levels of sensitivity. Despite this, virus separation is considered to be the gold standard for influenza testing.

Microarray technology is a state-of-the-art technology that was developed in the 1990s, and has a higher throughput and better accuracy than traditional test paper and blotting methods. DNA microarrays can provide high-throughput assays and rapid detection as well as data analysis and modeling through the application of high-

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density microarray probes, 2-D matrices, multicolor fluorescence marking, and highly specific binding [3, 4]. The microarray probes generate photosensitive signals by specifically binding to fluorescently-labeled targets, providing the identity and concentration of the targets in the sample. This provides a detection method for all biological organisms on a genetic level. DNA microarray technology is broadly applied in many fields, such as gene expression [5], gene function and drug evaluation [6]. They are also used to study pathogenic microorganisms, including viruses and bacteria [7, 8].

In this study, we performed a sequence analysis to identify a conserved gene segment of influenza A and B viruses. We then designed and synthesized specific primers and oligonucleotide probes to create a microarray for the testing of DNA from influenza A and B viruses. Specificity and sensitivity tests were performed for the prepared DNA microarrays and were validated using clinical specimens. This study provides a rapid, high-throughput technique for the identification of influenza A and B viruses, and for making the diagnosis of influenza.

Materials and Methods

Viruses and Clinical Samples

Influenza A virus A/California/7/2009 (H1N1), A/Victoria/210/2009 (H3N2) and A/chicken/JL/9/2004 (H5N1), AIV

isolate1 (H9N2); influenza B virus B/Brisbane/60/2008, Newcastle disease virus (LASOTA strain), foot and mouth disease virus (O/LZ strain), infectious bronchitis virus (H52), and infectious bursal disease virus (BC6) were used in this study. Thirty-five clinical specimens from patients with suspected influenza were collected at the First Hospital of Jilin University.

Design of Primer Probes and Preparation of Chips

The sequences of the M and HA genes of representative strains of influenza A and B viruses (Table 1) were retrieved from GenBank. The homological comparison was performed using v7.0 Laser Gene software (DNASTAR, Madison, WI) and the relatively conserved regions of the viral genes were identified using v4.2 Array Designer software (PREMIER Biosoft, Palo Alto, CA). The specific primers and probes were designed and Blastn analytic screening was conducted using software (Array Designer). All upstream primers were marked at the 5' terminus with hexachlorofluorescein (HEX). All oligonucleotide probes were amino-modified at the 5' terminus. The primers and probes were synthesized by Shanghai Sangon Biological Engineering Co., Ltd. The probes were diluted to 50 µM with Spotting Buffers (CapitalBio, Beijing, China), and 20 µL of each probe was placed in 384-well plates. The probes were dotted on the blank aldehydized slides with a spotter. The chips were put aside at room temperature for at

Table 1 The reference strains and genes in the design of primers and oligonucleotide probes

Type	Virus name	Gene	GenBank
Influenza A virus	A/mallard/Ohio/11OS2128/2011(H1N1)	M	CY132214
Influenza A virus	A/Hong Kong/2637/2004(H1N1)	M	CY125205
Influenza A virus	A/Boston/630/2009(H1N1)	M	CY089220
Influenza A virus	A/Philippines/3150/2007(H1N1)	M	CY031377
Influenza A virus	A/California/07/2009(H1N1)	M	CY121681
Influenza A virus	A/Taiwan/834/2007(H3N2)	M	CY031846
Influenza A virus	A/Korea/AF05/2008(H3N2)	M	CY037320
Influenza A virus	A/New York/3064/2009(H3N2)	M	CY050509
Influenza A virus	A/Netherlands/063/2011(H3N2)	M	CY114422
Influenza A virus	A/mallard/Ohio/11OS2128/2011(H1N1)	HA	CY132213
Influenza A virus	A/Hong Kong/2637/2004(H1N1)	HA	CY125204
Influenza A virus	A/South Korea/AF10/2008(H1N1)	HA	CY044349
Influenza A virus	A/California/07/2009(H1N1)	HA	CY121680
Influenza A virus	A/New York/3064/2009(H3N2)	HA	CY050508
Influenza A virus	A/Netherlands/063/2011(H3N2)	HA	CY114421
Influenza B virus	B/Taiwan/40/2007	M	CY040379
Influenza B virus	B/Brisbane/60-9/2010	M	CY098863
Influenza B virus	B/Brisbane/60/2008	M	CY115152
Influenza B virus	B/Malaysia/1710547/2007	M	CY118356
Influenza B virus	B/Wisconsin/01/2010	M	CY115184
Influenza B virus	B/Hubei-Wujiagang/158/2009	M	CY115384

least 12 h after spotting. Oligonucleotides not covalently bound to the chips were removed using 0.2 % SDS (Promega), and then the residual SDS was removed with distilled water. The chips were then air-dried for future use.

RNA Extraction, RT-PCR and Labeling

Viral RNA was extracted from specimens using the QIA-amp Viral RNA Mini Kit according to the manufacturer's instructions. The reaction is composed of 10 μ L 2 $\times$ One Step Buffer, 0.8 μ L PrimeScript 1-Step Enzyme Mix, 0.5 μ M of each upstream primer, 0.1 μ M of each downstream primer and 5 μ L RNA templates, and diluted to 20 μ L with ribozyme-free water. The reaction conditions include reverse transcription at 50 °C for 30 min and pre-denaturation at 94 °C for 2 min. PCR amplification conditions comprised 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. A final extension step at 72 °C was performed for 10 min. The target genes were labeled with HEX during the amplification procedure.

Hybridization, Washing and Detection of Microarrays

PCR products were denatured at 98 °C for 5 min and then placed in the ice bath for 5 min. A 10- μ L aliquot was added to the chip after the PCR products were isometrically mixed with the hybridization solution containing 2 μ L 99 % formamide, 2 μ L 50 $\times$ Denhardt's solution (Sigma-Aldrich), 2 μ L 1 $\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate), 2 μ L 2 % SDS (Promega), and 2 μ L diethyl pyrocarbonate–water (Sangon, Shanghai, China). The chips were then placed in a Microarray Hybridization Cassette (CapitalBio), incubated in 45 °C water and hybridized for 1.5 h. The hybridized chips were individually washed in Solution A (1 $\times$ SSC, 0.2 % SDS), Solution B (0.2 $\times$ SSC), and Solution C (0.1 $\times$ SSC) at room temperature for 20 s, and then dried at room temperature. The chips were scanned using GenePix 4100A personal scanner (Axon Instruments, Sunnyvale, CA) under the set parameters: PMT value 650, laser intensity 10 and monochrome excitation wavelength of 532 nm. The scanned results were saved in TIF format and subsequently analyzed using GenePix Pro 6.0 software (Axon).

Specificity Test of Chips

The specificity for the microarray was tested using RNA extracts of strains of influenza A virus, influenza B virus, Newcastle disease virus, foot and mouth disease virus, infectious bronchitis virus and infectious bursal disease virus.

Sensitivity Test of Chips

Using RNA from influenza A and B viruses as templates, the one-step RT-PCR approach was performed using the appropriate primers. The purified products were cloned into the pGEM-T easy vector, and the positive clones were screened by the blue/white colony method before sequencing. Plasmids from positive clones were extracted and linearized by enzyme digestion. In vitro transcription was performed using the RiboMAXTM Large Scale RNA Production System-T7 kit according to the manufacturer's instructions. The transcription products were extracted using the phenol–chloroform extraction method, precipitated with isopropanol, and re-suspended in ribozyme-free water, where they were quantified and the amount of initial copies were calculated. The solution was finally diluted to 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 and 1×10^1 copies/ μ L, and stored at –70 °C for future use. These dilutions were used to test the lower detection limit of the microarray.

Validation of Chips Using Clinical Specimens

Thirty-five clinical specimens suspected of being positive for influenza submitted by the First Hospital of Jilin University were tested according to the optimized chip testing conditions. The chip results were reexamined using American CDC real-time PCR testing method recorded in the literature [9].

Results

Design of Primers and Probes and Preparation of Chips

Forty specific oligonucleotide probes and amplification primers were originally designed to identify influenza A and B viruses using v4.2 Array Designer software. Four pairs of RT-PCR amplification primers and eight specific oligonucleotide probes were finally selected through exclusion of oligos with unreasonable GC content and hairpin structure (Tables 2 and 3). The probes and primers were blotted onto the slides using the MicroGrid TAS spotter in suture-styled contact-typed spotting method. Each chip contained 12 regions and each region contained a 7×6 probe array as shown in Fig. 1.

Specificity Test for the Chip

Five positive strains and four negative strains were selected in order to probe the specificity of the chip. The partial hybridization results of the microarrays are shown in

Table 2 Primers for the amplification of virus genes

Name	Target	Sequence (5'–3')	Size of product (bp)
FLUAF	M gene	TGGCTACTACCACCAATCC	297
FLUAR	M gene	CCCAATGATATTTGCTGCAATG	
FLUBF	M gene	TGAGAAGATGTGTGAGCTTTC	250
FLUBR	M gene	GCTGTGTTTCATAGCTGAGAC	
H1F	H1HA gene	CAATACAACCTGTCAAACACCC	371
H1R	H1HA gene	GGTTGAACTCTTTACCTACTGC	
H3F	H3HA gene	GGCCTGTCCCAGATATGTTAAG	200
H3R	H3HA gene	TGCTTTTGAGATCTGCTGC	

F forward primers, *R* reverse primers

Table 3 Oligonucleotide probes

Target	Probe no.	Sequence (5'–3')	Gene
Quality control probe	1	TGTTGATGGTCATCAGAAGCTT	
Influenza A virus	2	CCTGTTCACTCGATCCAGCCATCTGTTCCATAGCCTT	M
Influenza A virus	3	GCATGTACCATCTGCCTAGTCTGATTAGCAACCTCCAT	M
Influenza B virus	4	TTCCAGGATTTCAGGTACATGACCATGAGACAATATAGTAGCG	M
Influenza B virus	5	CTCTGCTATGAGCCCTGTGTGAATGTGATGCTTGTCTTCT	M
H1HA	6	GATAACCGTACCATCCATCTACCATCCCTGTCCACCC	HA
H1HA	7	AATTGTGATCGGATGTATATTCTGAAATGGGAGGCTGGTGTTT	HA
H3HA	8	GCCTCTAGTTTGTCTCTGCTGATTTTCGCATCCCTGTTG	HA
H3HA	9	CTGAAACCGTACCAACCATCCACCATTCCCTCCCAAC	HA
Antisense-QC	10	AAGCTTCTGATGACCATCAACA	

Fig. 2. The outcomes showed that the positive strains in the control group can be detected specifically, but no hybridization signal was found in the negative control groups. This indicated that the probes identifying the influenza A and B viruses were specific to influenza viruses.

Sensitivity of Chips

The RNA produced by in vitro transcription were made as templates for testing in 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 and 1×10^1 copies/ μ L dilutions. According to the results in Fig. 3, the threshold for detection appears to be 1×10^2 copies/ μ L.

Chip Testing with Clinical Specimens

Thirty-five clinical specimens were tested using chips and fluorescent PCR method (Table 4). Both methods concluded that 22 of 35 cases were positive and 13 negative.

Of the positive samples, nine were infected with influenza A subtype H virus, six with influenza A subtype H3 virus, and seven with influenza B virus.

Fig. 1 Schematic of a probe array on a chip. 1 Quality control probe; 2 influenza A virus probe 1; 3 influenza A virus probe 2; 4 influenza B virus probe 1; 5 influenza B virus probe 2; 6 H1 subtype probe 1; 7 H1 subtype probe 2; 8 H3 subtype probe 1; 9 H3 subtype probe 2; and 10 antisense-QC probe

Fig. 2 Assessment of chip specificity. **a** A/California/7/2009 (H1N1); **b** A/Victoria/210/2009 (H3N2); **c** A/chicken/JL/9/2004 (H5N1); **d** AIV isolate 1 (H9N2); **e** B/Brisbane/60/2008; **f** Newcastle disease virus;

g foot and mouth disease virus; **h** infectious bronchitis virus; **i** infectious bursal disease virus; and **j** control

Fig. 3 Results of the assessment of chip sensitivity

Discussion

Influenza outbreaks have shown a trend toward a global pandemic in recent years. Vaccination is the most effective way to control the influenza virus; however, testing for influenza is the most fundamental and necessary link between monitoring, prevention and control strategies. The rapid and precise laboratory diagnosis of influenza during pandemic periods is closely related to the control of influenza prevalence and the treatment of critically ill

Table 4 Detection and identification of influenza strains from 33 clinical samples using a microarray

Specimen	Chip test results	Real-time PCR test results
H1	9	9
H3	6	6
Influenza B virus	7	7
Negative	13	13
Total	35	35

patients. With the development of molecular biology techniques, rapid, flexible and accurate detection methods are being applied to influenza viruses. Several molecular diagnostic technologies have been broadly applied, such as RT-PCR, multiplex RT-PCR, MDD, real-time RT-PCR, duplex real-time PCR and DNA microarrays [9–12].

The present study explored the possibility of testing for the influenza virus using a microarray. Microarray technology can be divided into high-density and low-density DNA probe hybridization techniques. The high-density microarray, which has not been broadly applied in routine clinical diagnosis, can be used to test thousands of potential known or unknown pathogens. The low-density microarray analytic technology was rapidly developed by clinical specimen tests based on multiplex RT-PCR amplification [13–15]. Some low-density chips have been certified by CE Mark and applied to the in vitro diagnosis of human viral diseases, as viruses can be quickly detected using this method. The effective diagnosis of patients in hospitals with infectious diseases can be improved, and epidemiological surveys on infectious pathogens can be performed using this microarray technique [8, 13, 15].

In the case of low-density microarrays, specific fragments of the viral genome are amplified by multiplex RT-PCR, and the PCR products are subsequently tested by hybridizing with the specific oligonucleotide probes on the solid-phase vector surface. A variety of viruses in separate clinical specimens can be tested and identified using this technique. The specimens are fluorescently labeled during PCR amplification using chemically-modified primers. The amplified products are then hybridized with the specific probes on the microarray. The images are finally captured using a chip scanner and analytic software and the results are analyzed [15, 16].

Several PCR products were amplified in the multiplex RT-PCR simultaneously. However, several pairs of primers could generate primer dimers and resulting in non-specific amplification products. However, these problems can be excluded by optimizing primer design and RT-PCR conditions. This study analyzed the M gene of influenza A and B viruses and the HA gene of subtype H1 and H3 of influenza A virus. Primers and probes were designed and screened to exclude those with suboptimal GC content, hairpin structures, and other unfavorable properties, resulting in the selection of four PCR primer pairs. For each genotype, we selected two oligonucleotide probes from an original pool of 40 by optimizing for length, screening of T_m value, Blastn search, and other parameters. These oligonucleotide probes together with the appropriate primers ensure the specificity and sensitivity of the microarray. The results demonstrate that the method employed here is sensitive and specific.

Thirty-five throat swabs from the First Hospital of Jilin University were tested using the microarray technique

developed in the present study. The results were recorded using the real-time PCR method (CDC, USA). Using this method, 12 specimens could be tested on one chip in approximately 5 h, including: RNA extraction for 1 h, RT-PCR for 2 h, chip hybridization for 1.5 h and chip washing and scanning for 30 min. During a single test, eight chips detected 96 specimens.

This study primarily aimed to analyze influenza strains that directly affect human populations. In order to save costs, microarray analyses excluded viral strains that solely infect animals.

In conclusion, the influenza A and B viruses and subtype H1 and H3 influenza viruses were successfully tested using this novel technique, substantially reducing the time and complexity of influenza virus typing and subtyping. Furthermore, the test results demonstrated that our system displays higher sensitivity and specificity than other methods. Collectively, this study provides a rapid, convenient and accurate molecular biology diagnostic technique for routine monitoring as well as for emergency responses to influenza pandemics in the future.

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Conflict of interest The authors declared that they have no conflicts of interest.

References

1. Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, Fukuda K (2004) Influenza-associated hospitalizations in the United States. *JAMA J Am Med Assoc* 292:1333–1340. doi:10.1001/jama.292.11.1333
2. Khanna M, Kumar B, Gupta N, Kumar P, Gupta A, Vijayan VK, Kaur H (2009) Pandemic swine influenza virus (H1N1): a threatening evolution. *Indian J Microbiol* 49:365–369. doi:10.1007/s12088-009-0064-3
3. Stears RL, Martinsky T, Schena M (2003) Trends in microarray analysis. *Nat Med* 9:140–145. doi:10.1038/nm0103-140
4. Hughes TR, Mao M, Jones AR, Burchard J, Marton MJ, Shannon KW, Lefkowitz SM, Ziman M, Schelter JM, Meyer MR, Kobayashi S, Davis C, Dai H, He YD, Stephanian SB, Cavet G, Walker WL, West A, Coffey E, Shoemaker DD, Stoughton R, Blanchard AP, Friend SH, Linsley PS (2001) Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nat Biotechnol* 19:342–347. doi:10.1038/86730
5. Schurmann C, Heim K, Schillert A, Blankenberg S, Carstensen M, Dorr M, Endlich K, Felix SB, Gieger C, Grallert H, Herder C, Hoffmann W, Homuth G, Illig T, Kruppa J, Meitinger T, Muller C, Nauck M, Peters A, Rettig R, Roden M, Strauch K, Volker U, Volzke H, Wahl S, Wallaschofski H, Wild PS, Zeller T, Teumer A, Prokisch H, Ziegler A (2012) Analyzing illumina gene expression microarray data from different tissues: methodological aspects of data analysis in the metaxpress consortium. *PLoS One* 7:e50938. doi:10.1371/journal.pone.0050938

6. Debouck C, Goodfellow PN (1999) DNA microarrays in drug discovery and development. *Nat Genet* 21:48–50. doi:[10.1038/4475](https://doi.org/10.1038/4475)
7. Hwang TS, Jeong JK, Park M, Han HS, Choi HK, Park TS (2003) Detection and typing of HPV genotypes in various cervical lesions by HPV oligonucleotide microarray. *Gynecol Oncol* 90:51–56
8. Ruffing U, Akulenko R, Bischoff M, Helms V, Herrmann M, von Muller L (2012) Matched-cohort DNA microarray diversity analysis of methicillin sensitive and methicillin resistant *Staphylococcus aureus* isolates from hospital admission patients. *PLoS One* 7:e52487. doi:[10.1371/journal.pone.0052487](https://doi.org/10.1371/journal.pone.0052487)
9. Selvaraju SB, Selvarangan R (2010) Evaluation of three influenza A and B real-time reverse transcription-PCR assays and a new 2009 H1N1 assay for detection of influenza viruses. *J Clin Microbiol* 48:3870–3875. doi:[10.1128/JCM.02464-09](https://doi.org/10.1128/JCM.02464-09)
10. Wernike K, Beer M, Hoffmann B (2013) Rapid detection of foot-and-mouth disease virus, influenza A virus and classical swine fever virus by high-speed real-time RT-PCR. *J Virol Methods* 193:50–54. doi:[10.1016/j.jviromet.2013.05.005](https://doi.org/10.1016/j.jviromet.2013.05.005)
11. Zou S, Han J, Wen L, Liu Y, Cronin K, Lum SH, Gao L, Dong J, Zhang Y, Guo Y, Shu Y (2007) Human influenza A virus (H5N1) detection by a novel multiplex PCR typing method. *J Clin Microbiol* 45:1889–1892. doi:[10.1128/JCM.02392-06](https://doi.org/10.1128/JCM.02392-06)
12. Ellis JS, Smith JW, Braham S, Lock M, Barlow K, Zambon MC (2007) Design and validation of an H5 TaqMan real-time one-step reverse transcription-PCR and confirmatory assays for diagnosis and verification of influenza A virus H5 infections in humans. *J Clin Microbiol* 45:1535–1543. doi:[10.1128/JCM.02007-06](https://doi.org/10.1128/JCM.02007-06)
13. Frobert E, Escuret V, Javouhey E, Casalegno JS, Bouscambert-Duchamp M, Moulinier C, Gillet Y, Lina B, Floret D, Morfin F (2011) Respiratory viruses in children admitted to hospital intensive care units: evaluating the CLART(R) Pneumovir DNA array. *J Med Virol* 83:150–155. doi:[10.1002/jmv.21932](https://doi.org/10.1002/jmv.21932)
14. Huguenin A, Moutte L, Renois F, Leveque N, Talmud D, Abely M, Nguyen Y, Carrat F, Andreoletti L (2012) Broad respiratory virus detection in infants hospitalized for bronchiolitis by use of a multiplex RT-PCR DNA microarray system. *J Med Virol* 84:979–985. doi:[10.1002/jmv.23272](https://doi.org/10.1002/jmv.23272)
15. Renois F, Talmud D, Huguenin A, Moutte L, Strady C, Cousson J, Leveque N, Andreoletti L (2010) Rapid detection of respiratory tract viral infections and coinfections in patients with influenza-like illnesses by use of reverse transcription-PCR DNA microarray systems. *J Clin Microbiol* 48:3836–3842. doi:[10.1128/JCM.00733-10](https://doi.org/10.1128/JCM.00733-10)
16. Leveque N, Van Haecke A, Renois F, Boutolleau D, Talmud D, Andreoletti L (2011) Rapid virological diagnosis of central nervous system infections by use of a multiplex reverse transcription-PCR DNA microarray. *J Clin Microbiol* 49:3874–3879. doi:[10.1128/JCM.01214-11](https://doi.org/10.1128/JCM.01214-11)