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Multiple respiratory virus detection by DNA microarray

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Abstract

Current methods for detecting and identifying respiratory viruses in patient samples are based on viral amplification in cell culture followed by antibody detection. These methods are time-consuming, costly and depend upon complex and expensive equipment as well as resources. DNA-based microarrays have the potential and useful diagnostic tools that are able to detect and identify respiratory viruses in a rapid, sensitive, safe and cost effective manner. In order to transform this potential into reality, direct comparisons between the established cell culture and immuno-based methods and a DNA microarray method in a clinical setting are necessary. In this study, we developed a respiratory virus microarray that can be employed to detect eight different respiratory viruses at once, named: DR. Chip RV-chip. Throat swabs were taken from 433 patients with possible respiratory viral infections

and were analyzed using established cell culture and immuno-based assays and DR. Chip RV-chip, respectively. There were 92 positive tested specimens obtained in both assays. Although the respiratory virus microarray did not detect 20 of the 92 culture positive specimens (false negatives), the microarrays did detect 34 additional positive specimens in comparison to the traditional methods. Our observations demonstrate that in a clinical setting a rapid respiratory virus microarray assay can perform better than the slower cell culture and immuno-based assays.

Keywords: DNA microarray, Respiratory virus, Respiratory tract infection

1. Introduction

Respiratory viruses are the leading causative agents in respiratory infections worldwide. Common respiratory viruses such as influenza virus (IV), respiratory syncytial viruses (RSV), parainfluenza viruses (PIV), and adenoviruses (Adv) may co-circulate in the community, which cause acute respiratory infections that present with similar clinical signs and symptoms [1]. Therefore, a rapid and accurate identification of respiratory virus infection is crucial for efficient patient treatment, especially for acute respiratory infections as well as for outbreak control measures. As during the respiratory infection outbreak, it become very critical for the time it takes to identify the virus. The conventional diagnostic method for respiratory viral infections is to isolate viral particles from the patient, amplify the virus in cell culture and subjected to identify the virus using an immuno-based assay. However, this method is time consuming (at least 3-4 days) and is restricted to the availability and sensitivity of cell lines and monoclonal antibodies.

DNA microarrays are the technology that can be applied in many fields, including gene

expression in diverse organisms, drug discovery, genome mapping as well as mutation detection [2]. DNA microarrays are also the promising technology for the diagnosis of viral infections [3,4,5,6,7]. In addition, DNA microarrays have been used for the typing and subtyping influenza viruses [8,9] and for the species-specific detection of orthopoxviruses [10]. A method for the serotype-specific detection of enterovirus 71 (EV71) in clinical specimens by DNA microarrays has also been reported [4]. Altogether, DNA microarrays seem to be a powerful technology that merits further development of detection of pathogens in infectious disease.

Molecular diagnostic techniques, such as PCR or DNA microarray based methods, are an alternative approach for rapid and sensitive detection of respiratory viruses. Multiplex PCR methods have been developed in recent years for the detection of respiratory viral infections [1,11,12,13,14,15,16,17,18,19,20]. This approach has been demonstrated to be rapid and more sensitive in comparison to the established viral culture methods.

Many studies have also employed DNA microarray technology to detect one or multiple respiratory viruses [19,21,22]. These analyses demonstrate the potential for microarrays to be useful diagnostic tools that are able to identify respiratory viral infections in a rapid, sensitive, safe and cost effective manner. Direct comparisons in a clinical setting for the identification of respiratory viruses in patient samples are now necessary in order to compare the established and widely used cell culture and immuno-based methods with a DNA microarray based method. In this study, a DNA microarray was designed for simultaneous detection of eight respiratory viruses in less than 6 hours in a clinical setting. The microarray was directly tested using 433 clinical specimens (throat swabs) to determine the sensitivity and specificity of the DNA microarray based method in comparison to the cell culture and immuno-based methods.

2. Materials and Methods

2.1 Clinical specimens

A total of 433 clinical specimens (throat swabs) were collected from patients showing symptoms of respiratory viral infection and tested for respiratory viruses using the novel respiratory virus microarray (DR.RV^{TM} Chip, DR. Chip Biotechnology Inc., China). Specimens were collected at XuZhou Memorial Hospital from 2002 to 2005. Methods for the surveillance of respiratory virus infections via cell culture and immuno-based detection were employed as previously described [23,24].

2.2 Nucleic Acid Extraction

Viral DNA/RNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, USA) according to the manufacturer's directions. Briefly, 200 μL of specimen was added to 200 μL carrier RNA working solution (200 μL binding buffer and 4 μL carrier RNA), followed by adding 50 μL Proteinase K to the mixture and mixed well. The mixture was then incubated at 72˚ C for 10 min. A volume of 100 μL isopropanol was then added to each sample, and the suspensions were applied to spin columns. After centrifuging at 8000 *xg* for 1 min, the column was washed with 500 μL inhibitor removal buffer and 450 μL washing buffer twice. The DNA/RNA was eluted with 30 μL elution buffer.

2.3 RT-PCR

A Ready-To-Go RT-PCR Bead (Amersham Pharmacia Biotech Inc., U.S.A.) was dissolved in 20 μL DEPC-treated H_2O. A volume of 4μL of the dissolved enzyme mixture was transferred into two PCR tubes. A volume of 2 μL of RV Mix1 (including 7 sets of primers for PIV-1/2/3, RSV, IA, IB, adenovirus) and RV Mix2 (including 1 set of primers for internal PCR control) were added, respectively. The final volume of reverse transcription mixtures were 10 μL after adding 4 μL of the extracted nucleic acids. The RT mixtures were placed at 42˚ C for 45 min

and used as the templates for PCR. The PCR mixture of 39.5 μL RM (reaction mixture) (DR.Chip Biotech, HsinChu, China) and 0.5 μL DNA polymerase (2.5U, Promega, Madison, WI, USA) were added into RT mixture individually. A PTC-100™ Programmable Thermal Controller (MJ Research, Inc., USA) was used to perform the following program: 95˚ C for 5 min (1 cycle); 95˚ C for 20 sec, 50˚ C for 20 sec, 72˚ C for 40 sec (35 cycles), and extension at 72˚ C for 7 min. A volume of 5 μL of amplified products were analyzed by 2% agarose gel in TAE buffer and detected with ethidium bromide.

2.4 Hybridization on DR.RV™-chip

First, 490 μL DR.Hyb™ buffer was added into a new 1.5 mL Eppendorf tube. Second, 5 μL amplicons of tube 1 (from Mix1) and 5 μL amplicons of tube 2 (from Mix2) were transferred into the same tube and mixed. The tube was then chilled on ice for 2 min immediately after heating for 5 minutes in a boiling water bath. The mixture was transferred into a DNA microarray chip chamber (DR.RV™-chip, DR. Chip Biotechnology Inc., China) and covered with the lid. Then the chamber was incubated at 50˚ C in an oven (DR.Mini™ Oven, DR. Chip Biotechnology Inc., China) at maximal vibration for 60 min. The hybridization solution was then discarded, and each chamber was washed five times with 500 μL of Washing Buffer (Washing Buffer, DR.Chip). Streptavidin conjugated alkaline phosphatase (Strep-AP, DR.Chip) was diluted 1:1000 in the Blocking Buffer (DR.Blocking Buffer, DR. Chip Biotechnology Inc., China) followed by adding to each chip (500 μL/chamber). The chips were then incubated at room temperature for 30 mins. The chips were then washed five times with 500 μL of Washing Buffer. After the final wash, 500μL of the colorimetric substrate was added to each chip. The colorimetric substrate was prepared in diluting 20-fold the NBT/BCIP solution with Washing Buffer (NBT/TCIP, DR. Chip Biotechnology Inc., China) in the detection buffer (Detection Buffer, DR. Chip Biotechnology Inc., China). After 10 min of incubation in the dark at room temperature, the solution was discarded. The microarray

colorimetric signals were detected by the DR. AiM™ Reader. The results were analyzed with the DR.AiM Soft 2.0 program. The detailed microarray pattern is shown in Figure 1A and is described below.

3. Results

3.1 Traditional evaluation of clinical samples

A total amount of 433 throat swabs were collected from patients presenting with possible respiratory tract infections over a three-year period. Samples were collected and handled using standard techniques. Traditional cell culture and immuno-based assays yielded positive results in 112/433 samples (Table 2) [23,24]. While the goal of this study was to make a direct comparison in a clinical setting among these established cell culture and immuno-based methods and a DNA microarray based method for detecting respiratory viruses in patient samples. To this end, a low-density respiratory virus microarray was designed and produced in order to perform the detection of eight different respiratory viruses at once.

3.2 Design of a respiratory virus low-density microarray

The DR.RV™ IVD Kit (DR. Chip Biotechnology Inc., China) was designed with 25 spots arranged in five columns. The sequences of probes immobilized at each location are listed in Table1. The pattern of the low-density arrays is shown in Figure 1A. PIV1-3 (Parainfluenza virus) specific primers and probes were directed toward the HN (Hemagglutinin/Neuraminidase) region of the PIV genes of the three virus types, respectively. RSV (respiratory syncytial virus) specific primers and probes were designed from the NS (non-structure) genes of the A and B subtypes, respectively. Influenza virus specific primers and probes were targeted on NS and HA (Hemagglutinin) genes for A and B subtypes, respectively. SARS-CoV (Severe Respiratory Syndrome-Coronavirus) specific primers and

probe were directed toward the RNA polymerase. Adenovirus specific primers and probes were designed from the hexon gene. In addition to viral specific probes, there were one positive control probe for the PCR reaction, and 4 positive control probes (Probe-H) for hybridization were immobilized on the array along with a single buffer only negative control spot (Figure 1A).

3.3 Microarray evaluation of clinical samples

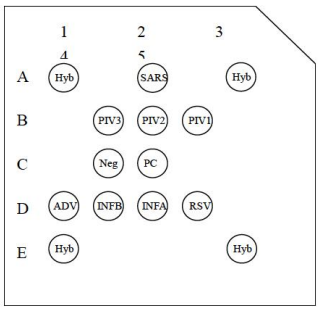
Samples (200 L) were prepared for microarray analysis from DNA/RNA extracts, followed by amplifying target genome sequences. Primers for the amplification step are listed in Table 1. PCR products of the correct size using each set of primers on representative samples were observed (Figure 2).

Amplicons from the 433 samples were incubated on the microarrays as described in detail in the Materials and Methods section. Probe-H was used as the hybridization positive control since the antisense oligonucleotide was adding in the hybridization buffer. The hybridization patterns for each specific respiratory virus were detected as shown in Figure 1B: The blank sample shows five gray spots; with four spots located on the corners (four hybridization control) and one located at the center of the array (the PCR internal control). On all 433 microarrays the internal PCR and hybridization positive controls were always observed. In all 433 analyzed samples, none of the sample displayed a positive signal in the buffer contained only negative control.

Positive results were observed in 120/433 specimens by the respiratory virus microarray (Table 2). The sensitivity and specificity of the respiratory microarray, in comparison to the viral isolation method, were 82.1% and 91.3%, respectively (Table 2). Ninety-two specimens tested positive in both the respiratory virus microarray and traditional isolation methods. Twenty-eight specimens tested positive in the microarray only, while 20 specimens were tested positive in the isolation method only. Overall agreement of both methods was 88.9%

(385/433). Subsequent analysis by RT-PCR on 41/48 of the discrepant samples demonstrated that the majority were false negatives for both detection techniques employed, with only 2/433 samples displaying a false negative result by microarray analysis (Table 2). Although the DR. RV-microarray did not detect 20 of the 92 culture positive specimens it did detect 34 more positive specimens in comparison to the traditional method.

Fig. 1A



A1.A5. E1.E5.	*Hybridization positive control*
C2.	*Hybridization negative contro*
C3.	*PCR Positive Control*
A3.	*Corona-virus (SARS)*
B4.	*Parainfluenza I*
B3.	*Parainfluenza II*
B2.	*Parainfluenza III*
D4.	*RSV*
D3.	*Influenza A*
D2.	*Influenza B*
D1.	*Adenovirus*

Fig. 1B.

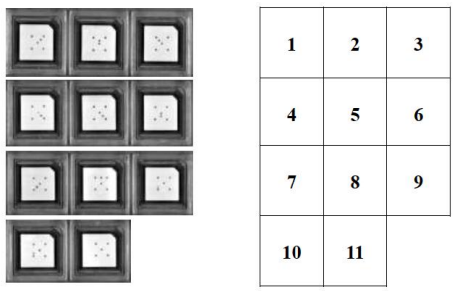


Figure 1. DR. RV-chip design for detection of respiratory viruses. **A)** Allocation pattern of specific oligonucleotide probes in the DR. RV-chip. **B)** Images of Dr. RV-chip arrays employed to detect positive clinical samples from PIV-1 (1), PIV-2 (2), PIV-3 (3), RSV-A (4), RSV-B (5), Inf A (6), Inf B (7), Corona-virus (SARS) (8), AdV-2 (9), AdV-3 (10) infected patients and an example of a negative throat swab (11) are shown.

Fig. 2.

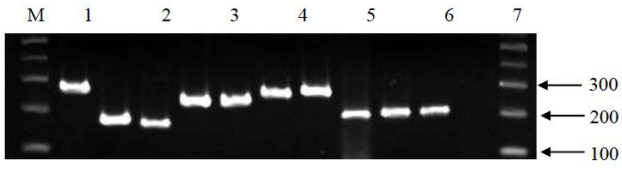


Figure 2. The RT-PCR products from different targets analyzed by electrophoresis in an 2% agarose gel. Positive clinical samples from PIV-1 (1), PIV-2 (2), PIV-3 (3), RSV-A (4), RSV-B (5), Inf A (6), Inf B (7), Corona-virus (SARS) (8), AdV-2 (9), AdV-3 (10) infected patients and a negative throat swab (11) are shown. M indicates molecular weight markers. The expected product lengths are given in Table1.

4. Discussion

This study aim to a direct comparison in a clinical setting among the established cell culture, immuno-based methods and a DNA microarray based method for detecting respiratory viruses in patient samples. To this end, we developed and employed a low-density respiratory virus microarray that can detect eight different respiratory viruses at once. Positive results were obtained in 120/433 specimens by the respiratory virus microarray while 112/433 viruses were isolated by cell culture and immuno-based methods. Although the DR. RV-chip did not detect 20 of the 92 culture positive specimens it did detect 34 more positive specimens in comparison to the traditional method. Our observations demonstrate that in a clinical setting the rapid respiratory virus microarray assay can perform better than the slower cell culture and immuno-based assays.

A total of 41 of the discordant specimens between the two methods were subjected to real-time PCR. This analysis demonstrated that the majority were false negatives for both detection techniques employed, with only 2/433 samples displaying a false negative result by microarray analysis (Table 2). These false negatives may due to RNA degradation or the detection limit of the respiratory virus microarray method. A previous report on the evaluation of an enterovirus 71 (EV71) microarray (DR.EV^{TM} Chip; DR. Chip Biotechnology Inc., China) revealed that the amount of template RNA corresponding to 10^2-10^3 virions was the least requirement in order to produce a visible specific amplicon on the agarose gel while the EV71-microarray can detect the amplicon derived from viral RNA corresponding to 1-10 virions [4]. This rate of false negatives can be minimized by including multiple oligos on the microarray for each virus, as was done recently [25]. Since the rate of false positives is low, only 2/433 samples, if any one of the multiple oligos is positive on the microarray then the virus might be considered likely to be present.

Respiratory virus microarrays offer several advantages over the current cell culture and

immuno-based assays. The whole procedure takes only 6 hours including the time required for PCR, which is faster than the traditional viral culture method, which takes 3-4 days. A rapid and accurate identification is crucial for efficient patient treatment, especially for acute respiratory infections and for outbreak control measures where the time it takes to identify the virus is critical. The microarray method also reduces the level of the biological hazard by avoiding the isolation of live virus [26,27, 28, 29, 30]. The relative short length of the amplified fragments in the proposed method also makes it appropriate for immediate hybridization without any fragmentation. The use of biotin labeled primers appears to display a highly efficient incorporation in the final PCR product, and the hybridization procedure takes only 2 hours. The microarray is also a very sensitive method to detect multiple pathogens simultaneously, which saves the amount of specimen as well as decreases the detection time. The RV-chip method exhibited the ability to detect co-infections in three specimens (Table 2). The DR. Chip RV-chip method employed here is also cost-effective, at about $10 USD per test. These advantages are enhanced by the flexibility inherent in a microarray assay.

There are many viruses that cause respiratory diseases. Some of these viruses have a predisposing effect on the outcome of the patients if they are not treated properly and efficiently. Therefore, microarray technology based on genomic differences between viruses may be a promising tool in diagnosis of respiratory virus infection. This study successfully designed and developed a respiratory virus (RV)-specific microarray, which may provide an early diagnosis to the common respiratory virus infections. Although the DR. RV-chip did not detect 20 of the 92 culture positive specimens, it detected 34 more positive specimens in comparison to the traditional method. Thus, a large numbers of positive specimens and a wide spectrum of respiratory viruses can be efficiently screened by combining rapid DR. Chip RV-chip method with the conventional viral culture method.. Further development of a

comprehensive microarray, by increasing the number of spots with little increase in cost for the detection, for emerging, reemerging and existing yet neglected respiratory viruses, such as bocaviurs, human metapneumovirus, rhinovirus and coronavirus is currently being investigated.

Figures and Tables:

Figure 1. DR. RV-chip design for detection of respiratory viruses. **A)** Allocation pattern of specific oligonucleotide probes in the DR. RV-chip. **B)** Images of Dr. RV-chip arrays employed to detect positive clinical samples from PIV-1 (1), PIV-2 (2), PIV-3 (3), RSV-A (4), RSV-B (5), Inf A (6), Inf B (7), Corona-virus (SARS) (8), AdV-2 (9), AdV-3 (10) infected patients and an example of a negative throat swab (11) are shown.

Figure 2. The RT-PCR products from different targets analyzed by electrophoresis in an 2% agarose gel. Positive clinical samples from PIV-1 (1), PIV-2 (2), PIV-3 (3), RSV-A (4), RSV-B (5), Inf A (6), Inf B (7), Corona-virus (SARS) (8), AdV-2 (9), AdV-3 (10) infected patients and a negative throat swab (11) are shown. M indicates molecular weight markers. The expected product lengths are given in Table1.

Table 1. Primers and probes used in this study

Species	Primer or Probe	Sequence (5'-3')	Target gene	Amplicon size	Reference Accession (Position)
PIV 1	PIV1-F	CTGTAATAGCTGCAGGAACAAG	HN	268	U70936 (832-1099)
	PIV1-R	CCTTGGAGCGGAGTTGTTA			
	PIV1-probe	GACATATTAGATCTCAAGGGAAAGACCA			
PIV2	PIV2-F	GATCTAGCTGAACTGAGACTTGC	HN	168	AF213352 (879-1046)
	PIV2-R	TATGAGACCACCATATACAGGAAA			
	PIV2-probe	TCATATCTCTTCCAAAYACAACAGGGCA			
PIV3	PIV3-F	AGTTGATGAAAGATCAGATTATGC	HN	156	M17641 (904-1059)
	PIV3-R	CCTGGTCCAACAGATGGGTAT			
	PIV3-probe	ATYATGATGGYTCAATCTCAACAACAAG			
RSV	RSV-F	GYATTGGCATTAAGCCTACAA	NS	222	U39661 (941-1162)
	RSV-R	AACTTGACTTTGCTAAGAGCCAT			
	RSV-probeA	AGGAGAGACATAAGATGAAAGATGGGGC			
	RSV-probeB	AAATATGACCTCAACCCGTAAATTCCAA			D00736
IA	INFA-F	CGAAATTTCACCATTGCCTTC	NS	252	AF055425 (500-

					751)
	INFA-R	GTCTCACTTCTTCAATCAGCCA			
	IA-probe	TCTACAGAGATTCGCTTGGRGAAGCAG			
IB	INFB-F	GTGGTCAAAACWGCTACTCAAGGG	HA	264	AY581955 (88-351)
	INFB-R	TGTTCTGTCGTGCATTATAGG			
	IB-probe	TGTGATACCACTGACAACAACACCWAC			
ADV	ADV-F	CTCCAGYAACTTYATGTCCAT	Hexon	199	J01917 (21510-21708)
	ADV-R	CAGGTASACGGYCTCGATGA			
	ADV-probe	GAAGTCTTTGACGTGGTCCGTGTGC			
SARS	SARS-F	ATGAATTACCAAGTCAATGGTTAC	orf1ab	190	AY864806 (18153-18342)
	SARS-R	CATAACCAGTCGGTACAGCTA			
	SARS-probe	TGTCATGCAACTAGAGATGCTGCGG			

Table 2. Summary of the DR.RV-chip and viral culture results for 433 clinical samples

Virus	No. of samples with positive result by:				
	DR.RV assay	Viral culture	Both DR.RV and Viral culture	Real-Time PCR positive on discrepant samples	
				DR.RV (+) / Culture (-)	DR.RV (-) / Culture (+)
PIV1	1	0	0	1	0
PIV2	3	4	3	0	1
PIV3	1	0	0	1	0
RSV	18	16	13	5	3
IA	39	32	28	11	3[b]
IB	30	25	20	8[a]	4[c]
ADV	28	35	28	0	ND[d]
Total (%)	120 (27.7)	112 (25.9)	92 (21.2)		

[a] Among 10 IB positive samples in DR.RV assay, 2 were negative by real-time PCR.

[b] Among 4 IA positive samples in viral culture, one was negative by real-time PCR.

[c] Among 5 IB positive samples in viral culture, one was negative by real-time PCR.

[d] The 7 ADV discrepant samples that showed culture positive and DR.RV negative, were not tested further by real-time PCR.

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