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ORIGINAL ARTICLE

Subtype identification of the novel A H1N1 and other human influenza A viruses using an oligonucleotide microarray

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Abstract A novel strain of influenza A (H1N1) virus was isolated in Mexico and the US in March and April 2009. This novel virus spread to many countries and regions in a few months, and WHO raised the level of pandemic alert from phase 5 to phase 6 on June 11, 2009. The accurate identification of H1N1 virus and other human seasonal influenza A viruses is very important for further treatment and control of their infections. In this study, we developed an oligonucleotide microarray to subtype human H1N1, H3N2 and H5N1 influenza viruses, which could distinguish the novel H1N1 from human seasonal H1N1 influenza viruses and swine H1N1 influenza viruses. The microarray utilizes a panel of primers for multiplex PCR amplification of the hemagglutinin (HA), neuraminidase (NA) and matrix (MP) genes of human influenza A viruses. The 59-mer oligonucleotides were designed to distinguish different subtypes of human influenza A viruses. With this microarray, we accurately identified and correctly subtyped the reference virus strains. Moreover, we confirmed 4 out of 39 clinical throat swab specimens from suspected cases of novel H1N1.

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Introduction

In March and April 2009, a novel strain of influenza A (H1N1) virus was isolated in Mexico and the US. By July 1, 2009, 77,201 cases of the novel influenza A virus infection, including 332 deaths, had been reported to the World Health Organization (WHO) [20]. On June 11, 2009, WHO raised the level of pandemic alert from phase 5 to phase 6, indicating that the world was now at the beginning of the 2009 influenza pandemic [21].

Previous studies indicate that the novel H1N1 is a "triple reassortant" combining genome segments from influenza viruses of humans, birds and swine [14, 16, 19]. The H1N1 appears to be more contagious than the seasonal influenza. The secondary attack rate of seasonal influenza ranges from 5 to 15%, while that of the novel H1N1 ranges from 22 to 33% [1]. Most confirmed cases of novel H1N1 have symptoms similar to those of seasonal influenza (cough, sore throat, rhinorrhea, headache and myalgia). However, approximately 38% of cases also presented with vomiting or diarrhea, neither of which is typical of seasonal influenza (Novel Swine-Origin Influenza A [11] Virus Investigation Team, 2009). Therefore, the accurate identification of the novel H1N1 virus and other human seasonal influenza A viruses is crucial for further treatment and control of their infection.

So far, influenza A viruses infecting humans have been limited to the subtypes H1N1 and H3N2. However, some avian subtypes, especially the highly pathogenic H5N1 [3, 12, 18], have crossed the species barrier and caused human infection in recent years. Multiplex reverse

transcriptase polymerase chain reaction (RT-PCR) and multiplex real-time RT-PCR (rRT-PCR) assays have been developed to detect influenza A viruses, and these show good sensitivity and specificity [2, 4, 13, 22]. On the other hand, microarrays have the advantages of high-throughput and less dependence on the sequences of the primer-binding sites.

In this study, we developed an oligonucleotide microarray to subtype human H1N1, H3N2 and H5N1 influenza viruses. Furthermore, this oligonucleotide microarray can distinguish the novel H1N1 from human seasonal H1N1 influenza viruses and swine H1N1 influenza viruses. The microarray utilizes a panel of primers for multiplex PCR amplification of hemagglutinin (HA), neuraminidase (NA) and matrix (MP) genes of influenza A viruses and includes 59-mer oligonucleotides for discriminating between different subtypes of human influenza A viruses.

Materials and methods

Influenza A virus strains and clinical samples

Our study used a panel of human influenza A viruses including the novel influenza virus A/Beijing/501/2009 (H1N1), seasonal influenza virus A/PR/8/34 (H1N1), influenza virus A/Beijing/30/95 (H3N2), influenza virus A/Beijing/01/2003 (H5N1), influenza virus A/duck/Taiwan/4201/99 (H7N7) and influenza virus A/Swine/Shandong/nb/2003 (H9N2). Novel A (H1N1) reference RNA and RNA extracted from the first confirmed novel H1N1 case in Chengdu Infectious disease hospital in China were kindly provided by China center for disease control. Clinical throat swab specimens from 39 suspected cases of novel H1N1 were collected from different infectious disease hospitals. Viral RNA was extracted either from the supernatants of cultures of infected MDCK cells or from throat swab specimens using an RNeasy mini kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions.

Oligonucleotide probe and PCR primer design

Viral sequences data were downloaded either from the GenBank database or from the Influenza Sequence Database. Novel H1, N1 and MP gene sequences, all other human seasonal influenza H1 and N1 sequences and swine H1 and N1 sequences were selected. Likewise, all human influenza virus isolates of subtypes H3N2 and H5N1 were selected. Viral sequences of each subtype were aligned using the software ClutalX (version 1.83) to select the most conserved regions of influenza virus segments HA, NA and MP. Probes for MP were designed as the positive control for the hybridization system. Oligonucleotide probes were selected from the most conserved regions and designed using the software Arraydesigner 4.0. The oligonucleotide 59-mer probes were designed to have similar annealing stabilities and melting temperatures (Tm) of 74 ± 3°C. Sequence alignment showed that each probe had at least 25 nt in common with the other members of the same subtype. Then, the probes were optimized by pairwise BLASTN analysis to select those sharing less than 50% sequence similarity with other viral subtypes. Primers for multiplex PCR amplification were designed for both sides of the regions including the oligo probes and selected from the most conserved regions of each subtype to minimize the number of PCR primers required. In addition, the primers were selected to amplify amplicons of 400–1,000 bp in length for convenience of subsequent hybridization to the microarray. The 400–1,000-bp amplicons covered the sequences of oligonucleotide probes of a certain subtype. All probes were synthesized by Shanghai Sangon Company (China).

Sample preparation

cDNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and influenza A virus universal primer AGCAAAAGCAGG [8]. PCR amplifications were done using multiplex PCR from first-strand cDNA. Multiplex PCR was carried out using two different reactions: one reaction contained the primers for seasonal H1, H3, H5, MP and novel H1, and the other reaction contained the primers for seasonal N1, N2, MA and novel N1. Briefly, the PCR reaction was performed with a denaturation step at 94°C for 5 min, followed with 35 cycles at a melting temperature of 94°C for 30 s, an annealing step at 50°C for 30 s, an extension step at 72°C for 45 s, and finally, an extension step at 72°C for 7 min. Furthermore, the reactions were supplemented with 2.5 units of Taq polymerase and amplified for an additional 20 cycles using the same PCR conditions in the presence of aminoallyl-dUTP. The resulting PCR product was purified using a QIAGEN QIAquick PCR purification kit (QIAGEN, USA).

Microarray hybridization and data analysis

The purified multiplex PCR product with incorporated aminoallyl-dUTP was further labeled with Cy3 or Cy5 fluorescent molecules at room temperature for 1 h and then quenched with 4 mol/l hydroxylamine. This product was purified using a QIAGEN QIAquick PCR purification kit before hybridization. Oligonucleotide probes were suspended in 3 × SSC (10 × SSC: 87.6 g of NaCl/l and 44.1 g of sodium citrate/l, pH 7.0) to adjust the final concentration to 40 μmol/l. Then, the probes were printed onto aldehyde-activated silylated microscope slides (CEL

Associates, Pearland, Texas, USA) using a SpotArray 24 Microarray Printing System (PerkinElmer Instruments, MA, USA). Each probe was printed in triplicate. Figure 1 shows the positions where the probes of the different subtypes are spotted on the microarray. Printed arrays were left to dry overnight at room temperature and blocked with 0.25% $NaBH_4$ at 37°C before hybridization. Microarrays were prehybridized in a solution containing 5 × SSC, 0.1% SDS and 0.1% BSA at 42°C for 1 h. Subsequently, 30 µl of Cy3- or Cy5-labeled PCR product was denatured at 95°C for 5 min and added to 60 µl of freshly prepared hybridization buffer containing 40% deionized formamide, 5 × SSC, 0.1% SDS and 0.5 µg/µl salmon sperm DNA. This mixture was then applied to the array and incubated at 42°C in a humidified chamber for 1.5 h and then washed at 50°C for 5 min in 1 × SSC containing 0.1% SDS and 0.1 × SSC containing 0.1% SDS. The arrays were then dried and stored in the dark. Hybridization results were analyzed using a ScanArray Gx PLUS (PerkinElmer). The fluorescence intensity was quantified by Genepix Pro5.0 software (PerkinElmer). Signal-to-background ratios of ≧ 2,000 were regarded as positive hybridization signals.

Quantitative real-time RT-PCR

For assessment of sensitivity, rRT-PCR assay was conducted to determine the viral load of the novel H1N1. rRT-PCR was carried out according to the protocols recommended by WHO. Briefly, TaqMan assay reactions were performed in a 30-µl volume (Applied Biosystems, USA) containing 4 µl of the cDNA, 15 µl 2 × TaqMan® Universal PCR Master Mix, 0.25 µM forward primer, 0.25 µM reverse primer and 0.125 µM probe in a fluorometric PCR instrument (LightCycler, Roche). Forty cycles of amplification (95°C for 15 s, 55°C for 30 s; fluorescence was recorded at 58°C) were undertaken after denaturing at 95°C for 10 min. Serially diluted RNA template of novel H1N1 (A/Beijing/501/2009) from 2×10^3 TCID50 to 0.002 TCID50 was used for the sensitivity test.

M1	M1	M1	M2	M2	M2	M3	M3	M3	E	E	E
1-1	H1-1	H1-1	H1-2	H1-2	H1-2	H1-3	H1-3	H1-3	E	E	E
NH1-1	NH1-1	NH1-1	NH1-2	NH1-2	NH1-2	NH1-3	NH1-3	NH1-3	E	E	E
H3-1	H3-1	H3-1	H3-2	H3-2	H3-2	H3-3	H3-3	H3-3	H3-4	H3-4	H3-4
H5-1	H5-1	H5-1	H5-2	H5-2	H5-2	H5-3	H5-3	H5-3	H5-4	H5-4	H5-4
N1-1	N1-1	N1-1	N1-2	N1-2	N1-2	E	E	E	E	E	E
NN1-1	NN1-1	NN1-1	NN1-2	NN1-2	NN1-2	NN1-3	NN1-3	NN1-3	NN1-4	NN1-4	NN1-4
N2-1	N2-1	N2-1	N2-2	N2-2	N2-2	N2-3	N2-3	N2-3	E	E	E
E	E	E	M1	M1	M1	M2	M2	M2	M3	M3	M3

Fig. 1 The printed position of oligo probes of different subtypes on microarray. *NH* and *NN* represent novel H and novel N, respectively. All the others represent different subtypes. *E* represent empty

Results

Oligonucleotide probes

In order to maximize the number of detectable strains of a certain subtype of human influenza A viruses, we selected the most highly conserved regions within the HA and NA segments of each subtype to design 59-mer oligonucleotides. The oligonucleotides designed from the conserved regions in the MP segment of influenza A viruses were selected as the positive control of the microarray. In order to discriminate the novel H1N1 from seasonal H1N1 and swine H1N1, we downloaded the sequences of all of the novel 2009 H1N1 outbreak strains, all seasonal H1N1 influenza A viruses and swine H1N1 sequences from GenBank to select the most conserved regions for each subtype. The selected oligos were subsequently aligned with NCBI database to ensure that each probe was unique to the respective subtype. Oligos of the novel H1N1 with a sequence similarity greater than 50% with seasonal H1N1 or swine H1N1 were removed to guarantee the discrimination of the novel H1N1 from seasonal H1N1 and swine H1N1. Finally, a panel of 25 oligonucleotide probes was designed: three for the novel H1, four for the novel N1, three for seasonal H1, two for seasonal N1, four for H3, two for N2, four for H5 and three for the influenza A virus MP segment. Table 1 shows the sequences of the oligonucleotides and the GenBank BLAST sequence alignment.

Multiplex PCR

The amplification of targeted genes of human influenza A virus isolates was done by multiplex RT-PCR using a pool of primers. The primers were designed from the most conserved regions of each influenza A virus subtype to minimize the number of PCR primers required. In addition, the primers were selected to amplify amplicons of 400–1,000 bp for convenience of subsequent hybridization to the microarray. The amplicons for each particular subtype were long enough to ensure the coverage of the detecting oligos. Table 2 shows the primers and their locations. The multiplex PCR were carried out in two different reactions: one reaction contained the primers for seasonal H1, H3,

Table 1 Oligonucleotide probes for identifying novel influenza A (H1N1) and other seasonal influenza viruses

Subtype	Oligo No.	Sequence (5′–3′)
Novel H1	Novel_H1_oligo_1	TGGGGCATTCACCATCCATCTACTAGTGCTGACCAACAAAGTCTCTATCAGAATGCAGAT
	Novel _H1_oligo_2	AGACCCAAAGTGAGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGCCG
	Novel _H1_oligo_1	ATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCACGATTGCAAT
H1	FLU_H1_oligo_1	AGCAAGGATCTGGCTATGCTGCAGATCAAAAAAGCACACAAAATGCCATTAATGGGATT
	FLU_H1_oligo_2	ATCAGAATGAGCAAGGATCTGGCTATGCTGCAGATCAAAAAAGCACACAAAATGCCATT
	FLU_H1_oligo_3	CAACAAGGATTTACCAGATTTTGGCGATCTATTCAACTGTCGCCAGTTCATTGGTACTG
H3	H3-oligo-1	GGGGTTACTTCAAAATACGAAGTGGGAAAAGCTCAATAATGAGATCAGATGCACCCATT
	H3-oligo-2	TGGGAAAAGCTCGATAATGAGATCAGATGCACCTATTGACACATGCATCTCTGAGTGCAT
	H3-oligo-3	TAGATGCTCTATTGGGAGACCCTCAGTGTGATGGCTTCCAAAATAAGAAATGGGACCTTT
	H3-oligo-4	CGTGACTATGCCAAACAATGAAAAATTTGACAAATTGTACATTTGGGGGGTTCACCACC
H5	H5_oligo_1	CTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCGA
	H5_oligo_2	TTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTGGTAT
	H5_oligo_3	GATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGAC
	H5_oligo_4	ATACCATCATAGCAATGAGCAGGGAAGTGGATATGCTGCAGACAAAGAATCTACCCAGAA
Novel N1	Novel _N1_oligo_1	CAATGGGGCAGTGGCTGTGTTAAAGTACAACGGCATAATAACAGACACTATCAAGAGTTG
	Novel _N1_oligo_2	ACCGATGGACCAAGTAATGGACAGGCCTCATACAAGATCTTCAGAATAGAAAAGGGAAAG
	Novel _N1_oligo_3	CACAAGAGTCTGAATGTGCATGTGTAAATGGTTCTTGCTTTACTGTAATGACCGATGGAC
	Novel _N1_oligo_4	AATTGGCTAACAATTGGAATTTCTGGCCCAGACAATGGGGCAGTGGCTGTGTTAAAGTAC
N1	N1-oligo-1	TGGTTGACAATTGGAATTTCTGGTCCAGACAATGGGGCTGTGGCTGTATTGAAATACAA
	N1-oligo-2	AGAACCTTAATGAGCTGTCCTATTGGTGAAGCTCCTTCTCCATACAACTCAAGGTTTGA
N2	N2_oligo_1	TGGACCTCAAACAGTATTGTTGTGTTTTGTGGCACCTCAGGTACATATGGAACAGGCTCA
	N2_oligo_2	ATGGAGTGAAAGGCTGGGCCTTTGATGATGGAAATGACGTGTGGATGGGAAGAACGATCA
	N2_oligo_3	TGGTGGACTTCAAATAGTATCGTCGTATTTTGTGGTACTTCTGGTACCTATGGAACAGGC
MP	FLUA_MP_oligo_1	GGCTCTCATGGAATGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAAGGGGATTT
	FLUA_MP_oligo_2	ACTGCAGCGTAGACGCTTTGTCCAAAATGCCCTCAATGGGAATGGAGATCCAAATAACA
	FLUA_MP_oligo_3	AAGAACACAGATCTTGAGGCTCTCATGGAATGGCTAAAGACAAGACCAATCCTGTCACC

H5, MA and the novel H1, and another reaction contained the primers for seasonal N1, N2, MP and the novel N1. Each specimen was amplified by two distinct multiplex PCR reactions. All reference strains were successfully amplified by the multiplex PCR systems.

Microarray validation by reference strains

In order to evaluate the detection specificity of the microarray, we tested different subtype reference strains of influenza A viruses. A panel of human influenza A viruses including novel influenza virus A/Beijing/501/2009 (H1N1), seasonal influenza virus A/PR/8/34 (H1N1), influenza virus A/Beijing/30/95 (H3N2), influenza virus A/Beijing/01/2003 (H5N1), influenza virus A/duck/Taiwan/4201/99 (H7N7) and influenza virus A/Swine/Shandong/nb/2003 (H9N2) were utilized for the test. All of the reference strains were accurately identified and correctly subtyped (Fig. 2). In addition, we did not detect any hybridization signals for H7N7 or H9N2.

Microarray validation with clinical specimens

To further test the discriminatory capacity of the microarray, we collected 39 clinical throat swab specimens from suspected cases of novel H1N1 from different infectious disease hospitals. RNA was extracted, and two separate multiplex RT-PCR reactions were carried out for each specimen with different pools of primers. The PCR products were mixed together for further labeling and hybridization. Hybridization results were interpreted as positive only when the fluorescence intensity for both the probable subtype and MP were greater than 2000. By using this microarray, four of the specimens (10.2%) were confirmed as novel A (H1N1) positive (Fig. 2). When testing clinical specimens of novel A (H1N1), cross-hybridization was observed with seasonal N1 and N2; however, based on the fluorescence intensity of each oligo, these weak signals could be interpreted as cross-hybridization. Similarly, 2 cases were identified as seasonal H1N1, and 12 cases were confirmed as H3N2,

Table 2 Primers for multiplex PCR amplification

Subtype	Primer no.	Sequence (5′–3′)	Product size (bp)
Novel H1	NAH1-F	GACAAGTTCATGGCCCAATC	494
	NAH1-R	CCCTTGGGTGTTTGACAAGT	
H1	AH1-F	AWGGTATGCYTTCGCAHTGA	810
	AH1-R	CGACWGTTGARTAGATCGCCA	
H3	AH3-F	GACCTTTTTGTTGAACGCAG	605
	AH3-R	TCCATTTGGAGTGATGCATT	
H5	AH5_F	TGCCCCAAATATGTGAAA	508
	AH5_R	AGAACTCGAAACAACCGT	
Novel N1	NAN1-F	CCATTGGTTCGGTCTGTA	816
	NAN1-R	CAGGAGCATTCCTCATAGTG	
N1	AN1-F	RCTTCAGTRACATTRGCNGGCA	810
	AN1-R	CSATYTTGAARATTCTGTAYGAG	
N2	AN2_F	TGTGCTCAGGACTTGTTG	452
	AN2_R	GCATGAGATTGATGTCCG	
M	AMP-F	CAGAGACTTGAAGATGTYTTTGC	450
	AMP-R	ATGCCTGATTAGTGGGTTGGT	

R = (A, G), Y = (C, T), W = (A, T), S = (C, G), H = (A, C, T), and N = (A, G, C, T)

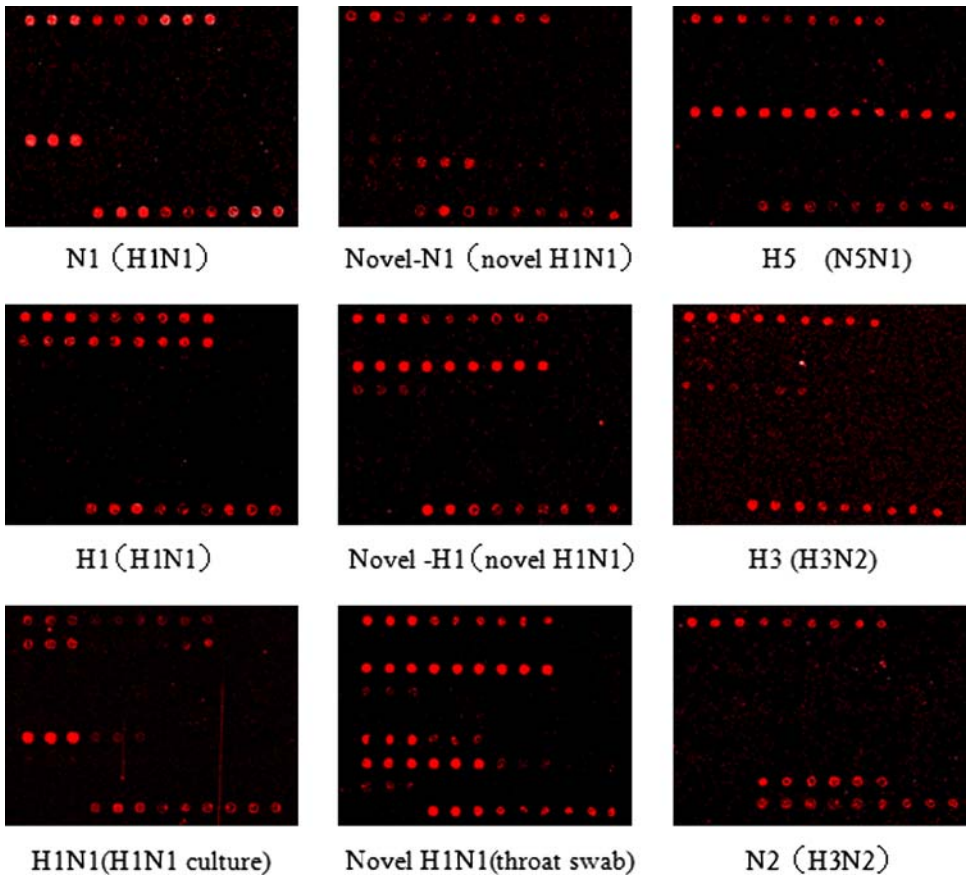

Fig. 2 The gene microarray hybridization scanning result for each reference viral segment and of clinical throat swabs specimens

which were identical in tests using the real-time PCR assay recommended by WHO and virus isolation (data not shown). The results indicated that these clinical specimens were also accurately subtyped.

Sensitivity of the microarray

The sensitivity of the microarray for the novel H1N1 (A/Beijing/501/2009) was comparable to that of the rRT-PCR

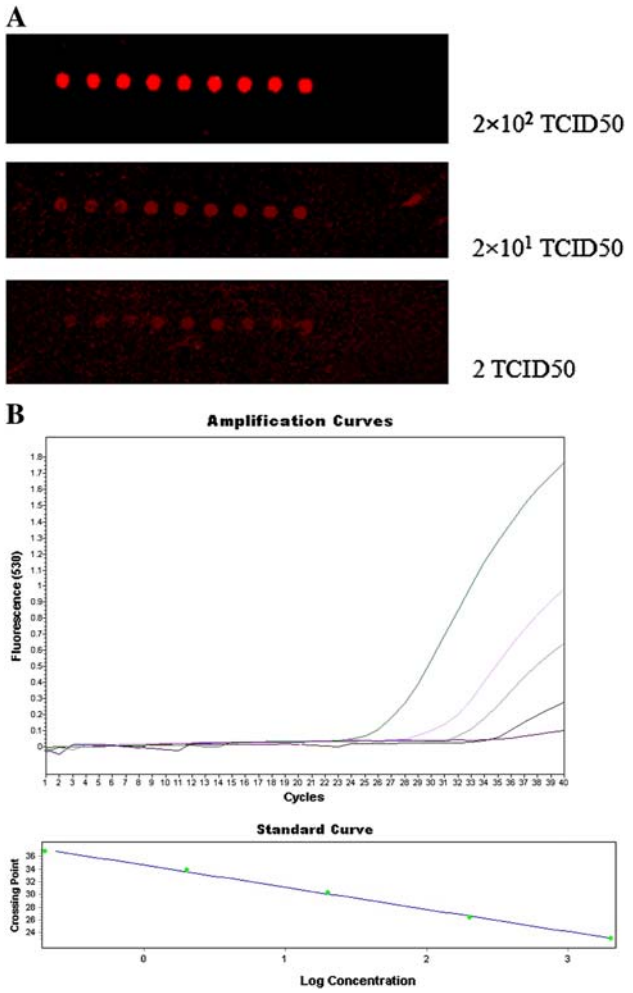

Fig. 3 Sensitivity of the microarray and real time RT-PCR assays. Serially diluted RNA template of novel H1N1 (A/Beijing/501/2009) from 2 × 103 TCID50 to 0.002 TCID50 was used for the sensitivity test. **a** Sensitivity of the microarray, the template was 2×10^2 TCID50, 2 × 10 TCID50 and 2 TCID50 respectively. **b** The amplification blot and the associated standard curve graph of real-time RT-PCR

recommended by WHO. A serial dilution of novel H1N1 (A/Beijing/501/2009) from 2×10^3 TCID50 to 0.002 TCID50 was used for the sensitivity test. The microarray had a detection limit of 2 TCID50 for the novel H1N1, while the rRT-PCR had a sensitivity of 0.2 TCID50 for the novel H1N1 (Fig. 3).

Discussion

The accurate diagnosis of novel H1N1 influenza and subtype identification for different human influenza A viruses is essential for improving patient management because the initial symptoms of the novel A H1N1 influenza are similar to those of other seasonal human influenza and infection with the highly pathogenic H5N1 avian influenza virus. Multiplex RT-PCR and multiplex real-time RT-PCR have been reported to simultaneously type and subtype different influenza viruses. However, these assays are limited by the number of pathogens they can detect, and moreover, the high similarity of primer sequences needed for these assays is difficult to achieve because of the large number of mutations within the HA and NA genes. As an alternative option, oligonucleotide microarrays have the advantages of high throughput and are less dependent on the sequences of the virus genomes, since the PCR primers used for microarrays can target more conserved regions for amplifying as many potential targets as possible. Several DNA microarrays have been developed for typing and subtyping of influenza viruses [9, 13, 15]. However, subtyping of the novel H1N1 influenza virus with other human seasonal influenza viruses (e.g. seasonal H1N1) has not been reported.

In this study, we designed 59-mer oligonucleotides for subtyping human H1N1, H3N2 and H5N1 influenza viruses. This microarray could specifically distinguish the novel H1N1 from the seasonal H1N1 influenza viruses and swine H1N1 influenza viruses. The 59-mer oligonucleotides did not require NH_2-modification or addition of a poly (T) spacer and could tolerate sequence mismatches better than shorter (15–25 mer) oligonucleotides. The tolerance of some sequence mismatches could significantly reduce the number of oligonucleotide probes needed, since each probe would potentially hybridize to several strains with high sequence similarity. The primers designed for multiplex PCR amplification were chosen for the most conserved regions of each subtype. This would also facilitate the amplification of all potential viruses with fewer primers.

As for design of oligonucleotide probes, we obtained the sequences of all of the novel 2009 influenza A H1N1 outbreak strains sequenced to date from GenBank, as well as all H1, H3, H5, N1 and N2 sequences of human influenza A viruses and swine H1N1 sequences to select the most conserved regions of each subtype. Moreover, MP genes were also included in this study to select the best consensus probes for all influenza A viruses, and these probes were employed as a positive control for influenza A viruses. The oligo probes were designed from these conserved regions and subsequently aligned with the NCBI database to ensure that each probe was unique to the respective subtype and would hybridize to the maximum number of variant sequences.

Several studies have investigated the potential of diagnostic microarrays for influenza virus detection and subtyping [5, 6, 10, 17], but all of the probes designed in these studies targeted the HA, NA and MP genes. The most recent DNA microarray (MChip) with 15 capture-oligonucleotides targeting the conserved MP segment can accurately subtype H1N1, H3N2 and H5N1 influenza A viruses [7]. In our study, highly variable hemagglutinin and

neuraminidase segments were targeted for designing subtyping oligonucleotides, and the relatively conserved matrix gene was utilized for influenza A virus positive probe design. This design would facilitate the discrimination of different subtypes by choosing the subtype oligos from the highly evolved genes, providing positive reference oligos for all influenza A viruses. The oligos could only be interpreted as positive signals when they were hybridized both to the influenza A matrix gene and a probes of a distinct subtype, which increased the diagnostic accuracy.

With this microarray, we correctly identified all of the HA and NA subtype strains. Moreover, we tested 39 clinical throat swab specimens from patients who were suspected to be infected with the novel H1N1 virus. Four of them (10.2%) were confirmed positive, which was consistent with the results of rRT-PCR assay and virus isolation.

Taken together, we have developed an oligonucleotides microarray to subtype human influenza A viruses as well as to distinguish the novel H1N1 from seasonal H1N1 and swine H1N1 influenza viruses. The array contains specific 59-mer probes for human H1, H3, H5, N1, N2 and novel H1 and N1 subtypes. We demonstrated that this array could screen and subtype the suspected novel A (H1N1) influenza virus and other influenza-like infections.

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