Human Influenza A Virus (H5N1) Detection by a Novel Multiplex PCR Typing Method[∇]

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We report the use of ResPlex III for genotyping influenza A viruses. The performance characteristics of the assay with regard to H5N1 are further evaluated. The ResPlex system incorporates a novel multiplex PCR technology, target-enriched multiplex PCR, to simultaneously amplify multiple molecular targets in one reaction. The ResPlex III assay targets the H1, H2, H3, H5, H7, H9, N1, and N2 genes from the influenza A virus as well as the NS genes from influenza A (NSA) and B (NSB) viruses, providing detection and genotyping of influenza A and B viruses. The analytical sensitivities for detecting the H5, N1, and NSA genes were 1, 10^{-1}, and 10 50% tissue culture infectious doses/200 μl/reaction, respectively. A total of 217 sequential clinical samples including 14 samples with human H5N1 infections were tested by the ResPlex III assay, and the results were compared to a reference standard combined with results of viral culture and conventional reverse transcriptase and real-time PCR. The clinical sensitivity and specificity for detecting H5N1 were 93.3% and 100%, respectively, indicating that different subtypes of influenza A virus can be quickly and correctly identified using the ResPlex III genotyping approach.

Since 1997, the H5N1 subtype of influenza A virus has been circulating in Asia, and now in European countries, causing widespread infection in multiple avian species. So far, human-to-human transmission has been suspected in only one case. If an outbreak of H5N1 occurs in humans, millions of people worldwide may die from the pandemic. Limited amounts of effective vaccines and drug treatments make the prevention and control of this disease difficult. Thus, the most effective measure for containing a potential outbreak is a large-scale quarantine of suspected patients.

To minimize the social and economic impact of a large-scale quarantine measure, a quick, accurate, and comprehensive diagnostic system is essential. Current diagnostic methods, such as culture, immunoassays, and PCR-based molecular tests, lack the ability to meet this challenge. We believe that an ideal diagnostic test for avian influenza virus pandemic surveillance and outbreak control should satisfy the following requirements: (i) high throughput, which allows the analysis of hundreds, even thousands, of samples per day; (ii) multiplex, to detect multiple relevant molecular targets using only one patient sample, conducting one experiment, in one reaction system; (iii) safety, to minimize the risk to health care providers by inactivating pathogens at the beginning of the test procedure; (iv) accuracy, to provide validated assay specificity and sensitivity; (v) speed (the entire procedure, from sample collection to result output, takes less than 5 h); and (vi) ease of use, to automate assay procedures, making it possible to

quickly establish a surveillance laboratory network without extensive training. The U.S. Food and Drug Administration has approved a real-time PCR assay for the identification of influenza A/H5 virus (Asian lineage). This diagnostic test, developed by the U.S. Centers of Disease Control and Prevention, was intended to be used in public health laboratory networks for the surveillance of H5N1 outbreaks (2). In this study, we report the development of the ResPlex III multiplex PCR assay for influenza A virus typing analysis. The analytical and clinical sensitivity and specificity of the assay were evaluated with regard to the H5N1 virus (Asian lineage).

MATERIALS AND METHODS

Patient samples and viral isolates. One important function of our National Influenza Center at the Chinese Centers for Disease Control (CDC) is to monitor outbreaks of influenza virus by collaborating with established provincial surveillance laboratories and testing samples submitted to us from provincial laboratories. From October 2005 to February 2006, The Chinese CDC received a total of 217 human respiratory samples from 16 provinces (AnHui, Liaoning, XinJiang, GuangXi, HeBei, JiangXi, FuJIan, XiZang, HuNan, ShanDong, SiChuan, JiLin, GuiZhou, HuBei, and GuangDong) to rule out avian influenza virus (H5N1). The majority of clinical samples were pharyngeal swabs (83%) or tracheal aspirates (17%). Samples were collected in 0.9% NaCl and shipped to Beijing at 4°C via express mail within 24 h of collection. If not shipped immediately, the samples were kept frozen at or below −70°C until shipping. Upon arrival, if the culture was being set up immediately, the samples were stored at 4°C; otherwise, samples were stored at or below −70°C. Viral isolation and culture were performed according to WHO guidelines (WHO/CDS/CSR/NCS/2002.5 Rev. 1; http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025.pdf). All samples were investigated with multiple methods, including viral culture, reverse transcriptase PCR (RT-PCR), and real-time PCR.

One human H5N1 viral isolate, A/Anhui/1/2005, was used for analytical sensitivity studies. The 50% tissue culture infectious dose ($TCID_{50}$) for this isolate was determined using a standard viral culture method (4). Briefly, viral isolates harvested from chicken embryo cultures were serially diluted in quadruplicate and added onto an MDCK (Madin-Darby canine kidney) cell monolayer in

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No	Samples	H1	H2	H3	H5	H7	H9	N1	N2	NSA	NSB	Reference
1	CnCDC1	24	17	53	532	17	11	455	77	123	74	H5N1
2	CnCDC2	9	5	14	590	13	12	1037	32	606	120	H5N1
3	CnCDC3	8	11	7	404	17	0	354	18	166	46	H5N1
4	CnCDC4	2	7	53	7	14	0	322	45	405	0	H5N2
5	CnCDC5	29	21	63	61	1	39	683	52	353	27	H5N1
6	CnCDC6	14	10	9	2352	11	18	1185	26	915	26	H5N1
7	CnCDC7	43	24	34	101	28	39	224	26	139	38	H5N1
8	CnCDC8	44	45	19	1112	14	19	543	40	307	43	H5N1
9	CnCDC9	6	4	16	370	18	0	257	31	137	41	H5N1
10	CnCDC10	4	19	17	1657	10	24	1500	17	811	43	H5N1
11	CnCDC11	20	8	5	837	7	20	276	42	246	44	H5N1
12	CnCDC12	27	0	36	81	0	28	381	91	316	21	H5N1
13	CnCDC13	9	7	83	147	16	0	192	140	126	12	H5N1
14	CnCDC14	23	10	91	133	8	13	228	106	188	46	H5N1
15	CnCDC15	10	2	26	27	21	25	13	21	68	1584	InfB
16	CnCDC16	15	15	9	24	30	18	31	10	31	667	InfB
17	CnCDC17	21	20	5	17	11	20	6	13	98	1348	InfB
18	CnCDC18	12	29	39	11	8	2	14	54	43	38	Negative
19	CnCDC19	28	17	17	24	7	40	11	33	46	34	Negative
20	CnCDC20	12	8	38	30	18	7	38	32	23	55	Negative
21	CnCDC21	708	16	5	16	0	13	298	9	589	97	H1N1
22	CnCDC22	807	8	9	13	13	19	342	19	611	38	H1N1
23	CnCDC23	2924	25	0	18	23	0	1792	37	3586	29	H1N1
24	CnCDC24	683	15	10	22	9	2	396	2	497	13	H1N1
25	CnCDC25	24	13	693	25	11	17	6	491	566	51	H3N2
26	CnCDC26	7	12	591	37	14	9	25	447	557	98	H3N2
27	CnCDC27	5	0	637	22	5	15	9	755	541	48	H3N2
28	CnCDC28	16	9	475	33	4	2	16	675	385	39	H3N2
29	CnCDC29	2	14	12	18	12	937	10	16	921	60	H9
30	CnCDC30	6	7	16	22	10	1044	19	0	633	34	H9
31	CnCDC31	19	12	13	40	578	9	517	12	28	65	H7N1
32	Blank1	19	17	22	18	28	24	15	11	24	65	Negative
33	Blank2	32	33	56	15	15	34	31	88	56	16	Negative
34	Blank3	14	16	12	32	35	16	20	29	21	36	Negative
	Cutoff	60	60	122	58	66	61	55	204	111	138	

FIG. 1. Representative results of ResPlex III genotyping analysis.

log-phase growth. The TCID$_{50}$ was determined according to a method described previously by Reed and Muench (7).

Determination of analytical sensitivity. The viral isolate (A/Anhui/1/2005) with a known TCID$_{50}$ was serially diluted in phosphate-buffered saline. At each concentration, three samples were prepared. Nucleic acid isolation was performed on each sample, and samples were then subjected to amplification with the ResPlex III method. The analytical sensitivity was determined to be the lowest TCID$_{50}$ at which all three duplicated samples were successfully detected.

Nucleic acid isolation and amplification with RT-PCR and real-time PCR. A QIAGEN QIAamp Viral RNA Mini kit (Valencia, CA) was used for the extraction of nucleic acid from all of the samples. The starting material for nucleic acid isolation was 200 μl of a respiratory sample (in 0.9% NaCl) or 200 μl viral isolate in dilution buffer (phosphate-buffered saline). RNA was eluted into 50 μl water.

Reference standard setup. Even though the ResPlex III assay can provide genotyping information for many influenza A virus strains, the most important intended use of this assay is to identify influenza A H5N1 virus (Asian lineage) as a confirmatory test. To evaluate the performance of ResPlex III in this regard, a reference standard was established to represent the combined results of the viral culture, RT-PCR, and real-time PCR. A sample was determined to be positive for H5N1 when at least two of the three reference methods were positive. For ResPlex III, a sample was determined to be positive for H5N1 only when all three targets (H5, N1, and NSA) were positive.

Conventional RT-PCR and real-time PCR. We have reported the development of RT-PCR and real-time PCR assays for the detection of H5N1 from human clinical specimens (9). The primer sequences used for these studies are as follows: RT-PCR primers were H5HA-F (GCCATTCCACAACATACACCC), H5HA-R (CTCCCCTGCTCATTGCTATG), N1-F (TTGCTTGGTCAGCAAG TGC), and N1-R (CAGTCACACCATTTGGATCC); real-time PCR primers were FluA-F (GACCRATCCTGTCACCTCTGAC), FluA-R (GGGCATTYTG GACAAAKCGTCTACG) FluA-p (TGCAGTCCTCGCTCACTGGGCACG), H5HA-F (TGGAAAGTGTAARAAACGGAACGT), H5HA-R (TGATTGCC AGYGGCTAGGGAACT), and H5HA-p (TGACTACCCGCAGTATTCAGAA GAAGCAAGACTAA).

Amplification and detection with target-enriched multiplex PCR (tem-PCR).

The analytical sensitivity of ResPlex III was evaluated with viral isolate strain A/Anhui/1/2005, which was established from a patient sample from Anhui Province (8). The TCID$_{50}$ were determined as described previously (4). Multiplex target amplification was carried out using the ResPlex III assay system from Genaco (Huntsville, AL) (catalog no. 011-03). The SuperPrimers from the assay system contain a mixture of specific primers capable of amplifying the hemagglutinin gene from subtypes H1, H2, H3, H5, H7, and H9 as well as the neuraminidase gene from the N1 and N2 subtypes. Amplification targets for the NS gene of influenza virus types A and B are also included in the mixture. The QIAGEN OneStep RT-PCR kit (Valencia, CA) was the enzyme and buffer system used for amplification. To set up the amplification reaction, 5 to 20 μl RNA template was mixed with 2 μl RT-PCR enzyme, 6 μl ResPlex III Super-Primers, 2 μl deoxynucleoside triphosphate mix, 10 μl 5× RT-PCR buffer, 5 to 10 units of RNase inhibitor, and RNase-free water to bring the final volume to 50 μl. Reverse transcription and amplification were carried out in a one-step reaction with a GeneAmp PCR system 9700 (Applied Biosystems) by using the specific cycling profile described in the Genaco protocol. A nontarget amplification control was included in each experiment.

After PCR amplification, 5 μl PCR product was directly mixed with 35 μl of Genaco detection buffer and 10 μl of Genaco ResPlex III bead mix. Hybridization was carried out at 52°C for 10 min. For detection, 10 μl of diluted streptavidin-phycoerythrin was added followed by an additional 5-min incubation at 52°C. Finally, 120 μl of prewarmed Genaco stopping buffer was added to the sample prior to detection on a Luminex instrument. The Luminex xMAP technology was described elsewhere previously (3).

RESULTS

Figure 1 shows representative results of a ResPlex III analysis. Each column represents a specific target, and each row represents a sample. The first 20 samples (samples 1 to 20) were clinical samples, including 14 human H5N1 samples iden-

TABLE 1. Analytical sensitivity of the ResPlex III assay for detecting H5N1 genotypes

Genotype	No. of positive samples/no. of samples tested with $TCID_{50}/200$ μl/reaction mixture of:									Analytical sensitivity ($TCID_{50}/200$ μl/ reaction mixture)
	100	10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	0	
H5	3/3	3/3	3/3	1/3	0/3	0/3	0/3	0/3	0/3	1
N1	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	10^{-1}
NSA	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	10

tified by the reference methods. Samples 21 to 31 were not clinical samples but were viral isolates. These samples were used to evaluate the usefulness of ResPlex III in detecting other H and N targets. The last three samples (samples 32 to 34) were blanks without any template in the reaction. The median fluorescent intensity of each target from these three blank samples represented the background. The cutoff values for determining the positive signal were determined specifically for each target. The cutoffs were calculated as the mean median fluorescent intensity plus four times the standard deviation of the blank samples. Positive results are indicated in Fig. 1.

Table 1 shows the analytical sensitivity of the ResPlex III assay. The analytical sensitivity was then determined by using serially diluted viral stocks. As shown in Table 1, the analytical sensitivity for H5, N1, and influenza A virus type-specific NS genes are 1 $TCID_{50}$, 10^{-1} $TCID_{50}$, and 10 $TCID_{50}$, respectively, per reaction mixture.

Table 2 shows the performance characteristics of the ResPlex III assay by comparing it against the reference standard. A total of 217 clinical samples were sent to the Chinese CDC during the study period and were included in the study. Among them, the reference methods identified 14 human H5N1-positive samples, while the ResPlex III assay identified 13 samples as being positive for H5N1. The assay's sensitivity, specificity, positive predictive value, and negative predictive value were calculated to be 93.3%, 100%, 100%, and 99.5%, respectively.

DISCUSSION

For the 13 H5N1-positive clinical samples identified by the ResPlex III assay (Fig. 1 and Table 2), positive results were also obtained by both the RT-PCR and real-time PCR assays. Viral isolation, on the other hand, failed in 3 of the 14 cases. Sample CnCDC4 was positive for the N1 and NSA target but negative for H5. Sequence analysis showed that a 1-bp mutation occurred at the 3′ end of the inside reverse primer for the H5 gene. This result suggested that the ResPlex III assay is vulnerable to viral mutations, even though primers were de-

signed by selecting conserved sequences. To reduce false-negative results, we need to monitor the viral mutations closely and add new primers and probes to adapt to the changes. Alternatively, the ResPlex III assay should amplify more than one target from the H5 gene to provide additional assurance and reduce false-negative results.

The principle of tem-PCR technology was described previously (1, 5, 6). Conventional multiplex PCR assays are difficult to develop because of three primary problems: incompatible primer sets, high background amplification, and poor reproducibility. The tem-PCR technology developed by Genaco successfully addresses these problems, improving and expanding the possibilities for multiplex PCR assay development.

For each target in the multiplex PCR, nested gene-specific primers were designed and included in the reaction (Fo [forward out], Fi [forward in], Ri [reverse in], and Ro [reverse out]). These primers are used at extremely low concentrations and are used only to enrich the targets during the first few cycles of PCR. The inside gene-specific primers have tag sequences that can be recognized by a universal set of primers, called SuperPrimers. Only the SuperPrimers are included at a concentration necessary for exponential amplification, and only the reverse SuperPrimer is labeled with biotin. Labeled PCR products are detected with a complementary capture probe that is covalently coupled to a color-coded bead. The concentration of the reverse SuperPrimer is higher than that of the forward SuperPrimer; the asymmetric PCR yields more reverse strand for detection. This also eliminated the need to denature PCR products prior to hybridization.

tem-PCR works because it addresses three of the most difficult problems inherent in multiplex PCR development. First, in a conventional multiplex PCR, each primer may require a different optimal annealing temperature or buffer formula. When the number of targets increases, it forces all of the primer sets to work under a single amplification profile, causing multiplex PCR to become difficult under these standardized conditions. With tem-PCR, during the enrichment stage, nested primers are used for each target. This design gives rise

TABLE 2. Performance of ResPlex III assay compared with the reference standard for detecting H5N1

ResPlex III result	Reference standard (no.)[a]			Performance characteristic (%)[b]			
	Positive	Negative	Total	Sensitivity	Specificity	PPV	NPV
ResPlex III				93.3	100	100	99.5
Positive	13	0	13				
Negative	1	203	204				
Total	14	203	217				

[a] The reference standard is the combined results of viral culture, RT-PCR, and real-time PCR.
[b] PPV, positive predictive value; NPV, negative predictive value.

to four possible forward and reverse primer combinations for amplification. Each combination may have its own optimum amplification profile, but given four amplification opportunities, a common condition satisfying all targets can be attained.

Second, conventional multiplex PCR uses multiple sets of high-concentration, labeled primers. These primers can associate with one another to form dimers or generate nonspecific background amplification. Reduced amplification efficiency can also occur because excess primers occupy active sites on the polymerase molecule. In addition, unused, labeled primers produce background signal and use up reagents during the detection portion of the assay. Because of these issues, post-PCR cleanup (such as spin column purification) is often required to remove these labeled primers before the products can be used as probes. These problems are unnecessary, because high-concentration primers are required only for the last cycles of a PCR. With tem-PCR, the amount of gene-specific primers used is only enough to "enrich" the targets and incorporate the SuperPrimer tag into the products. After enrichment and tag incorporation, exponential amplification is carried out with only one pair of primers. Because only one primer is labeled, the background is low; therefore, no post-PCR cleanup is required. tem-PCR is also very specific and sensitive. No posthybridization washes are necessary. These features make it feasible to fully automate laboratory procedures and perform high-throughput clinical studies.

Finally, a conventional multiplex PCR product is difficult to produce. Maintaining the quality of all biotin-labeled primers in a mix is a difficult task. Lot-to-lot variations often limit the performance of a multiplex reaction and require repeated optimization and adjustment. With tem-PCR, only a small amount of target-specific primers is used, and only one biotin-labeled primer is included; therefore, lot-to-lot variations are small, and quality control is more manageable.

The ResPlex III assay uses the Luminex instrument for detecting PCR products. The hybridization step requires the opening of PCR tubes and mixing PCR products with the bead mix. This step is prone to carryover contamination, and strict preventive procedures need to be established.

We have evaluated the performance of the ResPlex III assay using viral isolates and clinical samples. The assay is very easy to use and rapid. Up to 96 samples can be processed together. The test turnaround time is about 5 h, starting from sample preparation to obtaining the results, and the hands-on time is less than 1 h. We believe that the ResPlex III assay can be used as a medium-throughput confirmative test for those samples suspected to be H5N1 (Asian lineage).

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