A Simple Method for Molecular Detection of Swine-Origin and Human-Origin Influenza A Virus

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Abstract

Here we present a real-time one-step reverse transcriptase–polymerase chain reaction SYBR Green assay derived from the method reported by van Elden and collaborators (2001) that ensures the rapid, sensitive, and cost-effective detection of both seasonal influenza A virus and emerging (H1N1) swine-origin influenza A virus (S-OIV). In addition to this screening test, which successfully detected both seasonal influenza A virus and S-OIV in human clinical samples, we showed that the probe initially designed by van Elden and collaborators could detect seasonal influenza A virus, but not S-OIV; a new probe was designed and tested that specifically detects S-OIV, but not seasonal influenza A. Both probe-based assays were validated by testing human clinical samples and specifically detected either seasonal influenza virus or S-OIV. Finally, *in silico* analysis of databases predicted that minor modifications of the van Elden primers would facilitate the use of this assay for the broad spectrum detection of all currently characterized variants of influenza A virus, including avian strains.

Key Words: Arbovirus(es)—Chikungunya—Dengue virus—Tick-borne encephalitis—Vector-borne.

MOLECULAR DETECTION OF INFLUENZA A VIRUSES, in the form of real-time reverse transcriptase–polymerase chain reaction (RT-PCR), has been broadly adopted in medical diagnostic laboratories (Brown 2006), and thus routine diagnosis now includes these molecular techniques. However, the detection methods are directed at classical seasonal influenza viruses, currently, human H3N2 and H1N1, but not at the detection of avian or porcine viruses, which are rarely encountered in human medicine. Consequently, a large number of specific tests have recently been proposed for the detection of H5N1 avian viruses, after the significant emergence of human cases. However, assays designed to detect a large range of viral subtypes are rarely used for routine diagnosis. Nevertheless, such assays do exist and provided that they are precisely evaluated, merit more frequent use. Daum and collaborators (2007) proposed a panel of highly sensitive typing and subtyping protocols in a real-time RT-PCR format, and suggested that these tests offer significant utility in the context of a pandemic influenza outbreak. Other assays have been proposed (Whiley and Sloots 2005, Valle et al. 2006, Karlsson et al. 2007, Li et al. 2008), including

commercial kits (Gharabaghi et al. 2008) and tests derived from subtyping assays taking advantage of conserved nucleotide regions in the hemagglutinin gene (Gall et al. 2008, Yacoub et al. 2009).

Since 2002, we have been using a real-time PCR assay designed by van Elden and collaborators (2001) for the routine molecular detection of influenza A virus. In this test, a 189 nt fragment within the Matrix gene of the virus is amplified using the INFA-1 and INFA-23 primers and hybridized with the INFA-specific probe (see Fig. 1) using a two-step RT-PCR protocol (i.e., a reverse transcription reaction using random hexaprimers, followed by a real-time PCR reaction). This assay has proved to be robust and able to detect human influenza virus subtypes H1N1 and H3N2 in clinical samples. Moreover, it can be combined with the simultaneous detection of influenza B virus in a multiplex assay (van Elden et al. 2001).

The recent spread of the H1N1 swine-origin influenza A virus (S-OIV) led us to reconsider this assay. The Matrix gene of S-OIV is of porcine origin, and genetic analysis of sequences available in GenBank revealed an ~13% nt divergence

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In addition to this test, one-step real-time RT-PCR assays were performed using either the probe designed by van Elden and collaborators, or the equivalent probe (S-INFA) designed from the sequences available for S-OIV (see Fig. 1) and the following protocol. Amplification: Genetic amplification was performed in a one-step RT-PCR format and a 25 μ L final volume, using 5 μ L of RNA extract, 1 μ L of each 10 μ M primer solution, 0.4 μ L of 10 μ M probe solution, and the Super Script III Platinum One step qRT PCR System (Invitrogen, Cergy Pontoise, France). Two reactions were conducted using the Mx3005P QPCR System thermocycler and the following thermal profile. Segment 1: 48°C, 30 min; segment 2: 95°C,

A

Forward primer INFA-1 (panINFA-1)

Avian strains, N=3111
Human strains, N=2461
Swine strains**, N=344
Other strains, N=168
S-OIV strains, N=57

G	G	A	C	T	G	C	A	G	C	G	T	A	G	A	C	G	C	(N)	T	T
1	0	3	2	1	31	0	0	36	1	0	2	0	9	3	7	5	684	(0)	0	0
0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	4	(0)	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	(0)	0	0
0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	4	(0)	0	0
0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	(0)	0	0

Reverse primer* INFA-23 (panINFA-23)

Avian strains, N=3111
Human strains, N=2461
Swine strains**, N=344
Other strains, N=168
S-OIV strains, N=57

A	T	G	G	G	C (N)	C	T	C	A	T	A	T	A	C	A	A	C (Y)	A	G	R	A	T	G
0	0	0	0	0	2029 (0)	5	0	154	6	3	51	0	0	56	2	5	133 (3)	66	20	1	3	3	4
0	0	1	0	0	631 (0)	2	0	14	0	0	11	2	0	18	0	1	1234 (0)	1	1	0	0	0	1
0	0	0	0	0	1 38 (0)	0	1	17	1	0	8	0	0	17	0	0	42 (0)	21	0	0	0	0	0
0	0	0	0	0	93 (0)	0	0	50	0	0	1	0	0	1	0	0	1 (0)	5	0	0	0	0	0
0	0	0	0	0	1 (0)	0	1	1	0	0	0	0	0	2	0	0	0 (0)	0	0	0	0	0	0

Bstrains with >2 mismatches in the complete primer
INFA-1 (panINFA-1) INFA-23 (panINFA-23)

Avian strains, N=3111
Human strains, N=2461
Swine strains, N=344
Other strains, N=168
S-OIV strains, N=57

3	0	294	54
0	0	2	0
0	0	19	0
0	0	3	0
0	0	0	0

strains with >1 mismatches in the 6 terminal 3' nt
INFA-1 (panINFA-1) INFA-23 (panINFA-23)

11	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0

FIG. 2. (A) Number of nt mismatches observed between the primers used and sequences deposited in the Influenza Resource database for influenza A viruses of different origins. The sequence corresponding to the reverse primers is presented in the polarity of genomic sequence deposited in databases (reverse complement of the primer used for genetic amplification). **Swine strains do not include S-OIV strains, which are presented separately. The six terminal 3' positions of the primers (essential for a correct hybridization and initiation of polymerization) are in boxes. Nucleotides in parentheses correspond to modifications of sequences proposed for primers panINFA-1 and panINFA-23. Modifications were proposed only for variable positions among the six last 3' terminal nucleotides of each primer. The number of mismatches observed after introduction of these modifications are also indicated in parentheses. (B) Number of strains with a significant number of mismatches observed in the complete primers or in the six terminal 3' positions of the primers.

2 min; segment 3: 95°C, 15 s; 60°C, 1 min (45×). One reaction included the INFA-1/INFA-23 primer set and the INFA probe for the detection of the seasonal influenza A virus; the other included the same primers and the S-INFA probe for the detection of S-OIV. Synthetic RNA transcripts specific for human seasonal influenza A virus and S-OIV were used as positive controls for RT-PCR amplification.

Using both synthetic RNA transcripts and human samples positive for either H3N2 or S-OIV, it could be shown (see Fig. 3)

(i) that the van Elden and S-OIV probes could efficiently detect the H3N2 and S-OIV, respectively, but (ii) that the van Elden probe could not detect S-OIV, while the S-OIV probe could not detect seasonal influenza virus. A probe designed to detect specifically the synthetic positive control (NotI-P) was used to rule out the possibility of accidental amplification of the control.

Altogether, these results suggest that this SYBR Green one-step real-time RT-PCR assay is a simple, inexpensive, but very effective tool for screening clinical samples for the presence of

FIG. 3. Real-time reverse transcriptase-polymerase chain reaction amplification of synthetic controls and clinical samples. First line shows SYBR dissociation curves for influenza virus synthetic controls, H3N2-positive and S-OIV-positive clinical samples, and MS2 control. Second line shows specific probe detection of H3N2 and S-OIV in clinical samples using INFA and S-INFA probes that of S-OIV synthetic control using NotI-P probe (system designed for the detection of incidental contamination by synthetic controls).

influenza A virus. Most importantly, positive samples may be secondarily tested using the same primers and the van Elden (INFA) and S-OIV (S-INFA) probes to discriminate between seasonal and S-OIV. It should also be noted that the direct sequencing of the products resulting from the SYBR Green assay produced the definitive differentiation of seasonal and S-OIV strains.

In addition to this initial study, which provides a rapid and pragmatic solution to the detection of the newly emerging S-OIV, we analyzed in depth:

- (i) 6084 influenza A Matrix sequences available in the Influenza Virus Resource database (www.ncbi.nlm.nih.gov/genomes/flu/flu.html) for which hemagglutinin and neuraminidase subtyping was available. This data set included 3111 avian strains [H1N1 (73), H1N2 (5), H1N3 (4), H1N5 (2), H1N6 (4), H1N9 (1), H2N1 (14), H2N2 (8), H2N3 (25), H2N4 (1), H2N5 (10), H2N7 (7), H2N8 (4), H2N9 (14), H3N1 (8), H3N2 (57), H3N3 (5), H3N4 (3), H3N5 (7), H3N6 (44), H3N7 (3), H3N8 (96), H3N9 (2), H4N1 (3), H4N2 (15), H4N3 (5), H4N4 (2), H4N5 (2), H4N6 (91), H4N7 (1), H4N8 (35), H4N9 (8), H5N1 (980), H5N2 (126), H5N3 (50), H5N4 (4), H5N6 (1), H5N7 (6), H5N8 (4), H5N9 (5), H6N1 (145), H6N2 (92), H6N3 (4), H6N4 (6), H6N5 (19), H6N6 (9), H6N8 (47), H6N9 (2), H7N1 (43), H7N2 (121), H7N3 (91), H7N4 (4), H7N5 (2), H7N7 (29), H7N8 (4), H7N9 (4), H8N4 (18), H9N1 (6), H9N2 (524), H9N5 (4), H9N6 (2), H9N8 (2), H9N9 (1), H10N1 (2), H10N2 (2), H10N3 (3), H10N4 (3), H10N5 (6), H10N6 (2), H10N7 (37), H10N8 (6), H10N9 (2), H11N1 (8), H11N2 (9), H11N3 (7), H11N4 (1), H11N6 (5), H11N8 (2), H11N9 (42), H12N1 (1), H12N2 (1), H12N4 (3), H12N5 (23), H12N8 (1), H12N9 (2), H13N2 (5), H13N3 (1), H13N6 (11), H13N8 (1), H13N9 (3), H14N5 (1), H14N6 (1), H15N9 (1), H16N3 (5)], 2461 human strains [H1N1 (696), H1N2 (35), H2N2 (76), H3N2 (1532), H5N1 (119), H7N2 (1), H7N7 (2)], 344 porcine strains [H1N1 (158), H1N2 (56), H3N1 (2), H3N2 (92), H3N3 (2), H4N6 (2), H5N1 (5), H5N2 (1), H9N2 (26)], and 168 strains of diverse origin (environment, horse, tiger, leopard, cat, civet, musk rat, mink, etc.) [H11N6 (1), H11N8 (2), H11N9 (1), H1N1 (1), H3N2 (3), H3N6 (3), H3N8 (80), H4N6 (2), H5N1 (18), H5N2 (7), H7N2 (47), H7N3 (2), H7N7 (1)];
- (ii) 57 S-OIV influenza A Matrix sequences available in GenBank.

The performance of the van Elden primers was evaluated *in silico* for each group of influenza A viruses (see Fig. 2). This analysis revealed that these primers are indeed highly appropriate for the detection of human seasonal influenza A virus and for most porcine strains, including the newly emerging S-OIV. However, minor but important modifications among the six 3' terminal nucleotides of each primer would ensure the detection of viruses from other groups, in particular avian viruses (see Fig. 2B). Modified primers (panINFA-1 and panINFA-23) were predicted to be capable of detecting all Matrix sequences available to date, including strains of avian (including H5N1 viruses) and porcine (including H1N1 viruses) origin, irrespective of hemagglutinin and neuraminidase subtypes.

In conclusion, we propose a simple and cost-effective amplification protocol for the detection of both seasonal influenza

virus and newly emerging S-OIV strains. Its effectiveness relies on tried and tested amplification primers that were shown to be extremely reliable for the screening of clinical samples and on actual detection of both seasonal and H1N1 S-OIV in human clinical samples. *In silico* analysis predicts that minor modifications of these primers would allow the use of this assay for the broad spectrum detection of all currently known variants of influenza A virus, including avian strains.

Disclosure Statement

No competing financial interests exist.

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