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Short communication

Detection of pandemic influenza A H1N1 virus by multiplex reverse transcription-PCR with a GeXP analyzer

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A B S T R A C T

A novel application of the GeXP genetic analysis system for the differential detection of pandemic influenza A H1N1 from seasonal influenza A H1N1 and H3N2 is described. The assay was evaluated using identified influenza viruses and clinical samples. The results indicate that the assay is both highly sensitive and specific for the detection of the pandemic influenza A H1N1 virus with a detection limit of 10 copies per reaction superior to that of assays in use currently. The assay is able to detect potential mixed infections. This technique has the potential to provide both a powerful method to enhance surveillance of influenza and a platform for investigating the differentiation of other similar pathogens.

The emergence of a novel strain of influenza virus A H1N1 in April 2009 and the subsequent rapid global pandemic have focused attention on the methods for influenza surveillance worldwide (Fraser et al., 2009; Ortiz et al., 2009; Lipsitch et al., 2009). The methods for the accurate diagnosis and tracking of this novel pathogen are required to develop appropriate management strategies to reduce morbidity and mortality (de Wit and Fouchier, 2008; Gallaher, 2009; Tumpey and Belser, 2009). Some conventional and real-time RT-PCR protocols have been published recently for the detection of this virus (Dawood et al., 2009; Leo et al., 2009; Carr et al., 2009), but few of these can be used to detect simultaneously seasonal influenza A H1N1, H3N2 and pandemic influenza A H1N1 viruses in patients presenting with influenza-like illness by a single assay.

The Beckman Coulter (Brea, CA, USA) GenomeLab Gene Expression Profiler (GeXP) genetic analysis system was designed originally to allow for a high throughput, robust, and differential assessment of a multiplexed expression profile of up to 35 genes by a single RT-PCR (Alex et al., 2009). The analytical procedure includes a modified reverse transcription and PCR amplification followed by capillary electrophoresis separation based on the size of the amplified product (Fig. 1). The strength of the GeXP system lies in its improved sensitivity and specificity compared to other platforms. The combination of chimeric primer-based fluorescent-labeled PCR amplification and capillary electrophoresis separation minimizes non-specific reactions. Each of the intended targets is examined at both the hybridization and electrophoresis separation steps to confirm their identity.

The Center for Disease Control (USCDC, Atlanta, USA) and the China Center for Disease Control (CCDC) have provided a detailed method for real-time RT-PCR (qRT-PCR) amplification and detection of the pandemic influenza A H1N1 virus (Dawood et al., 2009). Currently, the real-time PCR diagnostic kits from both CCDC and US CDC are distributed domestically for the routine influenza surveillance network. However, this method has elaborate and complicated assay procedures, which involve four independent real-time PCR. The development of a novel application of GeXP genetic analysis system is described in which 8-plex reverse transcription-PCR is performed for differential detection of pandemic influenza A H1N1 from seasonal influenza A H1N1 and H3N2 viruses by a single assay.

Field isolates of human seasonal influenza A viruses including H1N1, H3N2, and influenza B virus from the China National Influenza Center, a part of the CCDC, were used as control viruses to evaluate the specificity of the GeXP assay. All isolates were verified previously by RT-PCR and sequencing. A/California/07/2009(H1N1), the circulating virus of this pandemic, from the US CDC was used as the reference virus. Throat swabs collected from 20 patients with seasonal influenza A H3N2 virus infection, confirmed previously by a single H3N2 q-RT PCR test kit

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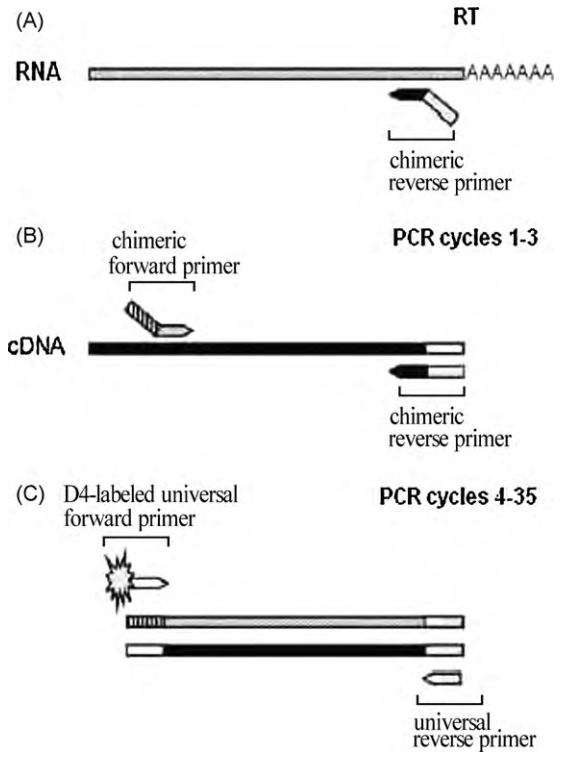

Fig. 1. GeXP multiplex RT-PCR strategy. (A) Extracted RNA was reverse transcribed using up to 35 chimeric reverse primers, each containing 20 nucleotides complementary to the target gene (solid black) coupled with a 19 nucleotide universal reverse sequence (solid gray). (B) The cDNA was PCR-amplified in cycles 1–3 using the specific sequence of the chimeric primers to synthesize up to 35 gene-amplification products, each of which contained universal tags at both termini. (C) Subsequent PCR amplifications (cycles 4–35) using universal forward and reverse primers to yield D4 fluorescent-labeled amplification products corresponding to each of the 35 specific genes tested.

from the CCDC, and 33 diagnosed cases of pandemic influenza A H1N1 infection, confirmed previously by qRT-PCR (US CDC test kit), from the Beijing CDC were included in this study.

The chimeric primers consist of a gene-specific sequence fused to a universal sequence at the 5′ end. The gene-specific portions of the primers were designed based on the sequence information obtained from the Influenza Sequence Database (http://www.flu.lanl.gov). All available sequences of human H1, H2, H3, B influenza viruses, swine H1N1 and pandemic influenza A H1N1 viruses from the database were analyzed comparatively. Three sets of primers were designed to correspond to the conserved regions of the HA gene segment for each of the HA types including pandemic H1N1, seasonal H1N1, seasonal H3N2 and two sets of primers for the HA gene of H1N1. One set of primers targeting the conserved regions of the matrix gene segment were designed to differentiate between human influenza A and B viruses. Two sets of primers were designed for the DNA and RNA quality control in clinical samples. All primers were HPLC purified (Table 1).

To test the specificity of the established GeXP assay, total RNA was extracted from 140 μl of various control viruses (HA titers: 1:32–1:1024) and the reference virus using the commercial QIAampViral RNA Mini Kit (Qiagen, Hilden, Germany). The extracts were centrifuged at 6000 × *g* for 1 min and the pellets were suspended in 50 μl of distilled water. The GenomeLab GeXP Start Kit (Beckman Coulter, Brea, CA, USA) was used for RT-PCR. Briefly, 20 μl of RT-PCR mixture, containing 5 μl of resuspended RNA and 0.05 μM of reverse primer set mix, was incubated at 48 °C for 1 min, 42 °C for 60 min and 95 °C for 5 min in a thermal cycler (Applied Biosystem, Foster City, CA, USA). Subsequently, a PCR, using 9.3 μl of RT

Table 1
GeXP primers designed for differential detection of pandemic influenza A H1N1.

Target virus	Forward primer (5′ → 3′)[a]	Reverse primer (5′ → 3′)[a]	Amplicon size with tags (bp)
Human RNase P_DNA	AGGTGACACTATAGAATAGAGGCCTGGCTTTTGAACTT[b]	GTACGACTCACTATAGGGAATCAAATTGAGGGCACTGGA[b]	125
Influenza A (FluA)	AGGTGACACTATAGAATAGARTGCCTAAAGACAAGACCAAT[c]	GTACGACTCACTATAGGGAGCTGCAGTCCTCGCTCACT[b]	134
Human RNase P_RNA	AGGTGACACTATAGAATAATGGCCGTGTTTGCAGAT[b]	GTACGACTCACTATAGGGATGATAGCAACAACTGAATAGCCA[c]	143
Pandemic H1N1 (novel H1)	AGGTGACACTATAGAATAGCATTCGCAATGGAAAGAAA[c]	GTACGACTCACTATAGGGATCCTCAATCCTGTGGCCAG[b]	236
Seasonal H1N1 (sH1N1)	AGGTGACACTATAGAATAGGTATGCTTTTGCAMTGARTAGAGG[c]	GTACGACTCACTATAGGGAAAGGGATATTCCTTARTCCTGTARCCAT[b]	250
Seasonal H3N2 (sH3N2)	AGGTGACACTATAGAATACAAATTGAAGTKACTAATGCTACTGAG[c]	GTACGACTCACTATAGGGATAGTGACCTAAGGCAGGCATAATC[b]	274
General H1N1 (gH1N1)	AGGTGACACTATAGAATAGCDGAYCAAAARAGCACACAAAAT[c]	GTACGACTCACTATAGGGACCARAGTTCTTTCATTTTCYAAT[b]	257
	AGGTGACACTATAGAATAGCSGACCWRAAGAGCACACAGAAT[c]	GTACGACTCACTATAGGGACCAAAGTCCTTTCATTTTCCAGT[b]	

Underlined oligonucleotides are universal sequences.
[a] Degenerate primer abbreviations are as follows: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; D, A/G/T.
[b] Primer is in the PCR primer mix.
[c] Primer is in the RT primer mix.

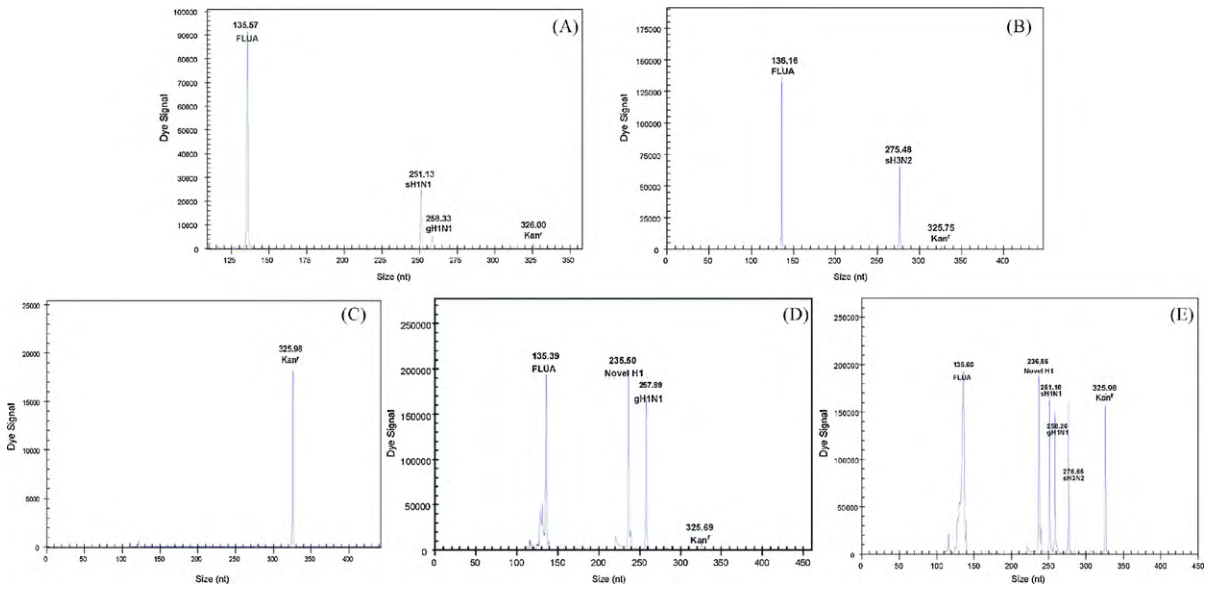

Fig. 2. Specificity analyses of GeXP detection of the pandemic influenza A H1N1 HA gene with multiplex primers. The GeXP assay was carried out using the following control and reference viruses: (A) human seasonal influenza A H1N1, (B) human seasonal influenza A H3N2, (C) human influenza B, (D) human pandemic influenza A H1N1 and (E) a mixture of seasonal influenza A H1N1, H3N2, pandemic influenza A H1N1. D4-labeled PCR products were separated by capillary electrophoresis and detected by fluorescence spectrophotometry given as a dye signal in arbitrary units on the *y*-axis. Each peak was identified by comparing the expected to actual size of the PCR product (*x*-axis). Peaks corresponding to general influenza A H1N1, seasonal influenza A H1N1, seasonal influenza A H3N2, pandemic influenza A H1N1 and the internal control, kanamycin-resistance gene, were detected accordingly.

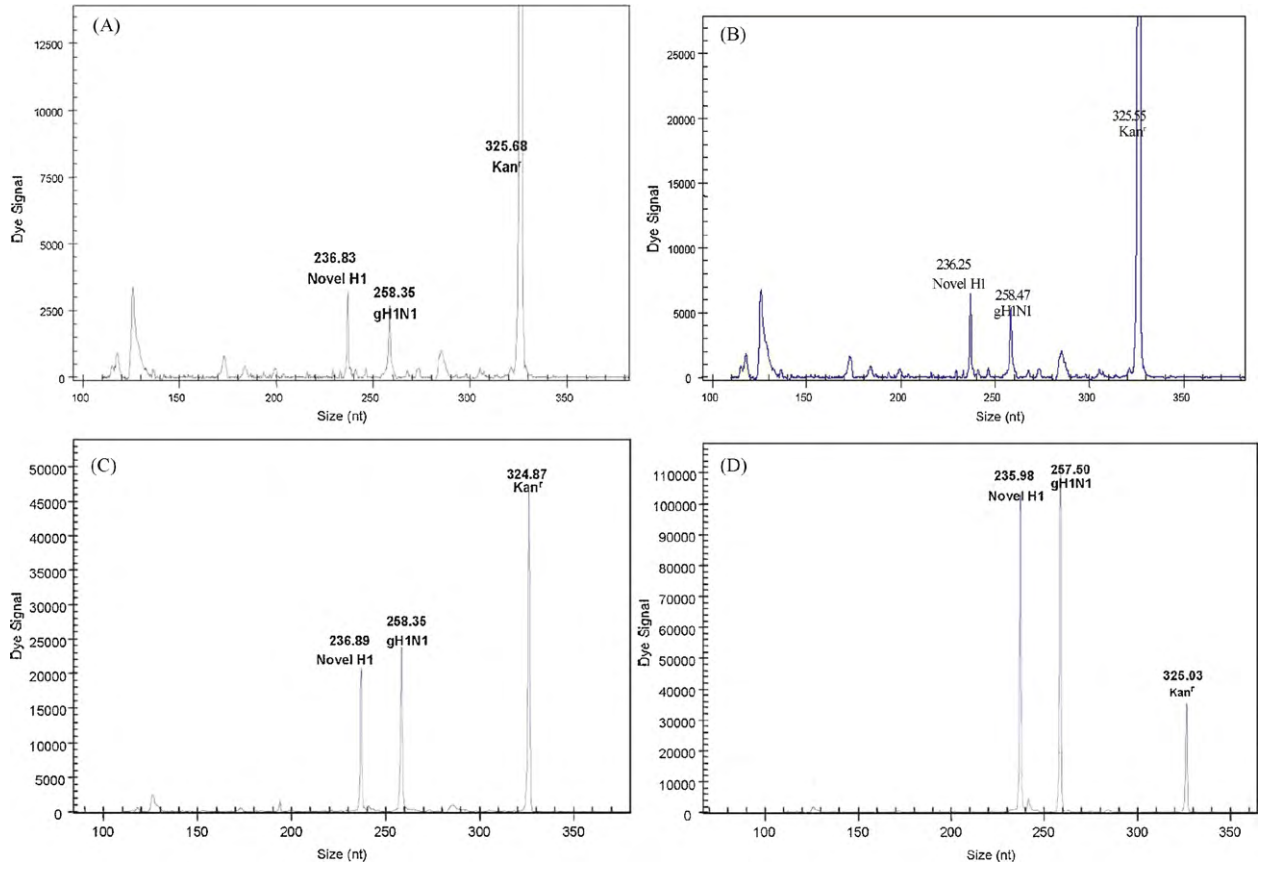

Fig. 3. Sensitivity analyses of GeXP detection of the pandemic influenza A H1N1 HA gene with multiplex primers. The GeXP assay was carried out using different concentrations of RNA transcripts with (A) 10 copies of RNA, (B) 100 copies of RNA, (C) 1000 copies of RNA, and (D) 10,000 copies of RNA per reaction transcribed *in vitro* from the recombinant plasmid pMD20 containing the full HA gene of human pandemic influenza A H1N1. D4-labeled PCR products were separated by capillary electrophoresis and detected by fluorescence spectrophotometry given as a dye signal in arbitrary units on the *y*-axis. Each peak was identified by comparing the expected to actual size of the PCR product (*x*-axis). Peaks corresponding to pandemic influenza A H1N1 and the internal control, kanamycin-resistance gene, were detected. The reaction at each concentration of the template was performed in triplicate and similar results were obtained each time (CV $\leq$ 10.7% for each concentration).

reaction and 0.02 μM forward primer set mix, was conducted at 95 °C for 15 min followed by 35 two-step cycles of 94 °C for 30 s and 55 °C for 30 s. The GeXP system was then used to separate the PCR products and analyse the data following the manufacturer's instructions. Each PCR amplicon labeled fluorescently was evaluated in two steps. First, the length or size of the amplicon was determined by the GeXP software, compared to known product sizes and then identified. Secondly, the fluorescent dye signal strength of the optical fluorescence for each PCR product was measured in arbitrary units (A.U.) and normalized to an internal control, a kanamycin-resistance RNA transcript. The GeXP assay, using multiple virus-specific primers, detected correctly and identified the presence of the pandemic influenza A H1N1, seasonal influenza A H1N1, H3N2 and mixtures of seasonal influenza A H1N1, H3N2 with pandemic influenza A H1N1. The PCR product corresponding to human influenza B virus was not detected (Fig. 2A–E).

To determine the detection limit of the GeXP assay, quantitative RNA transcripts were transcribed *in vitro* from the recombinant plasmid pMD20 containing the full HA gene of the reference virus with a commercial Riboprobe combination system-SP6/T7 kit (Promega, Madison, USA). A panel of RNAs with concentrations ranging from 1 to 10,000 copies per assay was prepared. The detection limit of the HA gene was 10 copies per reaction (Fig. 3A–D), which was superior to that of the qRT-PCR diagnostic kits supplied by both the CCDC and the US CDC using the same RNA panel as the template (data not shown). The reaction at each concentration of the template was performed in triplicate and similar results were obtained each time (CV $\leq$ 10.7% for each concentration).

Finally, the GeXP assay was evaluated using 53 throat swab specimens, including 33 confirmed cases of pandemic influenza A H1N1 virus infection from the Beijing CDC and 20 cases of seasonal influenza H3N2 infection from the CCDC. The results confirmed 33 out of 33 cases of pandemic H1N1 and 20 out of 20 cases of seasonal influenza H3N2. In addition, the GeXP assay was able to detect co-infections of pooled pandemic H1N1 and seasonal influenza H3N2 throat swab specimens. The normalized A.U. for samples was in the range of 9000–100,000, while the normalized A.U. for the negative control was less than 2000.

These results imply that the multiple GeXP is a sensitive and cost-effective assay for infection with the pandemic influenza A H1N1 virus and is able to detect seasonal influenza H3N2 co-infection, which the current qRT-PCR diagnostic kits used by the influenza surveillance network do not identify. The GeXP does not provide real-time analysis, but instead provides endpoint PCR readouts using multiplexed reactions by a single analysis for up to 35 targets. Further investigation and a larger sample are needed to confirm its value and modify the procedures to reduce the number of steps required. This proof of concept study indicates the potential use of a powerful method to enhance surveillance of influenza.

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