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Detection of seasonal H3N2 influenza A virus by type-specific TaqMan minor groove binder probe assay

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Abstract

Despite the emergence of the pandemic H1N1 influenza A virus in 2009, seasonal H3N2 viruses continue to co-circulate in the population, and may even predominate in the coming influenza season. We describe a specific minor groove binder Taqman assay for H3N2 viruses with a detection limit of 16.5 standard DNA copies.

Influenza A virus infection, as endemic ‘seasonal’ influenza or pandemic influenza, remains a significant public health threat (Morens et al., 2009). Human seasonal H3N2 influenza viruses are derived from the 1968–1969 pandemic that killed about one million people worldwide (Cox and Subbarao, 2000). Even with the re-emergence of seasonal H1N1 viruses in 1977 and their co-circulation with H3N2 viruses since then, H3N2 influenza viruses have been the dominant strains during most influenza seasons, and they have been the major cause of human influenza morbidity and mortality worldwide (Russell et al., 2008). While the 2009 pandemic H1N1 virus quickly became the dominant influenza A virus subtype soon after its emergence in early spring 2009 (Morens et al., 2010), H3N2 viruses continued to be isolated in the Southern Hemisphere during the 2009 influenza season, and the number of reports of seasonal H3N2 isolates started to increase again in the U.S. from late June and July, 2010 (<http://www.cdc.gov/flu/whatsnew.htm>). This correlates with recent worldwide surveillance data showing that H3N2 viruses currently are the dominant influenza A virus subtype being isolated in China and Chile (<http://www.cdc.gov/flu/international/activity.htm>). With the transition from the pattern of pandemic 2009 H1N1 to a mixed seasonal influenza A virus circulation pattern, H3N2 viruses may once again become the dominant subtype (Morens et al., 2010).

The co-circulation of influenza A viruses of seasonal H3N2, H1N1 and 2009 pandemic H1N1 strains, each with different antiviral sensitivity patterns (Layne et al., 2009), and the continuing risk of human infection with the highly pathogenic avian influenza A (HPAI) H5N1 virus, further stresses the need for rapid diagnostic tests that provide subtype-specific information, both for the initiation of appropriate therapy and for ongoing surveillance efforts. After successfully developing a rapid and sensitive one-tube method for subtype-discrimination of pandemic H1N1 and seasonal H1N1 influenza A viruses (Wang et al.,

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2009), we here applied an MGB probe for influenza A virus H3N2 detection using real-time PCR in this study.

MGB probes are now widely used in TaqMan-based virological testing (Jimenez-Clavero et al., 2006; Kulesh et al., 2004). These probes are conjugated with a minor groove binder (MGB) at the 3' end. Because the MGB moiety stabilizes the hybridization of the probe with single-stranded DNA targets, it can increase the specificity and sensitivity of the reactions by raising the melting temperature and reducing the length of the probe. The TaqMan MGB probe also contains a quencher dye that does not emit fluorescence within the detectable wavelength so that it can provide greater accuracy in quantitation.

In this study, 436 hemagglutinin sequences of human H3N2 viruses from 2007 to 2009 were retrieved from Genbank, aligned, and analyzed using the sequence analysis program, MacVector (Cary, NC). Conserved regions were identified for primer and probe design (Table 1) with ABI (Applied Biosystems) Primer Express 3.0 software (Foster City, CA). The primers and probe were synthesized at IDT (San Diego, CA) and ABI, respectively. The probe was labeled with the fluorescent reporter NED at the 5' end and conjugated with the minor groove binder (MGB) at the 3' end. Degenerate consensus primers were designed to amplify a selected 69 base pair hemagglutinin fragment (Table 1).

Viruses were propagated in MDCK cells and viral RNA was isolated using QIAamp viral RNA extraction kit (Qiagen Inc., Valencia, CA). For two-step Taqman-PCR, first-strand cDNA was synthesized using random primers as described before (Wang et al., 2009). Real-time TaqMan PCR was then carried out using an ABI 7500 Real Time PCR system (ABI, Foster City, CA) at concentrations of primers and probes at 900nm and 200nm, respectively. 2ul of target cDNA was used in a 25ul reaction at the cycling of 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. The one-step PCR assay was carried out using Taqman one-step RT-PCR master mix reagents kit following the manual instruction (ABI, Foster City, CA).

The specificity of the assay for H3N2 subtype influenza A viruses was initially evaluated using RNA from five cultured seasonal H3N2 viruses from 1968–2008, two 2009 seasonal H1N1 viruses, two 2009 pandemic H1N1 viruses (Memoli et al., 2010a; Memoli et al., 2010b; Memoli et al., 2009) and nine H1N1 swine and avian viruses. HPAI virus A/Viet Nam/1203/04 (H5N1) was also used for testing the specificity of H3N2 probe in this study. Although the H3N2 primers and probe were specifically designed from alignments of recent (2007–2009) H3N2 influenza A virus sequences, it was able to amplify a wide range of seasonal H3N2 viruses from 1995–2008 (Fig. 1A). Because of the substantial amount of antigenic drift that has occurred in H3N2 virus hemagglutinin since 1968, the H3N2 MGB assay did not detect 1968 pandemic H3N2 virus (A/Hong Kong/1/1968 (H3N2)).

RNA was also isolated from the primary clinical isolates in shell vial culture from patient nasal wash samples and also tested for H3N2 specificity in this study for ensuring the clinical applicability of this MGB-based real-time assay (Fig. 1A). Samples, including 3 confirmed H3N2 viruses, 3 confirmed seasonal H1N1 viruses, and 2 pandemic H1N1 viruses, were collected with the consent of participants under an IRB approved protocol, “Influenza in the Non-immunocompromised and Immunocompromised Host” (clinicaltrials.gov identifier: NCT00533182). Only the H3N2 samples were specifically amplified. Both one-step PCR and two-step PCR methods were used for the specificity detection on all cultured influenza A viruses and clinical samples and similar results were achieved for the specific amplifications.

The sensitivity and dynamic range test for this MGB-based H3N2 assay was evaluated by testing ten-fold serial dilutions (10^8 - 10^0 copies/ul) of standard template to create a standard

curve for quantitative analysis (Fig. 1B). The positive threshold for detection was determined as C_T value <35 . A strong correlation between the $\log_{10}$ of the DNA copy number and the C_T values were observed ($r^2 = 0.998$ and $r^2 = 0.996$ for two independent experiments). The detection limit was calculated to be 1.65×10^1 copy/reaction (Fig. 1B). Using H3N2 viral RNA, the detection limit was shown to be 6 fg RNA (two-step RT-PCR) and 0.6 pg RNA (one-step RT-PCR).

In summary, we report here a sensitive and specific method for the rapid detection of seasonal H3N2 influenza A virus. This assay should thus provide a useful tool for differential diagnosis of seasonal H3N2 virus from other currently circulating influenza A virus subtypes.

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Figure 1.

(A) Specificity of the assay for detection of H3N2 influenza A virus from (a) cultured seasonal H3N2 viruses from 1995–2010 and (b) clinical specimens. Amplification plots (c) represent amplifications from 2009 pandemic H1N1, seasonal H1N1 and HPAI H5N1 viruses.

(B) Standard curve of the H3N2 quantitative real-time PCR with the dilutions of standard plasmid DNA amounts from 1.65×10^1 to 1.65×10^8 copies/reaction.

Table 1

Nucleotide sequence of primers and probe used in the TaqMan-MGB assay

Sequence	* Nucleotide position
Forward Primer: 5'-GCACAGGGAATCTAATTGCTCCT-3'	788–810
Reverse Primer: 5'-AYCTCATTAYTGAGCTTTTCCCACTT-3'	856–831
TaqMan MGB Probe: 5'-(NED)-GGGTTACTTCAAATAC-(MGB)-3'	813–829

* The sequence location is based influenza A virus A/Pennsylvania/4/2007(H3N2), GenBank accession number EU516049.