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Broadly targeted triplex real-time PCR detection of influenza A, B and C viruses based on the nucleoprotein gene and a novel "MegaBeacon" probe strategy

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A PCR assay that covers animal and human influenza A, B and C viruses, i.e., most of *Orthomyxoviridae*, is needed. Influenza types are distinguished based on differences in the nucleoprotein (NP) present in the virus. Conserved NP regions were therefore used to design a TaqMan®-based triplex reverse transcription real-time PCR method. Variability of influenza A within the probe target region mandated the development of a novel molecular beacon, the "Mega" molecular beacon (MegaBeacon; MegB), for the detection of influenza A with this method. MegaBeacon is a mismatch-tolerant molecular beacon that is also a TaqMan® probe. The triplex method (3QPCR-MegB) was evaluated with influenza A isolates covering 18 HxNx combinations, two influenza B isolates, and five Japanese influenza C isolates, as well as influenza A, B and C synthetic DNA targets. One to ten viral RNA and cDNA genome equivalents were detected per PCR reaction for influenza A, B and C. Seventy-one human nasopharyngeal aspirates from respiratory infections yielded 30 influenza A, 11 influenza B and 0 influenza C with 3QPCR-MegB, where immunofluorescence (IF) found 28 influenza A and 10 influenza B. 3QPCR-MegB was more mismatch-tolerant than a variant PCR with an influenza A TaqMan® probe (3QPCR) and is a sensitive and rational method to detect influenza viruses of animal and human origin. MegaBeacon probes hold promise for variable target nucleic acids.

1. Introduction

Respiratory tract infections are caused by numerous viruses. In humans, viruses are major factors in the aetiology of respiratory infections. Among them are RNA viruses, such as orthomyxoviruses (influenza viruses A, B and C), paramyxoviruses (parainfluenza viruses 1–4, respiratory syncytial virus and metapneumovirus), coronaviruses (OC43, 229E, NL63, HKU1 and SARS viruses), human rhinoviruses (HRV), and some human enteroviruses (HEV). DNA viruses, such as adenoviruses (Heikkinen and Jarvinen, 2003) and, probably, the parvovirus Bocavirus (Allander et al., 2005), are also involved. Influenza A, B and C viruses belong to the *Orthomyxoviridae* family and are enveloped viruses with a segmented single-stranded RNA genome (Lamb and Krug, 2001). The detection of respiratory viruses can be improved through the application of molecular biology techniques. Numerous studies have developed and evaluated PCR- or reverse transcription-PCR-based methods for the detection and typing of respiratory viruses (Cram et al., 1999; Freymuth et al., 1997), although none combine three broadly targeted influenza PCRs in a real-time PCR format.

Influenza A and B virus infections are major causes of morbidity and mortality worldwide. The three influenza types, A, B and C, are divided based on antigenic differences in their nucleoproteins (NP). Matrix (M) proteins have also been used for wide-range classification (Widjaja et al., 2004). Influenza A viruses are further classified into subtypes based on antigenic differences of the surface proteins hemagglutinin (HA) and neuraminidase (NA). Currently, there are 16 distinct HA (H1–H16) and 9 NA (N1–N9) subtypes. Three subtypes of HA (H1–H3) and two subtypes of NA (N1 and N2) are found among influenza A viruses that have caused epidemics

Abbreviations: C_t, threshold cycle; FAM, 6-carboxyfluorescein; ROX, 6-carboxy-X-rhodamine; Cy5, 5′-disulfonatoindodicarbocyanine; FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction; RNA, ribonucleic acid; NP, nucleoprotein; RT-PCR, reverse transcription-polymerase chain reaction; 3QPCR-MegB, triplex reverse transcription real-time PCR; TD, touch-down temperature protocol; *r*, correlation coefficient.

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among humans (Gregory et al., 2002). Influenza A virus HA and NA glycoproteins undergo gradual point mutations known as *antigenic drift*. In contrast, *antigenic shifts* are saltatory evolutionary events due to reassortment of RNA segments from different subtypes in co-infected cells. Influenza B and C viruses display a lower variability than influenza A viruses, and human strains with antigenic shift have not yet been observed (Murphy and Webster, 1996). However, B and C viruses are represented by a smaller number of sequences than those from influenza A, which might bias the judgement of their variability. Although study of influenza C is lacking, it is known to cause both trivial and more serious respiratory diseases (Matsuzaki et al., 2005). Possible animal reservoirs of influenza B and C are not completely known and should be investigated.

The presence of all HA and NA subtypes in free flying birds and the diversity of their genetic information suggest that these animals, especially of the orders *Anseriformes* (ducks and geese) and *Charadriiformes* (shorebirds, gulls, terns and auks), may be the primordial reservoirs for all influenza A virus genes (Fouchier et al., 2000; Sharp et al., 1997; Swayne, 2008). The current subtypes of influenza A viruses found in humans include two HA subtypes (H1 and H3) and two NA subtypes (N1 and N2). During the past few years, sporadic human cases of infection with other subtypes of influenza A, including H5N1, H5N2, H7N7, H7N3 and H9N2, which are all hosted by birds, have been reported (Fouchier et al., 2004; Kuiken et al., 2005; Ogata et al., 2008; Saito et al., 2001; Tweed et al., 2004). It is feared that these occasional transmissions to humans could develop into a major influenza pandemic. It calls for comprehensive diagnostic techniques.

Traditionally, detection and identification of influenza viruses are based on virus isolation in tissue culture, embryonated eggs, immunofluorescence tests or enzyme immunoassays (Shorman and Moorman, 2003). Commercial immunofluorescence (IF) and immunoassay kits for detection of influenza viral antigens allow for rapid diagnosis. These are clinically useful, but are less sensitive and specific than appropriately designed PCRs (Herrmann et al., 2001; Schweiger et al., 2000), which are the most sensitive means of influenza virus detection and may also allow for subtyping of the virus (Donofrio et al., 1992; Herrmann et al., 2001). Virus isolation is less sensitive than PCR, but allows for a more complete characterisation of the virus, including its sensitivity to drugs, and is vital for ongoing global influenza surveillance.

Our goal in this study was to design a rational, broadly targeted system for detection of diverse influenza viruses with the ability to discriminate between influenza A, B and C. The three types were originally defined based on their nucleoprotein differences. This gene is, therefore, a logical target sequence. To achieve the goal of broad targeting the highly variable influenza A target sequence, we developed a new kind of probe, MegB. MegB allows for the use of very long and mismatch-tolerant nuclease-degradable probes. Such probes are hard to synthesise as conventional TaqMan® probes because of the necessity to introduce an internal quencher in a long oligonucleotide.

2. Materials and methods

2.1. Computer program for identification of conserved portions of variable viral genomes

The program "Consort©" (Blomberg, J., unpublished) uses alignments of 2–10,000 viral sequences from BLAST, Clustal, Megalign or Multalin. Areas of sequence conservation were identified, and the degrees of representation were computed for all sequences in the alignment or for selected groups included in the alignment. The program first shows the most common variant for each position, followed by less common variants in decreasing order. Insertions and deletions are marked. Each stretch of DNA sequence was automatically translated to protein sequence for the three reading frames (sense or antisense) with the least number of stops per 50 amino acids. The number of variant nucleotides (up to four) and the percent of nucleotides identical to the most frequent nucleotide (the consensus nucleotide) per position in an alignment can also be visualised graphically (see Fig. 1S in the Supplementary Material). This program has been used in several publications where it is described further (Bindra et al., 2007; Elfaitouri et al., 2006; Escutenaire et al., 2007; Forsman et al., 2003; Gyarmati et al., 2007; Mohamed et al., 2004, 2006; Muradrasoli et al., 2006, 2009; Yacoub et al., 2009).

The Visual OMP software (DNAsoftware, Ann Arbor, MI, USA) and the OligoAnalyzer version 3.0 online software (IDT SciTools) were used for assessment of oligonucleotide interactions.

2.2. Primers and probes

Initially, three fluorescently labelled TaqMan® probes were designed: one for influenza A (InfluANP-probe; see Supplementary Material), one for influenza B and one for influenza C. During the development, occasional false negative results occurred with the influenza A TaqMan® probe (data not shown), which were ascribed to variation in the probe target region. This variability in influenza A led to the development of a novel molecular beacon, the "Mega" molecular beacon (MegB), which is further described in Section 3. Primers were purchased from Thermo Hybaid, Interactiva Division (Ulm, Germany). The probes with LNA-modified nucleotides were purchased from Eurogentec (Seraing, Belgium) (see Table 1). Analyses of the predicted T_m and oligonucleotide interactions were performed using the OligoAnalyzer version 3.0 online software (IDT SciTools).

MegB was also tested in a virtual experiment against the 2005 and 2006 GenBank influenza A NP subsets, respectively, using the Visual OMP software.

2.3. Viral reference and control strains

The influenza 3QPCR-MegB assay was evaluated for its ability to detect influenza RNA from isolates of 19 influenza A, 2 influenza B and 5 influenza C strains. The 19 influenza A strains belonged to the H1N1, H2N3, H3N2, H4N6, H5N3, H6N2, H5N1 (LPAI), H5N1-Ty (HPAI), H5N2, H1N7, H7N7, H8N4, H10N8, H12N5, H6N8, H3N8, H9N2 and AIV-N3 subtype combinations. They included RNA from a recent Swine H1N1 isolate (A/Stockholm/28/2009) obtained from Dr. Maria Brytting of the Swedish Institute for Infectious Disease Control. The following preparations were obtained from the OIE and EU Community Reference Laboratory for Avian Influenza (Veterinary Laboratories Agency, Weybridge, UK): H7N1 A/African starling/England/1983/79 HA (titer 1:48), H5N1 A/chicken/Scotland/59 HA (titer unknown), H5N1 A/turkey/Turkey/05 (HPAI) HA (titer 1:64), H5N2 A/ostrich/Denmark/96 HA (titer 1:64), H7N7A/turkey/England/647/77 HA (titer 1:32) and H9N2 A/2003 HA (titer 1:64). The isolates were cultured in embryonated eggs, and the harvested allantoic fluids were inactivated with formaldehyde at a final concentration of 1/1000–1/4000. The following formalin-inactivated preparations were obtained from the Swedish Institute for Infectious Disease Control: H3N2A/IF60/01 HA (titer 1:64), H4N6/S90-0780 HA (titer 1:64), H5N3/S1174/05 HA (titer 1:64), H5N3/S90-436 HA (titer 1:64), H8N4/V121/9/87 HA (titer 1:64), H10N8/S90-0229 HA (titer 1:16), H12N5/S90-820 HA (titer 1:256), H16N8/S90-360 HA (titer 1:64), H3N8/S90-391 HA (titer 1:32), AIV-N3/S6-005 HA (titer 1:16) and several clinical H3N2 isolates of unknown titer.

Table 1
Primers and probes used for 3QPCR-MegB for detection of influenza viruses.

Primers and probes	Sequence 5′–3′	T_m (°C)
Influenza A		
Forward primer	GARRTYATAARRATGATGG	45.9
Reverse primer	ATTGTCTCCGAAGAAATAAG	48.2
InlfuANP-probe	[FAM]CGTCYGAGAGCTCRAARACTC CCC [Q]	69
InlfuANP-MegB	[FAM]**TTC**GAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTT TGACATGAAGAGCTCGAA[Dabcyl]	70
Influenza B		
Forward primer	ARCCAYAAKCAGYGAA	45.6
Reverse primer	TCATCTGGTTRTAGAATT-3′	47.3
InlfuBNP-probe (antisense)	[ROX]ACGCTYTTYTTTATCTCTGTYGGGGTYTGT[Q]	62.5
Influenza C		
Forward primer	TACATTGCCATTTGTAAR	46
Reverse primer	CATCACAACATATTCTTAT	45.8
InlfuCNP-probe	[Cy5]-TTAATGG**Y**GATGAAATGTTGAT[Q]	68

Notes: Sequences are shown as ordered for synthesis. IUPAC ambiguity codes are used for degenerate positions. Sequences in the NP segments are from the three reference strains of Influenza A (accession number AY684707), B (NC002208) and C (M17700). The positions of LNA residues are in underlined and in case of the MegaBeacon they are bold letters and the stem of the MegaBeacon is underlined. The forward and reverse influenza A primers were tested in two variants, see Section 2.2.

The two influenza B isolates were B/Russia/69 (ATCC # VR-790) and B/Lee/40 (ATCC # VR-101). The five influenza C clinical isolates, which were derived from Japanese patients, were kindly provided by Dr. Y. Matsuzaki. They were cultured in embryonated eggs, and the harvested allantoic fluids were inactivated with formaldehyde at a final dilution of 1/4000 (3 mM). A few non-influenza virus preparations were also tested. RNA was extracted from the following high-titered preparations: three human enterovirus isolates and three human calicivirus samples (one norovirus genogroup I and two norovirus genogroup II).

2.4. RNA extraction

RNA was extracted from 140 μl of the viral samples using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany). RNA was recovered in 60 μl of nuclease-free water and either used immediately or stored in aliquots at −70 °C in nuclease-free water.

2.5. Real-time PCR system

Initially, the One-Step EZ RT-PCR TaqMan® reagent kit (PE Applied Biosystems) was adapted for use with heat labile Uracil-N-Glycosylase (HK-UNG™; Epicentre Technologies Corporation, Madison, Wisconsin) in a one-tube format (Mohamed et al., 2004). This method is further referred to as 3QPCR. The 3QPCR method and its results are elaborated in the Supplementary Material. Due to a few false negative results with a few influenza A samples and the InfluANP-probe, a new broadly targeted probe (MegB) was designed. In further developments using 3QPCR-MegB, r*Tth* polymerase was replaced with the iScript one-step RT-PCR kit for probes (Bio-Rad, Hercules, USA) because a lower amplification efficiency and lower fluorescence signal was attained when r*Tth* was used. However, UNG cannot be used with iScript because it interferes with reverse transcription in this enzyme mixture. The iScript kit was used in further experiments with 3QPCR-MegB. Primer and

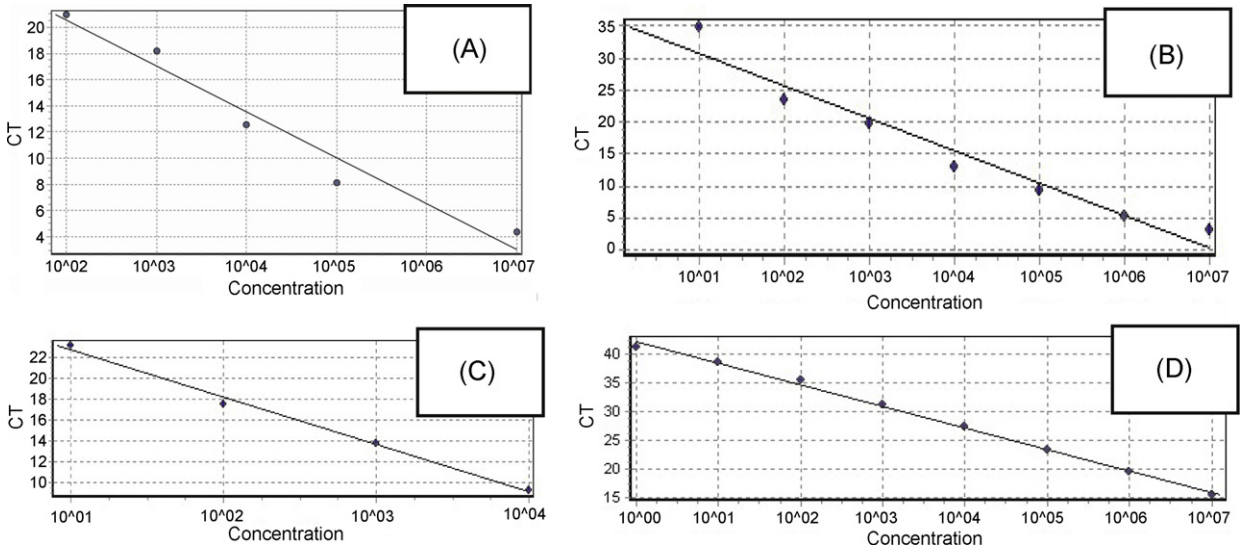

Fig. 1. Standard curves of 10-fold dilution series of synthetic influenza A, B and C target oligonucleotides. The Ct value is the cycle number at which a positive amplification reaction was measured; the straight line is the regression line. (A) Standard curve of a 10-fold dilution series of synthetic influenza A TaqMan probe (Influ ANP). (B) Standard curve of a 10 fold dilution series of synthetic influenza B antisense probe (Influ BNP). (C) Standard curve of a 10-fold dilution series of a perfectly matching synthetic influenza C probe (InfluCNP). (D) Standard curve of a 10-fold dilution series of a perfectly matching synthetic influenza A and mega beacon probe (InfluANP-MegB).

probes for influenza B and C were the same as for 3QPCR. The primers for influenza A were also the same, but the probe was changed to MegB. 3QPCR-MegB was carried out in a total volume of 50 μl containing 25 μl of 2× RT-PCR reaction mix for probes, 5 μl of RNA extract, a final concentration of 500 nM of each forward and reverse primer, 150 nM of a dual-labelled fluorogenic probe for influenza A, B and C viruses, respectively, and 1 μl of iScript reverse transcriptase. The subsequent RT step was performed at 50 °C for 30 min, immediately followed by an initial denaturation at 95 °C for 10 min. A total of 55 cycles were then performed, each consisting of a denaturation step at 94 °C for 30 s and an annealing-extension step in which the annealing temperature was 56 °C for 30 s in the first two cycles and was then decreased by 2 °C every second cycle down to 46 °C, according to the touch-down principle (Korbie and Mattick, 2008; Mohamed et al., 2006). Fluorescence data were collected during each annealing-extension step in the FAM channel for influenza A virus, the ROX channel for influenza B and the Cy5 channel for influenza C. Negative and positive PCR controls were included in each analytical round. 3QPCR-MegB was performed using the Corbett Research RotorGene Real Time Amplification system (RG 2000; Corbett Research, Mortlake, NSW Australia). RotorGene™ software version 6.4 (Corbett Research) was used for threshold selection and standard curve interpolation to derive approximate RNA concentrations relative to RNA and DNA standards. The term "genome equivalents" is preferable to the term "copies". Here, this term refers to the concentrations interpolated from the standard curve of 10-fold dilution series of synthetic influenza A, B and C target DNA oligonucleotides (Fig. 1A–D).

2.6. Reference methods

For comparison, a nested RT-PCR for influenza A and B was performed as previously described (Herrmann et al., 2001). In addition, virus isolation and indirect immunofluorescence (IFA) detection of influenza A and B and RSV antigens were performed, also according to Herrmann et al. (2001). Influenza antigen detection was performed by IFA, using either the respiratory panel kit (Cat. No. 3105; Chemicon International Inc., Temecula, CA, USA) or the influenza A and B kits (Cat. No. 5001 and 5002; Chemicon). To provide additional evidence for the specificity of the influenza triplex PCR, a pan-Corona QPCR (Escutenaire et al., 2007) was run on 77 of the 203 samples.

2.7. Clinical samples

From 1999 to 2005, 71 nasopharyngeal aspirates were collected from hospitalised patients with respiratory infections, using a baby feeding tube and an aspiration trap, as part of the routine diagnostic activities of the Academic Hospital Virology Department in Sweden. Both adult and child samples were included. The samples were a subset of the samples tested with 3QPCR (see Supplementary Material).

3. Results

3.1. Identification of conserved portions of influenza genomes using the ConSort© program

ConSort© analysis of over 1000 BLAST-aligned influenza A, B and C virus sequences, which encompassed the complete and partial human and animal influenza sequences available in GenBank in January 2006, indicated that the nucleoprotein segment of all three influenza types contained conserved regions that were suitable for design of broadly targeted diagnostic primers and probes for real-time PCR (see Fig. 1S in the Supplementary Material).

Although an unbiased evaluation of variability is not practical to achieve using archival data, which are biased with regard to sample, host species, number of observations and genetic subregion, a high variation (on average two variants per nucleotide position) was observed for all eight influenza A segments, while influenza B (1.3 variants per position) and influenza C (1.05–1.2 variants per position) were less variable. In this study, the NP gene was targeted because of its relative conservation and type-defining specificity (Obenauer et al., 2006).

3.2. The primer and probe design process

The nucleoprotein gene sequences of influenza A, B and C were used in the BLAST program to retrieve all of the influenza sequences available in GenBank in January 2005. This search was reiterated in January 2006 to check for new sequence variants. Another check was performed in 2008. The searches were made in the entire "Viruses" subset of GenBank. The presentation options "query anchored with identities" and "-v 8000 -b 8000" were specified to ensure that all sequence variants were included in the output alignment. The reference strains of influenza A, B and C that were used for querying the database were AY684707, NC002208 and M17700, respectively. The BLAST alignments were examined by ConSort© to detect conserved regions suitable for primer and probe construction. Conserved stretches were found in the NP gene. One pair of degenerate primers was designed for each influenza type to amplify a fragment of 152, 119 and 100 bp for influenza A, B and C, respectively. A score cutoff of 46 was set in order to include influenza sequence hits belonging to the same virus type and exclude hits from other influenza types and other viruses. Conserved regions were also aligned using CLUSTAL W 1.83 and were analysed with ConSort©. During this process, a few new influenza A variants with mismatches occurring at the 3′ end of both forward and reverse influenza A primers were found in GenBank. We then tested the primers with and without these 3′ nucleotides (i.e., forward 5′GARRTYATAARRATGATGGA3′ and 5′GARRTYATAARRATGATGG3′, and reverse 5′ATTGTCTCCGAAGAAATAAGA3′ and 5′ATTGTCTCCGAAGAAATAAG3′). The C_t values for 10 reference influenza A strains and ten clinical samples (H3N2) were the same, within half of a C_t step (data not shown). We cannot state that the shorter primers will perform as well as the original, one-nucleotide longer primers for all of the samples. However, an equal mixture of shorter and longer primers at the same final primer concentration, as reported in Table 1, also gave the same result. Thus, a safe way of handling this ambiguity seems to be using an equimolar mixture of the two primer variants for both forward and reverse primers. The primer sequences shown in Table 1 are the shorter variants.

MegB is a mismatch-tolerant molecular beacon that is also a TaqMan® probe (see Table 1). Molecular beacons (MBs) are single-stranded DNA probes that are useful for nucleic acid detection. MBs contain a fluorophore at the 5′ end and a quencher at the 3′ end and form a hairpin-like, stem-loop structure independently. The mismatch-tolerant of MegB is due to its long (55 nt) virus-specific sequence, which results in a high T_m for virus-specific annealing. MegB is a modified MB with a stem-loop structure that has TaqMan® properties, i.e., it can be released by the 5′–3′ exonuclease of the thermostable polymerase (Fig. 2A). MegB is not only complementary to the target sequence within the loop region, but it also has an additional 5′ stem of 10 self-complementary nucleotides. Taq polymerase can degrade MegB and cleave the dual fluorescent dye-labelled MegB during PCR through its 5′–3′ exonuclease activity. When the MegB is on its own, the quencher is bound to the fluorophore, and does not fluoresce below the T_m of the stem. Both the quenching of uncleaved probe at ~70 °C and the consumption of probe during the amplification process due to exonuclease

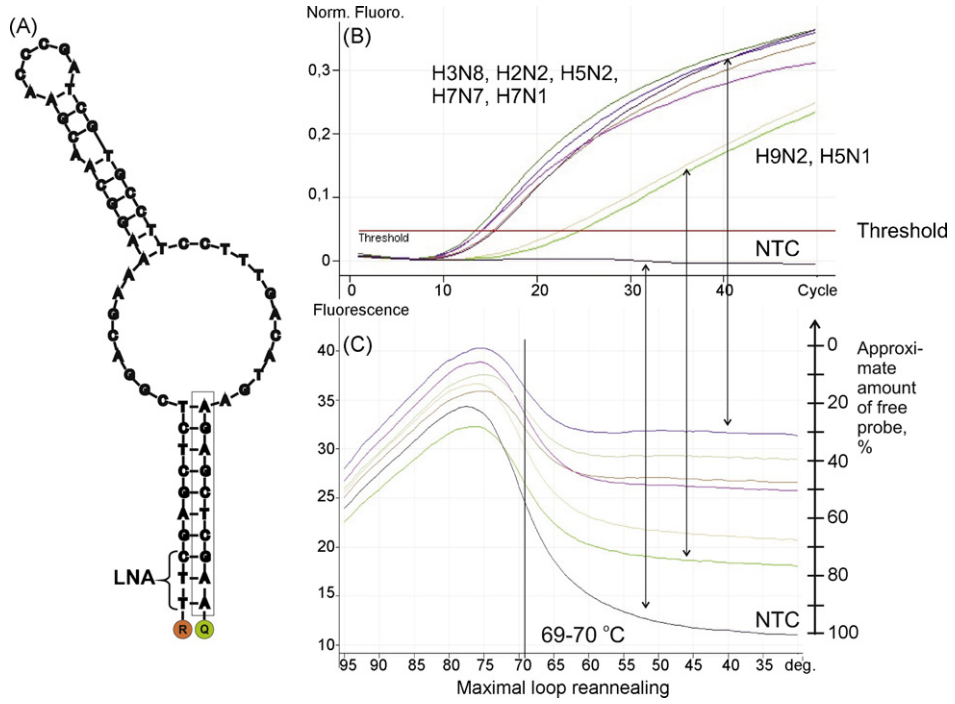

Fig. 2. (A) MegaBeacon influenza A probe construct. The FAM fluorophore and the Dabcyl quencher are shown in orange and green, respectively. The non-viral portion, added to create a hairpin loop, is shown in a box. (B) Amplification curves from seven different subtypes of influenza A from the FAM channel of 3QPCR-MegB. (C) Raw fluorescence data from reannealing the MegaBeacon probe. The correlation of residual fluorescence ("probe consumption") with amount of amplimer is evident and derives from cleaved probes as well as probes hybridising to amplimer. Negative samples occasionally gave an atypical amplification curve with the MegaBeacon influenza A probe. These curves could be discriminated from true positive amplification curves based on (i) curve form, if the curve started after a few cycles and remained low, (ii) if the sample was found to be negative in the probe consumption test and (iii) if the sample lacked a band of the expected size in agarose gel electrophoresis. All data were obtained using a Corbett Rotorgene© instrument and its software.

Table 2
Matching and mismatching influenza A, B and C synthetic oligonucleotides relative to the TaqMan® probes.

Target	Sequence 5′-3′
Influenza A	
InfluANP	GAAATCATAAGGATGATGGAAAGTGCCAGACCAGAAGATGTGTCTTTCCAGGG GCGGGGAGTT1TTC2GAGCTCTCA3GACGAAAAGGCAACGAACCCGATCGTGCC TTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAATGC
InfluANPmis	GAAATCATAAGGATGATGGAAAGTGCCAGACCAGAAGATGTGTCTTTCCAGGG GCGGGGAGTC1TTT2GAGCTCTCG3GACGAAAAGGCAACGAACCCGATCGTGCC TTCCTTTGACATGAGTAATGAAGGATCTTATTTCTTCGGAGACAATGC
Influenza B	
InfluBNP	AACCACAAGCAGTGAAGCTGATGTCGGAAGGAAAACCCAAAAGAACACA1AC CCCA2ACAGAGATAAAA3AAA4AGCGTCTACAATATGGTAGTGAAACTGGGCG AATTCTATAACCAGATGA
InfluBNPmis	AACCACAAGCAGTGAAGCTGATGTCGGAAGGAAAACCCAAAAGAACACG1AC CCCG2ACAGAGATAAAG3AAG4AGCGTCTACAATATGGTAGTGAAACTGGGCG AATTCTATAACCAGATGA
Influenza C	
InfluCNP	TACATTGCCATTTGTAAGGAAGTGGGCCTTAATGGCGATGAAATGTTGATCCTG GAAAATGGCATAGCAATTGAAAAAGCTATAAGAATATGTTGTGATG

Notes: In an assessment of 2757 Influenza A NP sequences from GenBank (2005), the frequency of variant (mismatching) nucleotides relative to the Influenza A TaqMan® probe was, at pos 1: 1183 T, pos 2: 183 T and pos 3: 1240 A. The mismatch positions in question are underlined, together with the mismatch position number. In an assessment of 95 Influenza B sequences, the frequency of variant (mismatching) nucleotides was, at pos 1: 15 A, pos 2: 15 A, pos 3: 6 A and pos 4: 4 A. There were no appreciable mismatches (counting >1 mismatch in the entire set of 88 sequences) in the set of Influenza C sequences. The oligonucleotides were purchased from Thermo Hybaid. Positions with occasional mismatches relative to probes are indicated in pink color, the primer positions are indicated in blue and the probe positions in yellow.

Table 3
Matching and mismatching influenza A synthetic oligonucleotides relative to the MegaBeacon probe.

Target	Sequence 5′-3′
InfluA_PerfectMatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTCGAGCTCTCGGACGAA AAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_1_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGCTCTCGGACGAA AAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_2_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATCTCGGACGAA AAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_3_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCGGACGAA AAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_4_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCAGACGAA AAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_5_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCAGACGAA AGGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_6_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCAGACGAA AGGGCCACGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_7_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCAGACGAA AGGGCCGCGAACCCGATCGTGCCTTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_8_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCAGACGAA AGGGCCGCGAACCCGATCGTGCCCTCCTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_9_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCAGACGAA AGGGCCGCGAACCCGATCGTGCCCTCTTTTGACATGAGTAATCTTATTTC TTCGGAGACAAT
InfluA_10_Mismatch	GAAATCATAAGAATGATGGAAAGTGCCAGATTTGAGATTCAGACGAA AGGGCCGCGAACCCGATCGTGCCCTCTTTTGACTTGAGTAATCTTATTTC TTCGGAGACAAT

Notes: A new Influenza A multiple alignment was generated: in an assessment of 2500 Influenza A NP sequences from GenBank (2008), the 10 displayed variant positions were found, Thus, the MegB target region (yellow shade) contained up to 10 mismatching nucleotides (magenta shade) versus the MegB probe. Primer targets are shaded cyan. Oligonucleotides of all 10 variants were purchased from Thermo Hybaid to test the mismatch tolerance of the MegB.

activity are demonstrated by the reannealing curve (Fig. 2C). The loop region of MegB was designed to be 45 bases in length, with arm length sequences of 10 nucleotides and complementarity to the target of 45 + 10 = 55 nucleotides (Table 1). Locked nucleic acid (LNA) residues were used to increase the T_m of the beacon hairpin (Johnson et al., 2004; Kumar et al., 1998; Mouritzen et al., 2003; Petersen et al., 2002; Ugozzoli et al., 2004; You et al., 2006).

Predicted T_m and oligonucleotide interactions of all oligonucleotides were examined using the OligoAnalyzer version 3.0 online software (IDT SciTools). During the development, a few new variants with one to two additional mismatches in the influenza A and B probe target sequences appeared in GenBank. In synthetic DNA form, they were also detected by 3QPCR-MegB (Table 2). An additional multiple alignment of 2500 newly reported influenza A strains with aberrant structures was generated, which revealed variation in 10 nucleotides in the MegB region. Synthetic target sequences for all 10 variants were ordered to test the ability of MegB to detect a broad range of targets (Table 3).

MegB was also tested in a virtual experiment against the 2005 and 2006 GenBank influenza A NP subsets, respectively, using the Visual OMP software.

3.3. Evaluation of specificity and sensitivity using reference strains

3.3.1. General performance of the assays, detection range and specificity

The 3QPCR-MegB fluorescence curves for selected influenza A reference strains demonstrate good signal strength and high efficiency (89, 91 and 81% for influenza A strains H5N1, H9N2 and H7N1, respectively, and 84 and 83% for one influenza B and one influenza C strain, respectively) (Fig. 2). For synthetic DNA targets that were perfectly complementary to their probes, efficiencies of 89, 82 and 81% were obtained for influenza A, B and C sequences, respectively. Synthetic A and B DNA targets with probe mismatches gave similar values as perfectly fitting targets (Fig. 1A–D). A set of 11 synthetic influenza A oligonucleotides at 10^6 copies/reaction

(Table 3) and 0–10 mismatches were detected. However, the 10-mismatch oligonucleotide was detectable at a rather late C_t value of 40.

When the influenza A MegB was tested in a virtual experiment against the entire 2006 GenBank influenza A NP sequence set using Visual OMP, predicted ΔG values at the PCR conditions varied from −41 kcal/mole (for a single, rarely occurring variant, which we detected in 2008, that had 10 mismatches, the maximum number of mismatches found) to −58 kcal/mole (for 7 mismatches, the maximum number of mismatches found in the GenBank sequence set from 2005) to −101 kcal/mole (for no mismatches). Judging from the experiment shown in Table 3, the 10 rarely occurring mismatches are close to the fault tolerance maximum of MegB. Thus, it is reasonable to assume that the MegB probe can detect most, but probably not all, upcoming influenza A strains.

The specificity of 3QPCR-MegB was evaluated by testing RNA from 3 enterovirus isolates and from 1 norovirus genotype I and 2 norovirus genogroup II isolates, as well as 10 negative controls. All of the samples were found to be negative in the three channels.

The specificity and range of detection of 3QPCR-MegB was evaluated by using reference strains of influenza A virus and synthetic DNA oligonucleotides covering the amplified nucleoprotein regions of influenza A, B and C (InfluANP, InfluANPmis, InfluBNP, InfluBNPmis and InfluCNP, respectively). Further, 3QPCR-MegB was evaluated for its ability to detect influenza RNA from isolates representing 18 influenza A subtype combinations, two influenza B strains from ATCC and five Japanese influenza C strains. Thus, H1N1, H2N3, H3N2, H4N6, H5N3, H6N2, H5N1 (LPAI), H5N1-Ty (HPAI), H5N2, H1N7, H7N7, H8N4, H10N8, H12N5, H6N8, H3N8, H9N2 and AIV-N3 were found to be positive. A recent swine influenza A H1N1 isolate was clearly positive. Amplification curves for seven of those combinations are shown in Fig. 2. B/Russia, B/Lee and the C strains AB064403/Yamagata/13/98/640 (Matsuzaki et al., 2002), AB099413/Yamagata/3/2000/2560, AB099414/Yamagata/6/2000/640, AB099416/Miyagi/2/2000/640 as well as AB099419/Miyagi/5/2000/640 (Matsuzaki et al., 2004) were also positive in the appropriate fluorescence channel. As mentioned, the synthetic influenza A, B and C sequences were found to be positive regardless of the indicated probe mismatches. The synthetic targets with three (InfluANPmis) and four (InfluBNPmis) mismatches versus the cognate probe were detected at equal C_t values, but had lower signal strengths (from a plateau value of 0.6 to a plateau value of 0.2) than their counterparts with no mismatches in the probe target sequence. The influenza B probe itself had a high T_m and did not contain LNA. As shown below, most clinical samples that were known to contain influenza A and B were detected by the appropriate 3QPCR-MegB component.

Addition of LNA was predicted to result in a large (around 15 °C) increase in T_m for the influenza C probe. This was tested both with and without LNA against synthetic template DNA. The plateau signals late in the temperature protocol for the LNA-free variants were low for a synthetic DNA target of influenza A (signal strength 0.2) and hardly measurable for that of influenza C (signal strength around 0.02), whereas all of the probes with a high estimated T_m (influenza C; LNA-containing, influenza B without LNA) gave stronger signals (0.6–0.4).

3.3.2. Sensitivity

3.3.2.1. Standard curve generation. Standard curves were generated for quantitation of assay sensitivities using stocks of influenza A, B and C PCR products. Their concentrations were determined spectrophotometrically using the Nanodrop ND_1000 instrument according to the manufacturer's instructions (Nanodrop, Wilmington, DE). DNA was serially diluted 10-fold in nuclease-free water containing carrier tRNA (100 ng/ml). Standard curves were also generated using 1×10^0 to 1×10^8 copies of PCR products per PCR reaction.

Tenfold serial dilutions of synthetic DNA oligonucleotides from influenza A, B and C ranging from 1×10^0 to 1×10^7 copies were run in 3QPCR-MegB. A linear relationship between C_t values and the log 10 of the amount of target, with efficiencies of 100, 84 and 96%, for influenza A, B and C target oligonucleotides, respectively, was obtained. The standard curves for influenza A are shown in Fig. 1A–D. Under these conditions, the limits of detection (analytical sensitivity) were 10 synthetic DNA target copies per PCR reaction for influenza A, B and C, respectively, for both QPCR variants. For influenza A and B, sensitivity was also estimated using titration of known amounts of the amplimers produced.

Sensitivity was also studied by parallel end-point titrations into the stochastic zone of 1–10 RNA copies per QPCR reaction, using six observations per dilution step. Three clinical samples that were positive for influenza A, B and C were titrated in 10-fold dilution steps for influenza A and C and in 2-fold dilution steps for influenza B down to complete negativity. An incomplete, stochastic, titration positivity (i.e., one to five positives per dilution step with six observations) at the last positive dilution step was observed for all three samples with 3QPCR-MegB. As expected, variation increased in the last two positive dilutions, just before the observed stochastic positivity in the last positive dilution. This result indicates that all three PCR assays have sensitivities of approximately 1–10 viral genomes per PCR reaction. The amplification efficiencies observed in the serial titrations of the three samples were 98, 92 and 96% for influenza A, B and C, respectively; illustrating that amplification from RNA has approximately the same efficiency as amplification from DNA in the two systems. However, it is likely that occasional mutations that compromise quantification can occur in practice. Absolute quantification can be somewhat erroneous. As stated above, we therefore prefer the term "genome equivalents". Using synthetic DNA as a standard in QPCR, the quantity of influenza RNA was shown to range from 10 to 10^7 influenza A, B and C genome equivalents per PCR reaction, which corresponds to around a 100-fold increase per ml of the nasopharyngeal specimens. No fluorescence signal was generated in the negative controls, which contained RNase-free water.

3.3.3. Comparison of the 3QPCR-MegB assay with other methods

Seventy-one samples (out of the 203 samples that are mentioned in the Supplementary Material) were analysed with 3QPCR-MegB; 25 of 26 influenza A-positive samples detected with IFA were also influenza-A positive with 3QPCR-MegB. 3QPCR-MegB detected two influenza A-positive sample that 3QPCR missed, but they were detected as influenza A-positive with IFA. Eight of ten influenza B-positive samples detected with IFA were also positive with 3QPCR-MegB. One influenza B-positive sample identified with IFA was found to be influenza A-positive with 3QPCR-MegB. One influenza B-positive sample detected with IFA was found to be negative with 3QPCR-MegB. However, one sample that was negative for influenza A and B using IFA was found to be influenza B-positive with 3QPCR-MegB. Additionally, one influenza A- and B-negative sample identified with IFA was found to be influenza A-positive in gel electrophoresis.

4. Discussion

The great variability of orthomyxoviruses is a problem for sequence-based nucleic acid detection methods. Broadly targeted methods that utilize the most conserved portions of the virus genome are needed. NP and matrix genes are conserved within, but not between, influenza types. A widely used, broadly targeted system for influenza A detection uses the matrix protein gene

(Fouchier et al., 2000; Kuiken et al., 2003; Whiley and Sloots, 2005). However, the high variability of influenza, which includes the frequent emergence of radically new variants such as the avian and swine influenzas, makes it important to have alternative broadly targeted PCR assays. As shown here, the NP gene is also relatively conserved, somewhat more so than the matrix gene for influenza B and C. In an analysis of conservation of influenza A genes (Obenauer et al., 2006), the nucleoprotein gene was among the more conserved genes; it had 1.81–1.85 variants per nucleotide position. The relative conservation of NP makes it a good choice for PCRs that cover a wide range of targets. In addition, influenza types A, B and C are based on the antigenicity of the NP gene, and it is, therefore, logical to detect and simultaneously type the three influenza types using NP as a target. The NP gene forms a complex with the polymerase subunits, which is necessary for RNA replication, and is probably conserved for similar reasons as the polymerase subunits. NP has been previously used for conventional PCR (Shu et al., 1993). However, our 3QPCR-MegB technique is unique in that it was optimised from a large sequence base of human and animal viruses in order to facilitate broad detection. In addition, its primers and probes were optimised to allow for simultaneous detection of a broad range of the three influenza types. A disadvantage of the high sequence conservation is that the NP amplimers are of limited use for molecular epidemiology. If further characterisation is needed, hemagglutinin PCR assays should be performed (Lu et al., 2005).

Target sequence variability is a significant problem for probe-based detection of orthomyxoviruses, but also of caliciviruses, retroviruses and flaviviruses. The increased fault tolerance of the MegB strategy has the potential to improve detection of variable viruses in general and to enable detection of entire virus families, such as *Coronaviridae* (Muradrasoli et al., in preparation). The basis of the MegB strategy is to extend the length of the probe to increase its T_m and to disperse hybridisation over a longer nucleotide stretch, encompassing variation in the entire region. As long as there are sufficient perfectly matching sequence regions, the probe will bind. This concept differs from the T_m-increasing LNA or Minor Groove Binding (MGB) oligonucleotide modifications, which increase the T_m of short probes that target conserved stretches, but remain sensitive to mismatches within those stretches. MegB differs from a traditional MB in that it also functions as a nuclease-degradable probe, like TaqMan® probes. This quality allows for a gradual accumulation of free fluorophore during amplification, thus enhancing the fluorescence signal. The signal from open MegB probes, which were bound to amplimers, but not degraded by the 5′ exonuclease activity of the polymerase, will be added to the total signal from the MegB probes. To ensure degradability of the 5′ end of MegB, the 5′ end should bind to a perfectly matching target sequence. We have placed LNA in this MegB region, which also ensures hybridisation of the 5′ end to its complementary 3′ end at temperatures below the hairpin T_m when MegB is not bound to amplimer. Thus, MegB is a hybrid between a MB and a TaqMan®. MegB probes can be much longer than traditional TaqMan® probes because the former do not rely on an intact oligonucleotide chain for FRET. Instead, FRET is delivered by the proximity of the 5′ and 3′ ends in the hairpin. Judging from theoretical predictions and from the model experiments shown here, the influenza A MegB probe should be able to detect most influenza A strains with an upper limit of 10 evenly spaced mismatches. An alternative design could be a partially double-stranded probe in which the quencher is situated on the strand opposite to the reporter (Huang et al., 2007).

A feature of our triplex system is the use of LNA nucleotides (Kumpf and Gauglitz, 2006; Mohrle et al., 2005; Nielsen et al., 2004). The proper role of LNA in diagnostic oligonucleotide design needs to be further defined. In our case, the introduction of LNA in theMegB influenza A and the influenza C probes allowed for the shortening of the probe in spite of maintaining a relatively high T_m. Normally, TaqMan® probes are around 30 nucleotides long. The use of LNA allowed us to focus the probe on a shorter (A: 25 nt; InflAprobe, see Supplementary Material, C: 22 nt), highly conserved NP region than for InflBprobe (which was 31 nt long), avoiding the more variable flanks. It is reasonable to assume that introduction of LNA nucleotide(s) surrounding a mismatch site could increase fault tolerance and allow for shortening of the TaqMan® probe, which is desirable for good-quality quenching, i.e., a close distance between the fluorophores. The influenza B probe was antisense to avoid 5′ G quenching. These versions gave a higher signal than the sense version of the same target sequence. As demonstrated here, the probes showed a remarkable tolerance to mismatches. The MegB influenza A probe was tested with and without LNA in the stem. These three LNA residues were necessary to generate a good signal. We cannot presently distinguish between possible better quenching of the LNA-containing version, higher 5′ degradability, or better binding to the amplimer as causes of this effect.

The 3QPCR signals from synthetic DNA targets with four mismatches were easily detectable. However, in the case of the highly variable influenza A, this fault tolerance did not prove to be enough. As discussed above, the influenza A MegB probe tolerated up to 10 mismatches, i.e., the entire recorded variation in the probe region. Evidently, the longer MegB length invited more possible mismatches than the shorter TaqMan® probes. However, it is unlikely that all possible mismatches would occur simultaneously in a wild-type virus. Therefore, the long MegB probe is more suitable to tolerate unanticipated variation than the shorter TaqMan® probes. More systematic tests of probe designs with LNA and the MegB principle are needed in order to encompass an even higher number of mismatches.

3QPCR and 3QPCR-MegB use nine more or less degenerate oligonucleotides, which increases the risk of inhibition due to interaction between them. However, the high amplification efficiencies, the observed high sensitivities, as well as the absence of primer–dimers observed in gel electrophoresis indicated that this increased risk is not a problem. When the complete 3QPCR and 3QPCR-MegB mixtures were run with influenza A, B and C samples, only one band was visible around 150 bp for influenza A and 100 bp for influenza B and C. When the three components of 3QPCR and 3QPCR-MegB were tested separately, the C_t values and amplification efficiencies were the same as when they were tested together in the same tube. Thus, neither interference nor false negative results from interaction among primers were observed (data not shown).

In this study, a single-step, multiplex reverse transcription-polymerase chain reaction (3QPCR-MegB) was designed for the simultaneous detection of influenza A, B and C agents of acute respiratory infection. The multiplex system was able to detect 1–10 influenza A, B and C nucleic acid equivalents. In the absence of another broadly targeted method for the detection of influenza virus, we compared the reactivity of 3QPCR-MegB (and 3QPCR, Supplementary Material) with a published RT-PCR assay evaluated for human clinical use (Herrmann et al., 2001). This assay uses primers that are, despite broad reactivity for influenza A and B, not adequate for a single-tube, multiplex technique.

Sensitivity was determined in three different ways: (i) dilution of known amounts of synthetic DNA targets and amplimers, (ii) dilution of positive samples into the range of stochastic positivity and (iii) determination of statistical relativity to nested PCR and IFA in a set of respiratory samples. The first two approaches indicated that 3QPCR-MegB (and 3QPCR) can detect a few target molecules of RNA from the three influenza types. The third approach indicated that 3QPCR-MegB (and 3QPCR) are more sensitive in clinical

samples than the two other influenza detection methods used for comparison.

The specificities of the primer/probe sets designed for detection and discrimination of influenza viruses were evaluated by using reference strains for influenza A and synthetic DNA oligonucleotides. The eight influenza A reference strains included the avian H5N1, H7N7 and H9N2 strains, which are known to have caused severe human infection. These strains were positive for influenza A, but not for influenza B or C, in 3QPCR-MegB. The specificity of the method was shown by the clear separation between influenza A, B and C signals in clinical samples. The correlation between nested PCR and 3QPCR-MegB (and 3QPCR) results was high. All samples found to be positive by nested RT-PCR were positive by the 3QPCR assay. Samples identified as positive for RSV by IFA were generally 3QPCR-MegB, (and 3QPCR) negative. Specificity and sensitivity data are presented in Tables 1S–8S in the Supplementary Material. In a future clinical version of 3QPCR-MegB, further quality control measures, such as inhibition control by "spiking" samples with known amounts of influenza virus or determination of RNA quality with housekeeping gene PCR (Muradrasoli et al., 2009), should be considered. However, we found no evidence for significant inhibition during the long developmental period of the method.

Influenza C has received relatively little diagnostic attention to date (Greenbaum et al., 1998; Hellebo et al., 2004; Matsuzaki et al., 2006). In order to cover most influenza strains from humans and animals, influenza C PCR was included in the 3QPCR method. Only one of the 203 nasopharyngeal aspirates was influenza C-positive by 3QPCR (Supplementary Material). As expected, it was negative by nested PCR and conventional diagnostic tests.

The 3QPCR-MegB system is unique in the utilisation of highly conserved NP sequences and should detect most influenza A, B and C strains. However, it is not possible to claim detection of all influenza viruses. Virus variability may give rise to occasional false negative results. However, our evaluation indicated that this system can tolerate a few mismatches in both probe and primer target sequences. Further development of 3QPCR was performed due to a few false negative results for influenza A. The 3QPCR-MegB assay could detect two IFA influenza A-positive samples, which 3QPCR missed, and the 3QPCR-MegB-positive influenza A sample was missed by both IFA and 3QPCR. We conclude that both nested RT-PCR and 3QPCR-MegB were suitable for detection of influenza viruses A and B, but that 3QPCR-MegB has several advantages over nested RT-PCR; including ease of set-up and interpretation, increased sensitivity of genome detection, reduction in potential PCR contamination and discrimination of influenza types in the same assay and a degree of quantification inherent to the method. PCR is an important tool in the diagnosis of influenza infections. Rapid, diagnostic, antigen-based methods are the first line of tests used for clinical management. However, because these tests have limited sensitivity, there must be a second line of detection with more sensitive techniques, such as 3QPCR-MegB. The quantitative dimension added by 3QPCR-MegB may be important in treatment and transmissibility studies and makes 3QPCR-MegB a valuable research tool. Quantification will also be clinically useful during patient follow-up. However, reliable quantitation requires attention to differences in amplification efficiency, which can otherwise distort the quantitation.

In summary, 3QPCR-MegB is the preferred triplex QPCR method. 3QPCR-MegB proved to be a rapid, specific and accurate assay for the detection and identification of influenza viruses. The combination of RT and PCR reactions in a single-step real-time PCR, along with typing, saves time and reagent costs. In addition, this assay can detect a broad range of influenza A viruses, including avian H5N1 and the new swine H1N1, and is, therefore, highly suitable as a rational screening tool for influenza viruses in humans and animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2009.10.017.

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