Arch Virol (2001) 146: 2275–2289

Archives of Virology

Universal primer set for the full-length amplification of all influenza A viruses

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Accepted August 29, 2001

Summary. To systematically identify and analyze the 15 HA and 9 NA subtypes of influenza A virus, we need reliable, simple methods that not only characterize partial sequences but analyze the entire influenza A genome. We designed primers based on the fact that the 15 and 21 terminal segment specific nucleotides of the genomic viral RNA are conserved between all influenza A viruses and unique for each segment. The primers designed for each segment contain influenza virus specific nucleotides at their 3′-end and non-influenza virus nucleotides at the 5′-end. With this set of primers, we were able to amplify all eight segments of N1, N2, N4, N5, and N8 subtypes. For N3, N6, N7, and N9 subtypes, the segment specific sequences of the neuraminidase genes are different. Therefore, we optimized the primer design to allow the amplification of those neuraminidase genes as well. The resultant primer set is suitable for all influenza A viruses to generate full-length cDNAs, to subtype viruses, to sequence their DNA, and to construct expression plasmids for reverse genetics systems.

Introduction

Influenza A viruses are segmented, negative-strand RNA viruses, that circulate worldwide and cause disease in a limited number of animals. Aquatic birds are the reservoir of all 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes identified so far [31]. H1N1 and H3N2 subtypes cause epidemics in the human population that result in excess morbidity and mortality, particularly in infants and in the elderly.

Interspecies transmission of influenza A viruses can occur after reassortment of two viruses, and pigs are thought to play an important role by acting as a "mixing

vessel" in the process of genetic reassortment [22]. However, the transmission of an H5N1 chicken virus and of an H9N2 quail virus to humans in Hong Kong in 1997 showed that direct avian-to-human transmission of influenza virus is possible without reassortment raising the possibility that land-based birds may also act as intermediate hosts [5, 6, 24, 25, 28]. Analyses of receptor specificity of HA have indicated that the HA is very important for virus transmission and a major determinant in host range [16, 20, 29]. In addition to HA, other viral proteins are important for host range and they might also be implicated in the direct transmission of viruses from birds to mammals [30, 33]. The HA of H5N1 viruses contains basic amino acids, which are characteristic of highly pathogenic avian viruses [2, 27, 32]. The fact that H9N2 viruses with genes encoding internal proteins similar to those of the H5N1 viruses were found in humans suggests that in addition to the HA, the internal proteins are important for transmission [12–14, 19, 21].

Simple and reliable methods of virus detection and identification would enable characterization of representatives of all of the circulating subtypes of influenza virus. Virus isolation is currently performed by infecting embryonated eggs or Madin-Darby canine kidney (MDCK) cells in culture with samples taken from the nose or throat of mammals or from the trachea or cloaca of avian species. Hemagglutination assays, hemagglutination inhibition (HI) assays, and immunofluorescence assays are standard diagnostic methods of detecting and subtyping influenza A viruses. Genetic analysis with RT-PCR is one of the most sensitive and specific methods for typing and subtyping influenza viruses. Virus isolates can be subtyped by sequencing, probe hybridization, PCR-enzyme immunoassay (EIA), restriction fragment length polymorphism (RFLP) analysis, single-strand conformational polymorphism (SSCP) analysis, and heteroduplex mobility assay (HMA) of the amplified products [3, 4, 8, 34].

Recently developed RT-PCR methods and primer design have been optimized only for H1 and H3 subtypes of human influenza A viruses, or each primer pair typically amplifies only one segment [11, 17, 34]. However, the amplification and characterization of the entire genome of all circulating influenza viruses would provide knowledge about the molecular basis of virus transmission, virulence and evolution of influenza A viruses. In addition, the application of the eight plasmid system for generating all influenza virus subtypes requires simple and fast methods for cloning full-length viral cDNAs into the plasmid vector pHW2000 [15]. We therefore attempted to establish a RT-PCR method that amplifies all eight segments of the vRNA of influenza A virus by designing primers with complementary sequences to the conserved vRNA-termini. Here, we provide evidence that this RT-PCR method is applicable to all subtypes of influenza A virus.

Materials and methods

Viruses

The virus strains given in the text and tables were obtained from the repository of St. Jude Children's Research Hospital and grown in embryonated eggs.

RNA extraction and RT-PCR

We used the RNeasy Kit (Qiagen, Valencia, CA) to extract vRNA from 100 or 200 μl of allantoic fluid from infected embryonated eggs. The RNA was eluted into 40 μl H_2O. Two-step RT-PCR was employed to amplify each of the viral gene segments. Briefly, the RNA (4 μl) was transcribed into cDNA by using AMV reverse transcriptase (Promega, Madison, WI) according to the protocol provided by using 500 ng of Uni12 primer (Fig. 2) in 30 μl. The RT reaction was performed at 42 °C for 60 min. 1 μl of the RT-reaction was used for each PCR reaction. The cDNA was amplified by using the Expand High-Fidelity PCR system (Roche Diagnostics, Mannheim, Germany) according to the protocols provided. The final concentration of Mg^{2+}-ions was 1.5 mM, the primer concentrations were 1–5 μM. The first cycle of the amplification program consisted of a 4-min period at 94 °C and was followed by 30 cycles with the following conditions: 94 °C for 20 sec, 58 °C for 30 sec, and 72 °C for 7 min. The program ended with one cycle at 72 °C for 7 min. The extension time of seven minutes was employed to increase the yield of the 'large' genes (P-genes). Using the relatively long extension time ensures that full-length PCR fragments are produced in the situation where one reaction resulting in a small fragment competes with a reaction that produces longer PCR fragments. Thus, the yield of HA-gene product is increased because by using the primer Bm-HA-1/Bm-NS-890R the generation of 'small' NS-fragments (900 bp) competes with generation of 'large' HA-fragments (∼1800 bp, Fig. 5B). The long extension times ensure that in addition to full length NS-fragment synthesis, the full length HA-products are produced in each PCR cycle. This standard PCR protocol allows the simultaneous amplification for all eight segments in parallel in one PCR machine (although for NS and M gene 2 min. extension time is sufficient). Thus, full-length PCR amplification of all eight segments can be performed within a few hours.

For sequencing of the segment specific noncoding regions of the N4, N5, N6, N7, and N8 subtypes, PCR-reactions were performed by using the forward primer Bm-fluA-1: TAT TCG TCT CAG GGA GCA AAA GCA GG which is complementary to the 12 conserved nucleotides (underlined) of the vRNA together with NA subtype specific reverse primers (data not shown) resulting in PCR-fragments of approximately 500 bp. The PCR products were cloned into pCR2.1-TOPO (Invitrogen) and two recombinant plasmids for each subtype were sequenced with M13 Reverse Primer and T7 primer. The 5′-end of the noncoding regions of the NA-vRNAs were determined by the same strategy by using subtype-specific forward primers (data not shown) and the reverse primer Bm-fluA-R: ATA TCG TCT CGT ATT AGT AGA AAC AAG G complementary to the 13 conserved nucleotides. The RT-PCR was performed under the same conditions as described above with the modifications that a lower annealing temperature (52 °C) and a shorter extension time (40 sec) was used. The primer design for the N3-neuraminidase gene was based on results from direct sequencing of viral RNA (Scott Krauss, personal communication). The primer design for the N9-neuraminidase gene was based on the previously published sequence of A/whale/Maine/1/84 (H13N9) (Genebank Accession Nr M17812).

Sequencing

The Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital determined the sequence of template DNA by using synthetic oligonucleotides and rhodamine or dRhodamine Dye-Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA polymerase FS (Perkin-Elmer, Applied Biosystems, Inc. [PE/ABI], Foster City, CA). Samples were subjected to electrophoresis, detection, and analysis on PE/ABI model 373, model 373 Stretch, or model 377 DNA sequencers.

Results

Design of oligonucleotides for RT-PCR

The influenza A genome is composed of eight vRNA segments with negative polarity. The genome of A/PR/8/34 (H1N1) is 13,588 nucleotides long: the polymerase (P) genes PB1 and PB2 each consist of 2341 nucleotides, and PA consists of 2233 nucleotides; the HA gene, 1778 nucleotides; the nucleoprotein (NP) gene 1565 nucleotides; the neuraminidase (NA) gene 1413 nucleotides, the matrix (M) gene, 1027 nucleotides; and the nonstructural (NS) gene, 890 nucleotides. The vRNA segments consist of noncoding and coding regions. The noncoding region at the 5′ termini of the vRNA are 20 to 58 nucleotides long; the length at the 3′ ends range from 19 to 45 nucleotides (Fig. 1). On the basis of sequence differences between each segment, the noncoding regions can be divided into two parts. The first part contains 13 conserved nucleotides at the 5′-end and 12 conserved nucleotides at the 3′-end. These nucleotides are highly conserved among all vRNA segments of all influenza A viruses [7, 26]. The second part of the noncoding region is unique for each segment. Using the sequences of the entire noncoding regions of the PR8-virus for each segment, a BLAST search in the Los Alamos Influenza database (http://www.flu.lanl.gov) showed that for the segments encoding the internal proteins (PB2, PB1, PA, NP, M, NS) the noncoding regions are identical in length. Alignment of the available sequences by this method revealed that one to three nucleotide differences are found among the different viruses. The segments encoding the HA and NA surface glycoproteins show a greater variability both in length and in sequence and most full-length sequence data is only available for H1 or H3 and N1 or N2 segments.

Since no substitutions were found in the 14 or 15 (3′-end) and 21 (5′-end) terminal nucleotides by these analyses, we sought to use only those regions for primer design (Fig. 1A). To evaluate whether those minimal segment-specific regions are sufficient for specific amplification of each segment by RT-PCR, we designed primers that are complementary to those conserved parts of the noncoding region at the 3′-end. To increase the annealing temperature used in PCR, we added noninfluenza virus sequences at the 5′-end of the primers. According to this design, the primers for amplification of different segments differ only by two or three nucleotides at the 3′-end (Fig. 1B, Table 1). We performed a two-step RT-PCR: The first step was reverse transcription, which was performed by using the Uni12 primer that is complementary to the 12 conserved nucleotides at the 3′-end of the vRNA. The second step was PCR in which the reverse-transcription product(s) was amplified by segment-specific primers (Fig. 2).

RT-PCR analysis of the internal genes (NS, M, NP, PA, PB1, and PB2)

To evaluate whether the primers with minimal nucleotide differences allow the specific full-length amplification of the corresponding gene, we performed RT-PCR with primers specific for each segment. The result by using this primer

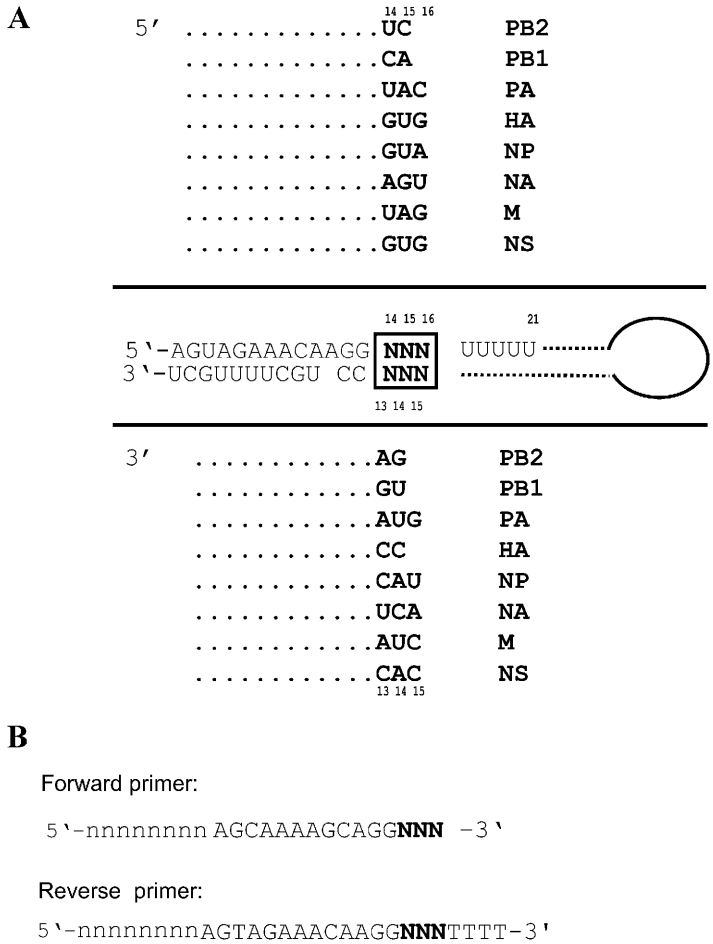

Fig. 1. Schematic representation of the conserved terminal regions of the eight segments of influenza A vRNA and the universal primers. **A** The noncoding regions of the vRNA segments differ in length and sequence, but are characteristic for each of the eight segments. The 5′ terminus of each influenza A vRNA segment has 13 conserved nucleotides, and the 3′ terminus has 12. The region from positions 14 through 16 of the 5′-end and positions 13 through 15 are shown for all eight segments and are (except for the NA sequence, see Fig. 4) conserved between all influenza A virus subtypes. Note that those regions are shown in a double-stranded conformation (Ns in the box). All eight segments contain an oligo-U sequence which is important for polyadenylation of the mRNAs. The dotted line represents the noncoding region characteristic for each segment. The half circle represents the coding sequence. **B** Primers were designed with sequences complementary to the conserved segment-specific influenza virus sequences (indicated by N) at the 3′-end and with noninfluenza nucleotides that increase the annealing temperature (represented by n′s) at the 5′-end. The sequences of the primers used to amplify each segment are shown in Table 1

set for amplification of A/Ck/HK/YU562/2001 (H5N1) is shown in Fig. 3. Under the PCR conditions applied (for details see Materials and methods) the differences at the 3′ end of the oligonucleotides are sufficient to specifically amplify the six internal genes. The use of seven minutes extension time is based on our

Table 1. Primer set used for RT-PCR amplification of the eight vRNAs of influenza A viruses

Gene	Forward primer	Reverse primer	Expected size [bp]
PB2	**Ba-PB2-1:** TATTGGTCTCAGGG**AGCGAAAGCAGGTC**	**Ba-PB2-2341R:** ATATGGTCTCGTATT**AGTAGAAACAAGGTCGTTT**	2341 + 29
PB1	**Bm-PB1-1:** TATTCGTCTCAGGG**AGCGAAAGCAGGCA**	**Bm-PB1-2341R:** ATATCGTCTCGTATT**AGTAGAAACAAGGCATTT**	2341 + 29
PA	**Bm-PA-1:** TATTCGTCTCAGGG**AGCGAAAGCAGGTAC**	**Bm-PA-2233R:** ATATCGTCTCGTATT**AGTAGAAACAAGGTACTT**	2233 + 29
HA	**Bm-HA-1:** TATTCGTCTCAGGG**AGCAAAAGCAGGGG**	**Bm-NS-890R:** ATATCGTCTCGTATT**AGTAGAAACAAGGGTGTTTT**	1778 + 29
NP	**Bm-NP-1:** TATTCGTCTCAGGG**AGCAAAAGCAGGGTA**	**Bm-NP-1565R:** ATATCGTCTCGTATT**AGTAGAAACAAGGGTATTTTT**	1565 + 29
NA	**Ba-NA-1:** TATTGGTCTCAGGG**AGCAAAAGCAGGAGT**	**Ba-NA-1413R:** ATATGGTCTCGTATT**AGTAGAAACAAGGAGTTTTTT**	1413 + 29
M	**Bm-M-1:** TATTCGTCTCAGGG**AGCAAAAGCAGGTAG**	**Bm-M-1027R:** ATATCGTCTCGTATT**AGTAGAAACAAGGTAGTTTTT**	1027 + 29
NS	**Bm-NS-1:** TATTCGTCTCAGGG**AGCAAAAGCAGGGTG**	**Bm-NS-890R:** ATATCGTCTCGTATT**AGTAGAAACAAGGGTGTTTT**	890 + 29

The sequences complementary to the influenza sequences are shown in bold. The underlined nucleotides at the 3′-end represent the segment specific sequences. The 5′-end has recognition sequences for the restriction endonucleases *Bsm*BI (Bm) or *Bsa*I (Ba). These sequences increase the annealing temperature and allow the cloning of the PCR products into the cloning vector pHW2000. The HA reverse primer is identical to the NS reverse primer. Abbreviations: polymerase genes, PB2, PB1, PA; hemagglutinin gene, HA; nucleoprotein gene, NP; neuraminidase gene, NA; matrix gene, M; nonstructural gene, NS. The expected size of the PCR-products are based on the genome of A/PR/8/34 (H1N1)(+ non influenza sequences) and may vary slightly for other influenza A viruses

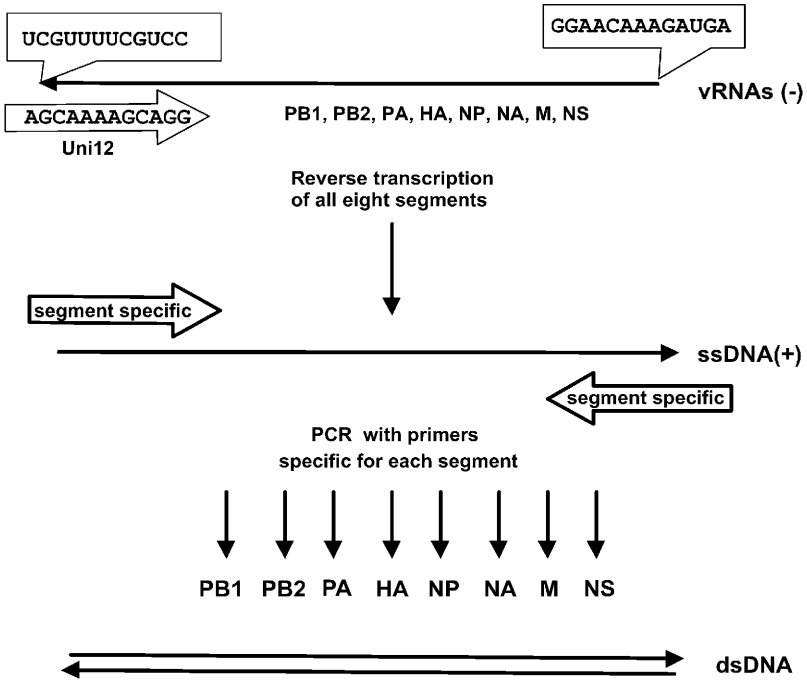

Fig. 2. Schematic representation of the RT-PCR methods. Viral RNA was purified from virus particles, and the eight negative-sense vRNAs were reverse transcribed with the Uni12 primer, which is complementary to the 12 conserved nucleotides at the 3′-end of the vRNA (the sequence is in the box on the left; the conserved sequence at the 5′end is in the box on the right). The single-stranded DNA (ssDNA) was then amplified by PCR using a primer pair complementary to the segment-specific regions. The sizes of the RNA segments range from 890 nucleotide (NS segment) to 2.341 nucleotides (PB1 segment)

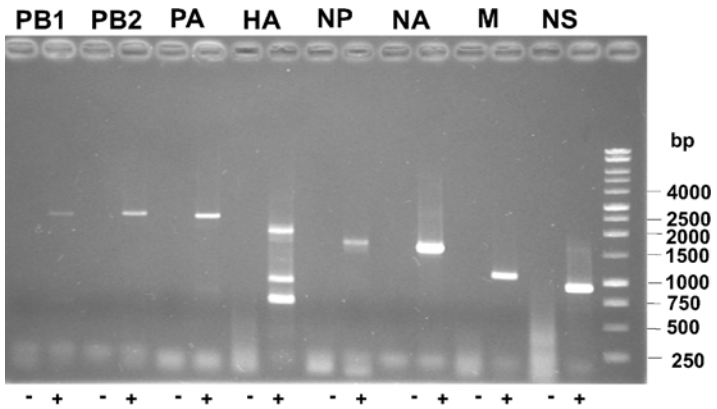

Fig. 3. Full length amplification of all eight segments by RT-PCR. RNA from A/Ck/HK/YU562 (H5N1) was isolated, and reverse transcription was performed with Uni12 primer. Subsequently, PCR reactions for each segment were performed by using the primer pairs presented in Table 1. As a negative control (−) 1 µl H_2O was added instead of 1 µl of the RT-reaction (+) to the PCR reaction. 10% of the PCR reactions were subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide

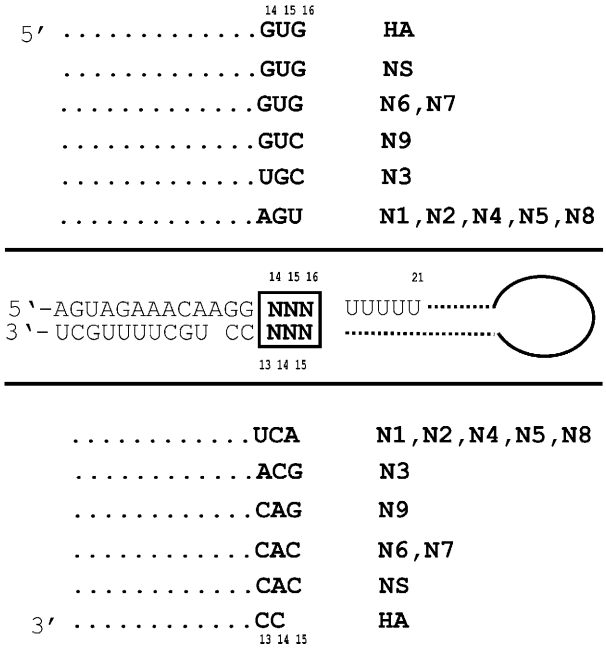

Fig. 4. Segment specific regions of the neuraminidase genes. The neuraminidase genes, unlike the other seven segments, differ in the segment specific regions (position 13–15 at the 3′ end and position 14–16 at the 5′ end) among the various NA subtypes. N3 and N9 subtypes have unique sequences, whereas N6 and N7 genes have identical sequences to the sequences found in the NS gene

observation that the yield of the P-genes is increased with longer extension times. These results demonstrate how the six internal genes of influenza A virus can be specifically amplified in six different reactions.

The noninfluenza virus sequences added at the 5′-end of the primers were engineered with restriction sites to facilitate cloning into the plasmid pHW2000, which is the cloning vector used for the eight plasmid system [15]. Alternatively to primers containing restriction sites of the type IIs restriction enzymes *Bsm*BI ($CGTCTCN_1/N_5$) and *Bsa*I ($GGTCTCN_1/N_5$) we have successfully employed primers which contain recognition sequences for the enzyme *Aar*I ($CACCTGCN_4/N_8$). Since many influenza virus genes have *Bsm*BI or *Bsa*I sites or both, digestion of PCR products with these enzymes results in multiple fragments that make cloning cumbersome. The use of 'the rare cutting' *Aar*I eliminates the problem of having multiple fragments and thus improves the efficiency of cloning. The use of the *Aar*I site adds 5 nucleotides to the primers presented in Table 1 having little effect in the yield of PCR product obtained compared to those containing the *Bsm*BI site (data not shown).

Amplification of the genes encoding hemagglutinins

The genes encoding the surface glycoproteins HA and NA are the most heterogeneous. Alignment of the noncoding regions of all HA sequences available in the Influenza Database at Los Alamos revealed that only the sequence CC (positions 13 and 14) in the 3′ noncoding region is conserved among all 15 HA subtypes (Fig. 1). The sequence GUG (positions 14 through 16) at the 5′-end of the HA gene is also found at the 5′-end of the NS gene. The results from PCR reactions using the primer pair Bm-HA-1/Bm-NS-890R are shown in Fig. 5B. For all nine

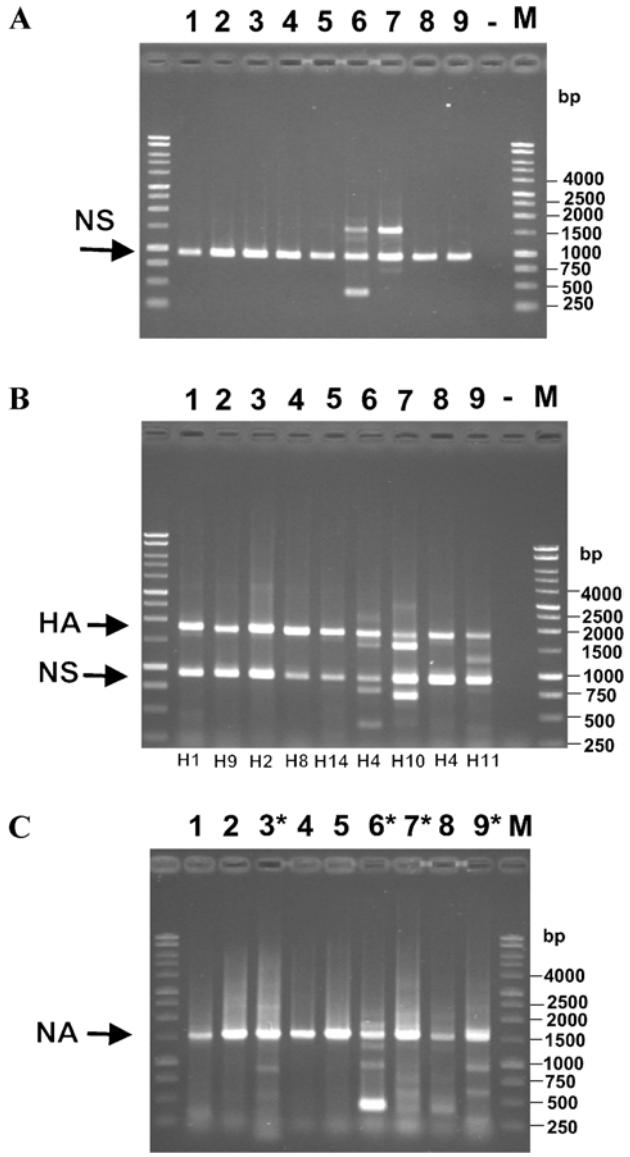

Fig. 5. Amplification of the NS, HA and NA segments for viruses representing nine different neuraminidase subtypes. The virus strains representing the nine neuraminidase subtypes (1–9) used for RT-PCR are shown in Table 2. **A** PCR reactions using primer pair Bm-NS-1/Bm-NS-890R. **B** PCR reactions using primer pair Bm-HA-1/Bm-NS-890R. **C** PCR reactions using primer pair Bm-NA-1/Bm-NA-1413R (*1, 2, 4, 5, 8*). For N3, N6, N7 and N9 (*3, 6, 7, 9*), specific primers for each subtype were used (Table 3)

analysed viruses full length HA products were generated. In addition, to the HA-fragments, NS fragments were produced, because NS and HA have the identical GUG sequence between the conserved part and the oligo-U sequence (Fig. 1A). These results demonstrate that the designed primers specific for HA genes in all influenza A viruses can be used to amplify the full-length cDNA of HA. To overcome the co-amplification of the NS gene we have successfully employed

Table 2. Influenza A viruses used for RT-PCR

NA subtype	Virus isolate
1	A/New Caledonia/20/99 (H1N1)
2	A/Quail/HK/G1/97 (H9N2)
3	A/Duck/Germany/1215/73 (H2N3)
4	A/Ty/Ontario/118/68 (H8N4)
5	A/Mallard/Astrakan/263/82 (H14N5)
6	A/Duck/Czech/56 (H4N6)
7	A/Chicken/Germany 'N'/49 (H10N7)
8	A/Duck/HK/Y264/97 (H4N8)
9	A/Duck/HK/P50/97 (H11N9)

forward primers that have additional HA-subtype-specific nucleotides at their 3′-ends. Only one full-length HA-product is generated because those additional nucleotides do not hybridize with the NS sequences (data not shown). The latter approach is very useful in situations where the HA subtype was already determined by HI-assay. However, the advantage of the 'universal' HA-primer pair (Bm-HA-1/Bm-NS-890R) is that all HA genes can be amplified without determining the subtype before the RT-PCR.

Amplification of the genes encoding neuraminidases

Analysis of NA sequences indicated that the three nucleotides AGU at the 5′-end (positions 14 to 16) and the complementary nucleotides UCA at the 3′-end (positions 13 to 15) are conserved among all of the N1 and N2 subtypes. With the primer pair Ba-NA-1/Ba-NA-1413R, we were able to amplify the NA genes of the N1, N2, N4, N5 and N8 subtypes. However, we could not generate specific products of the expected size (∼1.5 kb) for N3, N6, N7 and N9 subtypes (data not shown). Because these results were obtained by using exactly the same reaction conditions, failure of the PCR amplification was caused most likely by mispriming having the latter subtypes different sequences in these regions. Indeed, the additional ∼1.5 kb fragments obtained by using the primer pair Bm-NS-1/Bm-NS-890R in reactions where N6 and N7 viruses were used, suggested that the N6 and N7 neuraminidase genes might have similar or identical sequences to the NS segment in these regions (Fig. 5A, lane 6, 7). Therefore, we sequenced the noncoding regions of the N3, N4, N5, N6, N7 and N8 neuraminidase genes. As shown in Fig. 4 four different sequences can be distinguished in the positions 14–16 at the 5′ and 13–15 at the 3′ end, respectively.

Based on those sequences, specific primers for N3, N6, N7 and N9 neuraminidase genes were designed (Table 3). Because in N6 and N7 subtypes the sequences at 3′-termini of the vRNA are the same as in the NS gene (Fig. 4) as a reverse primer the primer Bm-NS-890R was used. To reduce the amplification of the NS gene and increase the specificity for the N6 and N7 neuraminidase genes, the sequence of the forward primer was extended to include the start codon. PCR

Table 3. Primers used for the amplification of the Neuraminidase genes

NA subtype	Forward primer	Reverse primer
N1	Ba-NA-1 TATTGGTCTCAGGG**AGCAAAAGCAGGAGT**	Ba-NA-1413R ATATGGTCTCGTATT**AGTAGAAACAAGGAGTTTTTT**
N2	Ba-NA-1	Ba-NA-1413R
N3	Bm-N3-1 TATTCGTCTCAGGG**AGCAAAAGCAGGTGC**	Bm-N3-1420R ATATCGTCTCGTATT**AGTAGAAACAAGGTGCTTTTT**
N4	Ba-NA-1	Ba-NA-1413R
N5	Ba-NA-1	Ba-NA-1413R
N6	Bm-N6-1 TATTCGTCTCAGGG**AGCAAAAGCAGGGTGAAAATG**	Bm-NS-890R
N7	Bm-N7-1 TATTCGTCTCAGGG**AGCAAAAGCAGGGTGATTGAGAATG**	Bm-NS-890R
N8	Ba-NA-1	Ba-NA-1413R
N9	Bm-N9-1 TATTCGTCTCAGGG**AGCAAAAGCAGGGTC**	Bm-N9-1473R ATATCGTCTCGTATT**AGTAGAAACAAGGGTCTT**

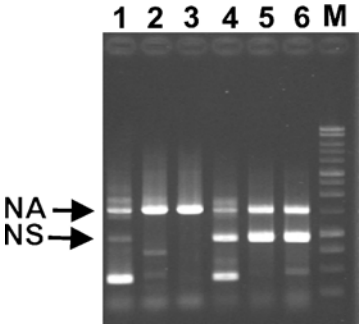

Fig. 6. Amplification of N6 neuraminidase genes. *1–3* PCR reaction with primer pair Bm-N6-1/Bm-NS-890R, *4–6*: PCR reaction with Bm-NS-1/Bm-NS-890R. The following viruses were used: *1, 4* A/Duck/Czech/56 (H4N6); *2, 5* A/Duck/HK/526/79 (H3N6); *3, 6* A/Duck/HK/365/78 (H9N6)

reactions performed with those subtype specific primers resulted in a specific amplification of N3, N6, N7 and N9 neuraminidase genes (Fig. 5C lane 3, 6, 7, 9). The PCR result by using the primer pair (Bm-N6-1/Bm-NS-890R, Table 3) for three different strains of N6 subtypes are shown in Fig. 6. The generation of full-length NA PCR fragments for all three viruses suggests that this strategy is suitable for all N6 subtypes. These results show that with five different primer pairs all nine neuraminidase genes can be amplified.

Discussion

Choosing primer sequences is the most important variable for determining the sensitivity and specificity of RT-PCR based methods. In this report, we demonstrated that all eight segments of the influenza A genome are amplified by RT-PCR using a set of universal primers, that differ by only two or three residues at their 3′-end. Three lines of evidence indicate that the universal primer set is applicable to all influenza A virus subtypes. First, the primer set was used to successfully amplify genes from a variety of influenza A viruses isolated from different species (i.e., humans, ducks, chickens, quail). Second, the Influenza database shows unique sequences in the termini of each gene segment of the influenza A viruses; only C or U residues are found at position 4 of the 3′-end. Our primers for the NP, PA, PB2, and PB1 segments were designed with a G-residue and the primers for the NS, M, NA, and HA gene have an A-residue. Third, mutational analyses have shown that the vRNA promoter forms a secondary structure with a double-stranded conformation in the segment-specific region [9, 10, 18]. One point mutation that disrupts this double-strand conformation probably results in nonviable variants. Therefore, to compensate for a point mutation, a second mutation would have to occur simultaneously during replication to retain the double-stranded conformation, an event that is highly unlikely. Thus, there is selection pressure for retaining those sequences for optimal virus replication and transcription.

HI and neuraminidase inhibition (NI) assays are commonly used to determine the subtype of influenza A viruses. The preparation of antisera used for HI or NI assays is time-consuming and the separation into different subtypes by these assays is sometimes error prone. Our RT-PCR results (Fig. 3A, lane 6 and 7) suggest that the primer pair Bm-NS-1/Bm-NS-890R originally designed for amplification of the NS-gene can now be used to identify N6 and N7

subtypes. For HA subtyping the universal HA-primer can be used to amplify the hemagglutinin gene, subsequently the subtype can be determined by sequencing. Alternatively, TaqMan-PCR [23] could be employed by using a universal HA-primer pair (i.e., Bm-HA-1/Bm-NS-890R) and subtype-specific HA primers. Because the RT-PCR-based techniques are amenable to automation and provide alternatives to less efficient HI and NI assays, these techniques may be used in the future for rapid identification and characterization of all 15 HA and 9 NA subtypes.

Adeyefa et al. [1] have described a multiplex RT-PCR method in which 12 mer and 13 mer oligonucleotides complementary to the conserved regions were used resulting in simultaneous amplification of all of the eight RNA segments. Unlike those primers, the primer set that we used, allow the specific amplification of each of the eight segments. The RT-PCR method based on the 12 and 13 conserved nucleotides is not applicable for cloning the eight segments because PB1 and PB2 have the same size and cannot be separated by gel electrophoresis. The primers that we developed overcome this disadvantage by adding segment-specific nucleotides which now result in the amplification of a targeted segment. Only for HA or certain NA subtypes multiple PCR products are produced which can be separated by gel electrophoresis. Unlike the primers described by Zou [34] the primers in our experiments have minimal length of the segment specific region at the 3′ end and contain non-influenza virus sequences at the 5′-end that allow the cloning of the amplified products into plasmids after digestion of these fragments with restriction enzymes (*Bsm*BI, *Bsa*I or *Aar*I). Thus, the products can be used for sequence analysis or subtyping or for cloning all segments into the plasmid pHW2000 [15]. Because cloning is the most time-consuming aspect of this technology, the use of universal primers suitable for all influenza A viruses simplifies this procedure. After cloning the eight plasmids, we can generate recombinant infectious viruses within one to three days [15]. Universal primers could be used to create a representative collection of plasmids representing all different influenza subtypes and their variants. In pandemic situations where a new virus subtype emerges, we could either use the plasmid collection already available or quickly generate plasmids with the same or similar sequences, and use it to develop antiviral therapies (i.e., vaccines). Thus, a universal primer set is a powerful tool that can be used in classic and reverse genetics methods to prevent and contain future influenza A epidemics and pandemics.

Acknowledgements

This study was supported by Public Health Research Grants AI-95357 and AI-29680 from the National Institute of Allergy and Infectious Diseases, by Cancer Center Support (CORE) Grant CA-21765, and by the American Lebanese Syrian Associated Charities (ALSAC). We thank Alan Hay for providing the influenza A virus A/New Caledonia/20/99 (H1N1) and Kennedy F. Shortridge for providing the avian H5N1 virus (A/Ck/HK/YU562/2001). We gratefully acknowledge the excellent technical support provided by Scott Krauss and David Walker.

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Received July 2, 2001