Detection of Influenza A and B in Respiratory Secretions with the Polymerase Chain Reaction

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Influenza A and B are RNA-containing viruses that frequently infect humans. Currently, sensitive detection of these viruses requires fresh respiratory secretions and special facilities for culture. To facilitate diagnosis of influenza, the polymerase chain reaction (PCR) was used in the present studies to detect DNA produced by reverse transcription of influenzal RNA in vaccines, tissue culture fluids, and stored respiratory secretions. Primers were directed at targets on the highly conserved segment 7 (matrix gene) of influenza A (212-bp product) and B (365-bp product). The primers were completely type specific. Critical variables in the assay were the concentration of pleotropic salts used during preparation of samples, the use of carrier RNA and RNase inhibitors during sample preparation, and the use of optimum K⁺ and Mg²⁺ levels at each step. Studies of 33 patients with symptoms of viral respiratory infection whose nasal washes had been cultured and frozen for up to 1 year before assay showed that PCR provided type-specific detection of influenza with a sensitivity comparable to that of culture of the fresh secretions. The assay offers a powerful test for detection of devitalized influenza viruses and may be useful in both diagnostic work and epidemiological studies of influenza.

Influenza A and B are major causes of respiratory infection. Because the surface proteins of influenza A are highly mutable, infection with this type is associated with pandemic disease in humans. In contrast, influenza B causes sporadic, endemic disease. Type-specific identification of the viruses in clinical material is important because specific prophylaxis and treatment are available only for influenza A.

Influenza viruses are readily identified by culture of respiratory secretions, but culture requires fresh, unfrozen respiratory samples and appropriate culture facilities, which are often available only at major medical centers. Devitalized influenza virus, such as that in frozen respiratory secretions, can be detected by immunological techniques, but these methods are much less sensitive than culture.^(1,2) The polymerase chain reaction (PCR) has the potential to detect nonviable influenza virus with a sensitivity comparable to culture by amplifying DNA obtained from influenzal RNA in respiratory secretions. We report here results with a PCR assay designed to detect influenza A and B viruses in a variety of samples, including frozen human respiratory secretions.

METHODS

Virus

Whole virus vaccines of influenza A/Leningrad/x-91 (H3N2), A/Taiwan/89 (H1N1), A/Bangkok/1/79 (H3N3),

B/Ann Arbor/1/86, B/Singapore/84, and B/Yamagata/89 were kindly provided by Connaught Laboratories (Swiftwater, PA). Influenza A/Weiss/43 and B/Lee/40 were obtained from the American Type Culture Collection (Rockville, MD).

Clinical Samples

Nasal washes in normal saline solution were obtained by Dr. Robert Betts (University of Rochester) from 33 individuals with symptoms or viral upper respiratory infection in December, 1989. Viral cultures of the washes were obtained and the remainder of the samples then were stored at -20°C. Influenza A was cultured from washes of 14 of the patients, whereas 5 patients grew other respiratory viruses (2 adenovirus and 3 respiratory syncytial virus; 14 patients had sterile cultures. In December, 1990, the stored washes were shipped to Lexington, Kentucky, in dry ice for assay by PCR. Bronchoalveolar lavage fluids from 1 patient with culture-documented influenza B and 2 patients who had sterile viral cultures were provided by Dr. Calvin Linemann of the University of Cincinnati (Cincinnati, OH).

Primers

Primers were selected from data on multiple isolates of influenza A and B on file in GenBank and the European Molecular Biology Sequence Database (Gene/Protein Sequence Database,

Hatachi Software, Egen Co. Ltd., and America Ltd., San Bruno, California). The Oligo 4.0-2.1 program (National Biosciences, Hamel, MN)⁽³⁾ was used for analysis of the primers, and particularly for estimating their potential for inter- and intramolecular bonding.

Sample Processing, RNA Extraction, and Reverse Transcription

Nine parts of denaturing solution containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol was added directly to one part of frozen or unfrozen sample. Yeast tRNA was added during thawing and mixing. Phenol-chloroform extraction, followed by ethanol precipitation and vacuum centrifugation of the RNA-containing aqueous phase was carried out by the single step method of Chomczynski and Sacchi.⁽⁴⁾

DNA was produced from viral RNA by reverse transcription. The reaction mixture contained the following: 4 μ l of 5x buffer (Reverse Transcriptase Buffer, Life Technologies, Inc., Bethesda Research Laboratories, Gaithersburg, MD, pH 8.3, with 250 mM Tris-HCl, 375 mM KCl, 50 mM DTT, and 15 mM $MgCl_2$), 100 pmoles of complementary primer, up to 30 μ g of RNA (including carrier), 20 units of RNasin (Promega Corporation, Madison WI), 0.5 mM of each dNTP, and 200 units of reverse transcriptase (Mo-MLV; Life Technologies, Inc., BRL). The mixture was heated at 37°C for 1 hr and the reaction was terminated by heating at 95°C for 5 min.

PCR

For PCR, the entire volume of the reaction mixture from reverse transcription (20 μ l) was mixed with the following: 10 μ l of 10x buffer (PCR Buffer, Perkin-Elmer Cetus, Norwalk, CT, containing 100 mM Tris-HCl, pH 8.3, with 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin), 0.1 mM of each dNTP, 100 pmoles of each primer, and 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). Final concentrations of the reactants were: 20.3 mM Tris-HCl, 2.1 mM $MgCl_2$, 65.5 mM KCl, 2 mM DTT, 100–200 μ M for each deoxynucleotide, 1–2 μ M for each primer, 1 mM NaCl, 1% glycerol, and 0.0015 mM EDTA.

The optimal concentration of Mg^{2+}

for use in the final reaction mixture was determined by titration with primers for both influenza A and B and RNA extracted from A/Leningrad/x-91 and B/Ann Arbor/1/86 vaccines. Mg^{2+} concentrations of 0.6–10.0 mM were studied (requirements of the enzyme in the reverse transcription reaction imposed a minimum Mg^{2+} concentration of 0.6 mM). With primers for influenza A and Mg^{2+} concentrations of 0.6–1.25 mM, no specific PCR products were noted on gels after 40 cycles, whereas abundant specific product resulted when 1.5–2.1 mM Mg^{2+} was used. At 2.5–10 mM Mg^{2+} , there was declining but still discernable specific product formation; however, there was a large increase in nonspecific product formation above 2.5 mM Mg^{2+} . For influenza B, specific product formation peaked above 1.0 mM Mg^{2+} with noticeable nonspecific product formation above 2.5 mM Mg^{2+} . A Mg^{2+} concentration of 1.5–2.1 mM was selected as optimal for assays in which both sets of primers were present in a single tube.

Potassium was tested in a concentration range of 40.5 mM to 115.5 mM with the system for influenza B. Sensitivity increased throughout this concentration range. A level of K^+ of 65.5 mM was selected for routine use.

Based on analysis of optimal denaturing, annealing, and extension temperatures, routine PCR for detection of influenza was performed for 35 cycles with denaturation at 94°C for 1.5 min, annealing at 54°C for 2 min, and extension at 72°C for 1 min in a Perkin-Elmer Cetus Thermal Cycler (Norwalk, CT). Amplification products were identified by electrophoresis on 2% agarose (DNA grade, Biorad, Richmond, CA) by use of a DNA ladder of 123 bp (Life Technologies, Inc., BRL), followed by staining with ethidium bromide (1 μ g/ml) and visualization with ultraviolet light (UV Transilluminator; Fotodyne Corporation, New Berlin, WI).

Hybridization with ^{32}P -labeled Probe

DNA was denatured by shaking the submerged gel for 1 hr in 2 volumes of 1.5 M NaCl and 0.5 M NaOH. The gel was neutralized for 1 hr in 1 M Tris-HCl (pH 8.0) in 1.5 M NaCl. After Southern transfer,⁽⁵⁾ products of PCR on nitro-

cellulose filters were air-dried and heated at 80°C for 2 hr. Probes to the inner region of the amplification target were end-labeled with ^{32}P and hybridization was carried out as described elsewhere.^(6–8)

Sequencing of PCR Products

Sequencing was carried out with an asymmetric PCR reaction in which *Taq* polymerase was used to incorporate fluorescently labeled terminator deoxynucleotides (TAQ Cycle Sequencing Kit; Applied Biosystems, Foster City, CA). The sequence of the DNA was determined by use of DNA sequencing gels and a 373 DNA sequencer (Applied Biosystems, Foster City CA). PCR primers and hybridization probes served as primers for sequencing. PCR products were sequenced without cloning and in both directions.

RESULTS

Primers

The surface proteins of influenza A characteristically show genetic drift, and for this reason the more stable matrix gene⁽⁹⁾ was selected for amplification by PCR. The primers chosen were two unique sequences (A1, 25-mer; A2, 23-mer) that defined the boundaries of a highly conserved target of 212 bases on segment 7 of A/Bangkok/1/79 (H3N2) (Fig. 1) (AC# KO1140).⁽¹⁰⁾ A search of the pertinent files on influenza in the database indicated primer A1 had 100% homology with 5 of 9 available sequences [i.e., A/Udorn/72 (H3N2) (AC# JO2167), A/Camberra/77 (H3N2) (AC# JO2097), A/Ri/5/57 (H2N2) (AC# JO2155), A/Loyang/4/57 (H1N1) (AC# JO2128), and A/Fort Warren/50] (H1N1) (AC# JO2125)] and at least 94% homology with the remaining sequences in the file [A/Puerto Rico/34 (H1N1) (AC# XO5905), A/WSN/33 (H1N1) (AC# M19374), A/Rostock/34 (H1N1) (AC# M12699), and A/Mallard/NY/78 (H2N2) (AC# M12699)]. The introduction of a single nucleotide degeneracy in A1 (adenosine for thymidine at position 124) provided 100% homology with all 10 influenza A isolates in the database when both the original and degenerate forms of the primers were used in PCR.

The A2 primer selected (Fig. 1) was

INFLUENZA A/BANGKOK/1/79, SEGMENT 7

FIGURE 1 Schematic representation of segment 7 (matrix gene) of influenza A/Bangkok/1/79 showing the position of two primers, A1 and A2, used for amplification of a target of 212 bases by PCR. To maximize homology with known influenza A isolates, each primer was used in PCR in combination with a degenerate form containing adenosine (as illustrated). AH2 is the structure of the hybridization probe of the inner region of the PCR target.

100% homologous with A/Bangkok/1/79 and A/Udorn/72 (AC# JO2167). By substitution of adenosine for guanosine at position 320 (Fig. 1), a degenerate form was created which had 100% homology with A/Puerto Rico/34 (AC# JO2145) and A/WSN/33 (AC# M193), and the homology for A/Rostock/34 (AC# XO5905) was increased from 91% to 96%. The homology of both forms of primer A2 for A/Mallard/NY/78 (AC# M12699) was 87%.

To permit assay of both influenza A

and B in the same tube for the PCR, primers for influenza B were selected that had minimum molecular bonding with A1 and A2 and gave a larger product size (365 bases) than that of influenza A. The target selected was position 100-464 on the matrix gene of segment 7 of influenza B/Lee/40 (AC# JO2094) (Fig. 2).⁽¹¹⁾ The primers for this target (B1, 23-mer; B2B, 26-mer) were 100% homologous with the three isolates of influenza B in the database [i.e., B/Lee/40, B/Ann Arbor/1/86 (AC# M20175-6), including wild-type and

INFLUENZA B/LEE/40, SEGMENT 7

FIGURE 2 Schematic representation of segment 7 (matrix gene) of influenza B/Lee/40 showing the position of two primers, B1 and B2B, used for amplification of a target of 365 bases by PCR. HybB365 was used as a radiolabeled probe of the inner region of the target.

cold-adapted viruses, and B/Singapore/79 (AC# M14909)]. Therefore, the use of degenerate primers was unnecessary.

The potential for cross-reactivity between primers for influenza A and B was studied. A search for the 100 best homologies to segment 7 of B/Lee/40 indicated that the B1 primer had only 44% homology with 5 of 7 isolates of influenza A. It had only 40% homology with A/Rostock/34 (AC# XO5905) and 48% homology with A/USSR/90/77 (H1N1) (AC# K01330). Similar results were obtained with primer B2B, where homologies for all isolates were less than 43%. Homologies between A primers and influenza B sequences were all below 50%.

Detection of Influenza A

RNA was extracted from influenza vaccine (A/Leningrad/x-91), from monkey kidney cell cultures infected with A/Weiss/43, and from nasal washes of a patient with culture-proven influenza (H1N1 isolate of influenza A), as well as a variety of controls. Viral DNA was produced by reverse transcription with the A2 primer, and PCR was then performed for 35 cycles. The amplification products and controls were electrophoresed on 2% agarose, stained with ethidium bromide and visualized with ultraviolet light. Results (Fig. 3) showed that products of about 212 bp were obtained with influenza A vaccine, with infected monkey kidney cells, and with nasal secretions of a patient infected with influenza A. No such products were obtained with various controls, including samples containing all reactants except reverse transcriptase (Fig. 3) and samples containing influenza A but reacted with primers for influenza B rather than A (data not shown). The specificity of the products of 212 bp was established with a ³²P-labeled probe (Fig. 3).

Detection of Influenza B

RNA was extracted from influenza vaccine (B/Yamagata/89), monkey kidney cells infected with B/Lee/40, bronchoalveolar lavage fluid of a patient with culture-documented influenza B, and a variety of controls. After reverse transcription with B2B, PCR, and gel electrophoresis, amplification products of the expected size (365 bp) were found with the samples containing in-

FIGURE 3 (Left) Amplification products of influenza A on gels stained with ethidium bromide. (Lane 1) DNA "sizing" ladder (123 bp); (lane 2) nasal washes from a patient without influenza; (lane 3) influenza A-infected kidney cells as in lane 7 but without reverse transcriptase; (lane 4) uninfected monkey kidney cells; (lane 5) vaccine as in lane 8 but without reverse transcriptase; (lane 6) nasal wash from a patient with culture-documented influenza A; (lane 7) monkey kidney cells infected with A/Weiss/43; (lane 8) whole virus vaccine, A/Leningrad/x-91. (Right) Autoradiogram of the gel after Southern transfer and hybridization with a ^{32}P -labeled probe, AH2, of the inner region of the influenza A target.

Sequencing of Specific Products

Sequencing confirmed that the size of the specific PCR product of influenza A (H1N1) in infected nasal secretions was 212 bp. The size of the product of B/Ann Arbor/1/86 vaccine was confirmed as 365 bp. The sequence of the product for influenza A differed from the model sequence (i.e., A/Bangkok/79) by 3 nucleotides, whereas the products of influenza B/Ann Arbor/1/86 differed from the model sequence (B/Lee/40) by 20 nucleotides.

Clinical Trial of PCR in Influenza A

Frozen nasal washes were available from 33 patients who had had symptoms of viral respiratory infection and viral cultures taken approximately 1 year earlier. Disassociation buffer was added directly to the frozen samples and they were assayed for both influenza A and B in PCR. Of 14 patients whose washes grew influenza A, 13 gave products of about 212 bp in PCR

fluenza B (Fig. 4). Negative controls (Fig. 4) included samples with influenza B and all reactants except reverse transcriptase or those reacted with A primers rather than B primers. Bronchoalveolar lavage fluids from 2 patients whose viral cultures were negative for influenza were negative in PCR (data not shown). An autoradiogram with a ^{32}P -labeled probe (HybB365) confirmed the specificity of all products of 365 bp as influenza B (Fig. 4).

Simultaneous Detection of Influenza A and B in a Single Tube

Samples of DNA derived from influenza A/Taiwan/89 vaccine and influenza B/Ann Arbor/1/86 vaccine were mixed in a single tube containing primers A1, A2, B1, and B2B and reacted in PCR. A tube containing DNA of A/Taiwan/89 vaccine and primers A1 and A2 and a similar tube with DNA of B/Ann Arbor/1/86 and the B1 and B2B primers were assayed as positive controls. Results (Fig. 5) showed that the tube with influenza A and B and both sets of primers yielded products of about 212 bp and 365 bp, indicating independent amplification of the targets of influenza A and B (Fig. 5).

FIGURE 4 (Left) Ethidium bromide-stained gel with amplification products of PCR of influenza B. (Lane 1) 123-bp ladder; (lane 2) lung washes from a patient with culture-documented influenza B but with influenza A primers substituted for B primers; (lane 3) lung washes as in lane 2 with A primers but without reverse transcriptase; (lane 4) uninfected monkey kidney cells; (lane 5) B/Yamagata whole virus vaccine with B primers but without reverse transcriptase; (lane 6) lung washes from a patient with culture-documented influenza B with B primers; (lane 7) monkey kidney cells infected with B/Lee/40; (lane 8) B/Yamagata vaccine. (Right) Autoradiogram of the gel after Southern transfer and hybridization with a ^{32}P -labeled probe to influenza B, HybB365.

FIGURE 5 Ethidium bromide-stained gel with amplification products of A/Taiwan/89 and influenza B/Ann Arbor/1/86. PCR of the two viruses was performed separately with type-specific primers (lanes 2 and 4, respectively) or with both viruses together in the same reaction tube with both sets of primers (lane 3). The sizing ladder is in lane 1.

and the specificity of the products was established by use of the ^{32}P -labeled probe for type A influenza (AH2) (Table 1). Washes of 5 patients that had grown other respiratory viruses (2 adenovirus and 3 respiratory syncytial virus) gave no such products. Washes of the remaining 14 patients whose cultures had been sterile were negative for both influenza A and B in PCR, except in 2 cases. Results with these 2 individuals showed products with strong bands of 212 bp, and these products reacted strongly with the AH2 probe for influenza A. The findings suggested that PCR could detect influenza A in some cases where cultures failed to yield the virus.

DISCUSSION

In the present studies, a target on segment 7 (matrix gene) of influenza A was used for amplification in PCR. There is frequent antigenic variation in the envelope proteins of influenza A,

whereas the sequence of the matrix gene is more conserved. To obtain maximum homology of the target with existing sequences for influenza A in the database, a single degeneracy was introduced into the A1 and A2 primers. This maneuver has been used to broaden the reactivity of primers in other systems.⁽¹²⁾ Here, it not only broadened the potential reactivity of primers and desired targets, but also may have decreased dimer formation in the case of the A2 primer. With the original A2 primer, dimer formation resulted in a molecular species with significant complementarity at the 3' ends:

The introduction of the degeneracy into A2 decreased the stability of this dimer as follows:

An alternative maneuver would have been to exclude the terminal base at the 3' end of the primer, which also would have decreased the stability of a dimer formed at the 3' end. However, the smaller primer might have been less effective in reverse transcription. In the case of influenza B, 100% homology was achieved without the use of a degeneracy. The primers for both influenza A and B appeared versatile in that they worked well in transcription and PCR with four different influenza A viruses and with three different influenza B viruses.

Optimum conditions for PCR were established by titration with representative isolates contained in influenza A (A/Taiwan/89) and influenza B (B/Ann Arbor/86) vaccines. As noted by others, the concentrations of Mg^{2+} and K^+ were important variables in the assay.⁽¹³⁾ Our findings with K^+ differ from those reported for an asymmetric PCR system used in sequencing. There, *Taq* polymerase was inhibited when the K^+ concentration was $\geq 75 \text{ mM}$.⁽¹³⁾ Such inhibition was not noted in our system, perhaps because it was not asymmetric, was not optimized for sequencing, and had little gelatin

TABLE 1 Detection of Influenza A in Nasal Washes of 33 Patients with Symptoms of Viral Respiratory Infection

PCR	Culture	
	positive	negative
Positive	13	2 ^a
Negative	1	17 ^b

^aPCR products in both cases were strongly reactive with the hybridization probe for influenza A.

^bThree patients grew rhinovirus and 2 grew respiratory syncytial virus. PCRs of all 33 washes were negative with primers specific for influenza B.

(0.0011%). In fact, specific product formation in our assay actually increased in direct proportion to the K^+ concentration up to the highest level tested (i.e., 115.5 mM).

The sequence of the PCR product of the H1N1 isolate of influenza A in clinical samples was found to differ from that of the model sequence (A/Bangkok/79) by only 3 nucleotides. In contrast, the PCR product of influenza B/Ann Arbor/1/86 differed in 20 of 365 nucleotides from the model sequence (B/Lee/40). This result was not unexpected, in that a comparison of published sequences of the same region of other, more recent isolates of influenza B shows appreciable genetic drift. For example, in the database, B/Ann Arbor/1/86 differs from B/Lee/40 by 9 nucleotides (each confirmed in the present study) and B/Singapore/222/79 differs by 17 nucleotides (13 of which were confirmed in the present work).

The present assay provided type-specific identification of influenza. The primers for type A were completely nonreactive with products of influenza B viruses and vice versa. Type specificity was established by the characteristic size of the PCR products on gels and by binding of type-specific probes. Type-specific identification of influenza is very important clinically because amantadine and its congeners, which are the only antimicrobials available for prophylaxis and treatment, affect only type A viruses. Typing is also important epidemiologi-

cally because influenza A is subject to major antigenic shifts and can cause pandemics while influenza B causes only sporadic disease.

The present assay detected influenza in respiratory secretions stored for up to 1 year at -20°C . The addition of disassociation buffer for inactivation of RNases just before the samples were thawed appeared satisfactory for routine use with frozen respiratory secretions in PCR. The success of the assay with such samples suggests that respiratory secretions can be collected without disassociation buffer and stored frozen until assay. The ability of the assay to detect influenza in stored samples makes it potentially very useful for epidemiological studies and for the diagnosis of influenza in areas that lack facilities for culture.

A clinical trial to assess the sensitivity of PCR in routine clinical use showed that PCR detected influenza A in 13 of 14 patients with positive cultures for influenza A and in 2 additional patients whose cultures did not grow any respiratory virus. Because of the strong bands of the products at 212 bp on gels and their strong binding of the type A hybridization probe, the products appeared specific for influenza A. It seems unlikely that positive results in culture-negative samples were due to contamination of the samples during assay because controls, including nasal washes of 5 patients with respiratory infection due to other kinds of respiratory viruses, remained negative. Taken together, these results indicate that the PCR method presented here can detect influenza in frozen samples with a sensitivity comparable to culture of the fresh material.

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A patent application has been submitted for primers and hybridization probes.

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