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Isolation and Characterization of a Naturally Occurring Parainfluenza 3 Virus Variant

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A parainfluenza 3 virus variant which failed to react with parainfluenza 3 virus-specific monoclonal antibodies from two commercial sources was isolated from a 14-month-old boy. Analysis of the coding region of the hemagglutinin-neuraminidase gene identified 36 nucleotide changes and 4 amino acid changes compared with a consensus sequence derived from strains isolated from 1957 through 1983. Two unique amino acid changes occurred at positions 174 and 283, which are close to identified epitopes in the hemagglutinin-neuraminidase protein. Ongoing viral surveillance to detect variants is important, particularly in regard to vaccine development.

Parainfluenza viruses are frequently recovered from pediatric patients with upper and lower respiratory tract infections (8). In our institution, 73% of all parainfluenza virus isolates are parainfluenza 3 virus (PIV3) (1). During the summer of 1994, we recovered a PIV3 variant that failed to react by immunofluorescence (IF) with monoclonal reagents from two commercial sources. This report details our identification and characterization of this isolate, designated MO/1/94, and discusses the importance of conventional viral isolation techniques for recognition of emerging variants.

MATERIALS AND METHODS

Patient history. In June 1994, a 14-month-old boy was presented to Cardinal Glennon Children's Hospital with a 3-day history of fever, cough, and wheezing. He was admitted with diagnoses of bronchiolitis and left otitis media, treated with a bronchodilator and antibiotics, and discharged after 2 days.

Virus isolation. A nasopharyngeal swab specimen collected at admission was inoculated into a human neonatal kidney (BioWhittaker, Walkersville, Md.), an MRC-5 (Viromed, Minneapolis, Minn.), and three primary rhesus monkey kidney (RhMK) cell culture tubes (Viromed and BioWhittaker). One tube of MRC-5 cells and two tubes of RhMK cells were incubated at 33°C, while one tube each of HNK, MRC-5, and RhMK, cells was incubated at 36°C. Cell culture tubes were examined daily for a cytopathic effect for the first 7 days (except Sunday) and once more at 10 days postinoculation (p.i.). Hemadsorption with 0.5 ml of a 0.5% guinea pig erythrocyte suspension at 4°C for 20 min was routinely performed on RhMK tubes at days 2, 7, and 10 p.i. for all respiratory specimens, regardless of the presence of a cytopathic effect.

IF staining. IF staining was performed with monoclonal antibody (MAb) preparations for PIV1 to PIV3 (Baxter Diagnostics, Deerfield, Ill., and Chemicon International, Inc., Temecula, Calif.), PIV4 (Chemicon), influenza A and B viruses (Imagen; Dako Corp., Carpinteria, Calif.), and measles and mumps viruses (Chemicon). Staining was performed in accordance with each manufacturer's instructions. MAbs 2E9, specific for a PIV3 protein of approximately 67,000 Da, and 4E5, specific for the 29,000- and 31,000-Da proteins of PIV3 (15), were also tested by IF against this isolate.

Enzyme immunoassay (EIA). The MO/1/94 isolate and prototype strain WASH/47885/57 were reacted with a panel of previously characterized MAbs to the PIV3 hemagglutinin-neuraminidase (HN) protein by EIA (6). Antibody blocking studies indicated that a minimum of three distinct epitope groups could be distinguished with this panel (data not shown).

Consensus sequence. Eight sequences of the PIV3 HN gene obtained from GenBank, including the prototype strain, Wash/47885/57 (5, 14), and seven strains isolated between 1973 and 1983 (10, 14), were used to construct a consensus sequence for comparison with the MO/1/94 strain. Published sequences (5, 14) of the prototype strain agreed best and were given a single weight in the consensus. For comparison, a base change was indicated if four or more (≥50%) of the isolates possessed the same mutation.

Primers. Oligonucleotide primers used for sequencing of the human PIV3 HN gene are listed in Table 1. Primer sequences PF and PR were taken directly from Karron et al. (7). All other primers were selected to conserved sequences of the HN gene of the prototype sequence WASH/47885/57 (5, 14) and optimized by using Oligo Primer Analysis Software (National Biosciences, Plymouth, Minn.); base changes from the prototype sequence were made to accommodate consensus differences among multiple virus isolates (10, 14).

Oligonucleotide primers were prepared by the β-cyanoethyl phosphoramidite method (9) on an ABI 380A DNA synthesizer (Applied Biosystems, Foster City, Calif.) and synthesized with the 5′-dimethoxytrityl group attached to facilitate purification by reverse-phase, high-performance liquid chromatography as described elsewhere (2).

Reverse transcription-PCR. For RNA extraction, 1 ml of RNAzol B (Cinna/Biotecx Laboratories Inc., Houston, Tex.) and 100 μl of chloroform were added to packed cells from a 25-cm^3 flask infected with MO/1/94, vortexed for 1 min, and left on ice for 5 min. The lysate was then centrifuged at 10,000 × *g* for 15 min at 4°C. The aqueous phase was recovered and extracted once with an equal volume of chloroform, and the RNA was precipitated with an equal volume of isopropanol for 30 min at −20°C. The pellet was then washed once with 1 ml of 75% ethanol, vacuum dried, and dissolved in 20 μl of diethyl pyrocarbonate-treated water.

To prepare cDNA, 6 μl of the sample was brought to a total volume of 10 μl containing 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 2 pM primer P1 or P2 in diethyl pyrocarbonate-treated water and incubated at 50°C for 45 min. Ten microliters of the annealing reaction was then reversed transcribed into cDNA by incubation at 42°C for 1 h in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 6 mM MgCl, 1 mM dithiothreitol, 1 mM each deoxynucleoside triphosphate, 40 U of RNase inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and 50 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) in diethyl pyrocarbonate-treated water. The reaction was heated to 95°C for 5 min and then chilled on ice. PCR amplification of the cDNA was performed as previously described (7), with primer pairs P1-PR and P9-PF.

Sequencing. The PCR products were purified either by gel electroelution or by centrifugation through Wizard PCR Prep Minicolumns (catalog no. A7170; Promega, Madison, Wis.) by following the manufacturer's instructions. Both strands were cycle sequenced on an automated DNA sequencer (Applied Biosystems) by using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were confirmed from two cDNA preparations.

RESULTS

Virus isolation and identification. At day 2 p.i., hemadsorption of one RhMK cell culture tube was positive. At day 5 p.i., both remaining RhMK cell culture tubes were cytopathic effect and hemadsorption positive. IF staining with MAb prepara-

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TABLE 1. Oligonucleotide primers used for sequencing of the human PIV3 HN gene

Primer	5′ to 3′ sequence	Nucleotide location[a]
P1	ACGCAATCCAACTCTACTCATA	13–34
P2	ACAAGGCTTCTTACAATTCAG	362–382
P3	CTGAATTGTAAGAAGCCTTGT	382–362
PF	CTCGAGGTTGTCAGGATATAG	705–725
P4	CTATATCCTGACAACCTCGAG	725–705
P5	AACTGTGTTCAACTCCCAAAG	873–893
PR	CTTTGGGAGTTGAACACAGTT	893–873
P6	CAAGTTGGCATAGCAAGTTAC	1350–1370
P7	GTAACTTGCTATGCCAACTTG	1370–1350
P8	TGCAGCCTGTTGTTGTATATC	1679–1659
P9	CTATTGTCTGATTGCTGATTAC	1867–1846

[a] Bases numbers are based on the gene sequences of Storey et al. (11).

tions against PIV1 to PIV4, influenza A and B viruses, and measles and mumps viruses was negative.

To rule out the possibility that the positive hemadsorption result was due to the presence of a simian virus contaminant of RhMK cell cultures (4), the original nasopharyngeal swab specimen, which had been stored in viral transport medium at −70°C, was reinoculated into RhMK tubes of different lot numbers. The reinoculated RhMK cell culture tubes were cytopathic effect and hemadsorption positive on day 7 p.i. Attempts to type this isolate by IF with the same reagents were again unsuccessful. Passages of the virus were hemadsorption positive but also not typeable by IF. A different isolate of PIV3 recovered from another patient 3 days after the variant had been initially detected reacted appropriately with the commercial PIV3 reagents.

The isolate, designated MO/1/94, was therefore sent to the Missouri State Public Health Laboratory, where it was identified as PIV3 by hemagglutination inhibition with horse antiserum VS-0368 prepared against PIV3 strain C243 (Biological Products Branch, Centers for Disease Control and Prevention [CDC], Atlanta, Ga.).

Reactivity with MAbs. MO/1/94 reacted by IF with both MAbs 2E9 and 4E5 (Table 2). Antibody 2E9 had been shown to be specific for a viral protein of approximately 67,000 Da, while antibody 4E5 reacted with 29,000- and 31,000-Da proteins (15). The MO/1/94 isolate and prototype strain WASH/47885/57 were reacted with a panel of previously characterized MAbs to the PIV3 HN protein of strain C243 by using an indirect EIA (6). Antibody blocking studies (data not shown) indicate that a minimum of the following three distinct epitope groups could be distinguished with the panel: 1, 342-11A; 2, 341-7H, 343-5G, and 240-12D; 3, 241-1H, 242-7A, 261-7H, and 260-10B. Only MAb 342-11A reacted with both the prototype and the MO/1/94 isolate. Results are shown in Table 2.

HN gene sequence. The MO/1/94 strain contained 36 nucleotide changes and 4 amino acid changes over the coding region for the HN protein (nucleotides 74 to 1789; amino acids 1 to 572) compared with the consensus sequence (Table 3). The ratio of nonsynonymous to synonymous changes was approximately 1:10. Twenty base changes and two amino acid changes had no historical precedent among the strains composing the consensus sequence. The two unique amino acid changes occurred at positions 174 and 283; HN epitopes have been previously identified at positions 171 and 281 (13).

DISCUSSION

Laboratory diagnosis of parainfluenza virus is commonly performed by conventional isolation in cell culture tubes, centrifugation culture in shell vials, and IF staining (shell vial culture) and by direct IF staining of nasopharyngeal specimens. Excellent-quality immunofluorescent reagents are available from a number of commercial sources for this purpose. However, neither direct IF of the nasopharyngeal swab specimen nor shell vial culture using PIV3-specific reagents from two different commercial sources would have detected this variant; the specimen would have been considered virus negative. Our experience underscores the problem with relying exclusively on MAb-based detection systems in a diagnostic virology laboratory. Viral variants that occasionally fail to react with monoclonal reagents do occur (12).

Sequencing data clearly demonstrate the presence of a new PIV3 strain. Two unique amino acid changes occurred at positions 174 and 283, which are near previously identified HN epitopes (13). Our finding is consistent with epidemiological studies which demonstrated that HN mutations do not accumulate over time but genetically heterogeneous populations of PIV3 can cocirculate (14). It is not known whether the variant we isolated will become predominant in our patient population. Since its detection, all of the PIV3 strains that we subsequently isolated could be readily typed with commercial reagents. To determine if PIV3 variants have emerged in other laboratories, PIV3 isolates are being solicited from diagnostic laboratories in the United States for HN sequence analysis.

TABLE 2. Reactivity of MO/1/94 PIV3 with antibodies

Source[a]	Designation	Antibody	PIV3 strain	Specificity	Reactivity	Test method
CDC	VS-0368	Horse antiserum	C243	Whole virus	+	HI[b]
Baxter		MAb pool	Not available	Not available	−	IF
Chemicon		MAb pool	C243	HN protein	−	IF
OKUHSC	2E9	MAb	NW	67-kDa protein	+	IF
OKUHSC	4E5	MAb	NW	29-, 31-kDa proteins	+	IF
CDC	241-1H	MAb	C243	HN protein	−	IF
CDC	242-7A	MAb	C243	HN protein	−	EIA
CDC	261-7H	MAb	C243	HN protein	−	EIA
CDC	341-7H	MAb	C243	HN protein	−	EIA
CDC	343-5G	MAb	C243	HN protein	−	EIA
CDC	240-12D	MAb	C243	HN protein	−	EIA
CDC	260-10B	MAb	C243	HN protein	−	EIA
CDC	342-11A	MAb	C243	HN protein	+	EIA

[a] Chemicon reagent contains CDC MAbs 341-7H and 343-5G. OKUHSC, Oklahoma University Health Sciences Center.
[b] HI, hemagglutination inhibition.

TABLE 3. HN nucleotide substitutions for MO/1/94[a]

Nucleotide no(s).	Amino acid no.	Nucleotide position(s)	Amino acid	Consensus sequence	Base(s) in MO/1/94
115		3	Syn	T/c	C
124		3	Syn	G	A
139		3	Syn	T/c	C
160		3	Syn	T/c	C
256		3	Syn	C/t	T
271		3	Syn	G/a	A
289		3	Syn	G	A
413		1	Syn	T/c	C
430		3	Syn	A/g	G
460		3	Syn	A	G
594	174	2	Leu→Ser	T	C
607		3	Syn	G	A
644, 646	191	1, 3	Val→Ile	G/a, T/c	A, C
655		3	Syn	G/a	A
742		3	Syn	A/c	C
748		3	Syn	G	A
796		3	Syn	T/c	C
802		3	Syn	C	T
892		3	Syn	A	G
920	283	1	Ser→Pro	T	C
940		3	Syn	A	G
1006		3	Syn	T	C
1015		3	Syn	A/t	T
1120		3	Syn	C	T
1153		3	Syn	G/a	A
1156		3	Syn	A	G
1369		3	Syn	A	G
1531		3	Syn	C	T
1555		3	Syn	C	T
1567		3	Syn	A/g	G
1660		3	Syn	A	G
1708		3	Syn	T	C
1762		3	Syn	C	T
1772	567	1	Ile→Val	A/g	G
1777		3	Syn	A	C

[a] HN nucleotide substitutions for MO/1/94 compared with the consensus sequence are shown. An uppercase letter indicates a consensus base; a lowercase letter indicates a base present in other strains. Syn, synonymous.

Sequencing studies are ongoing to analyze HN sequence variation in other recent PIV3 isolates from St. Louis. Continued viral surveillance is important to monitor for antigenic changes which may occur in nature, particularly in regard to selection of strains for vaccine development (3).

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