JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1996, p. 334–337 Vol. 34, No. 2
0095-1137/96/$04.00+0

Strain-Specific Reverse Transcriptase PCR Assay: Means To Distinguish Candidate Vaccine from Wild-Type Strains of Respiratory Syncytial Virus

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Received 19 July 1995/Returned for modification 7 September 1995/Accepted 7 November 1995

Candidate live-virus vaccines for respiratory syncytial virus are being developed and are beginning to be evaluated in clinical trials. To distinguish candidate vaccine strains from wild-type strains isolated during these trials, we developed PCR assays specific to two sets of candidate vaccine strains. The two sets were a group A strain (3A), its three attenuated, temperature-sensitive variant strains, a group B strain (2B), and its four attenuated, temperature-sensitive variant strains. The PCR assays were evaluated by testing 18 group A wild-type strains, the 3A strains, 9 group B wild-type strains, and the 2B strains. PCR specific to group A wild-type strains amplified only group A wild-type strains, and 3A-specific PCR amplified only 3A strains. PCR specific to group B wild-type strains amplified all group A and group B strains but gave a 688-bp product for group B wild-type strains, a 279-bp product for 2B strains, a 547-bp product for all group A strains, and an additional 688-bp product for some group A strains, including 3A strains. These types of PCR assays can, in conjunction with other methods, be used to efficiently distinguish candidate vaccine strains from other respiratory syncytial virus strains.

Human respiratory syncytial virus (RSV), a leading cause of severe lower respiratory tract diseases in infants and young children worldwide, is a high-priority candidate for vaccine development (10, 11). Progress in developing a live RSV vaccine, however, has been hindered by genetic instability and difficulties in achieving optimal levels of attenuation (1, 16). Concerns about enhancement of disease have hindered development of inactivated or subunit vaccines. RSV strain diversity is also a concern. The two major groups of RSV strains, A and B, have sufficient antigenic differences that vaccination or infection with one is unlikely to confer adequate protection from the other (3, 9, 13–15). Thus, to maximize RSV vaccine efficacy, it is likely that both groups will need to be represented. Despite these problems, work is continuing toward development of both live and subunit RSV vaccines (8, 16).

Several attenuated RSV mutants have been generated as potential vaccines (6, 17), and efforts to assess their safety and efficacy are under way. Thorough evaluation of such vaccines will require the means to determine if RSV isolates from vaccine recipients are vaccine or wild-type strains. In this study, we describe the development and initial evaluation of reverse transcriptase (RT) PCR assays designed to distinguish two sets of attenuated, temperature-sensitive (*ts*) variant strains of RSV (17) from wild-type strains. The G protein gene was chosen for development of these assays because it is the most genetically diverse of the RSV genes (4, 12, 19, 20).

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MATERIALS AND METHODS

Cells and viruses. The wild-type RSV isolates tested in the PCR assays are listed in Table 1. Attenuated, *ts* variant strains developed by Lederle-Praxis Biologicals (Pearl River, N.Y.) have been previously described and include three group A strains, four group B strains, and the respective 3A and 2B parent strains (17). All strains were propagated in Vero cells with minimum essential medium (GIBCO/BRL, Life Technologies, Inc., Gaithersburg, Md.) plus 2% fetal calf serum. To determine the group (A or B) and the monoclonal reactivity pattern, each isolate was tested against a panel of anti-G and anti-F monoclonal antibodies in an enzyme immunoassay as previously described (2).

RNA extraction. Virus-infected monolayers of Vero cells were washed with phosphate-buffered saline and then lysed with extraction buffer (4 M guanidine, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). The lysate was either extracted immediately or stored at −70°C for future extraction. The cell lysate (400 μl) was extracted with an equal volume of acidified phenol (pH 4.0) and a chloroform-isoamyl alcohol mixture (24:1), the aqueous phase was removed, and the RNA was precipitated by the addition of 2 volumes of ethanol plus 10 μg of dextran as a carrier.

Oligodeoxynucleotides. Synthetic oligodeoxynucleotides were prepared, purified, and analyzed as previously described (18).

Reverse transcription. The extracted RNA template and the RT primer FV (2 pmol) were resuspended in 10 μl of annealing buffer (300 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA), heated at 95°C for 3 min, and prehybridized at 50°C for 15 min. This mixture was then combined with reverse transcription buffer (50 mM Tris-HCl [pH 8.3], 10 mM $MgCl_2$, 10 mM dithiothreitol) containing 1 mM each deoxynucleotide triphosphate (dNTP), 200 U of Moloney murine leukemia virus RT (GIBCO/BRL), and 20 U of RNase inhibitor (Boehringer Mannheim Corp., Indianapolis, Ind.), brought up to a volume of 50 μl by the addition of RNase-free water, and incubated for 1 h at 37°C. The reaction products were heated for 5 min at 95°C to inactivate the remaining RT.

PCR. PCR was run at a volume of 100 μl with buffer containing 20 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg of bovine serum albumin per ml, 0.5 pmol of each primer, 50 μM (each) dNTPs, and 1 to 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif.). The reaction was run for 30 to 35 cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 50°C for 1 min, and an extension temperature of 72°C for 1 to 3 min. The products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

TABLE 1. Reactivities of PCR primer pairs to RSV wild-type and vaccine candidate strains from both A and B groups

Strain	Origin	Yr isolated	MAb reactivity pattern[a]	Reactivity of PCR primer:				
				BWC-FV			AWC-AWV (616-bp product)	AVC-AVV (616-bp product)
				688-bp product	279-bp product	547-bp product		
8/60	Sweden	1960	B/1	+				
1776A	Ga.	1984	B/1	+				
2045-4	Minn.	1984–1985	B/2	+				
2045-41	Minn.	1985–1986	B/2	+				
2334-4	Wis.	1976	B/3	+				
2334-5	Wis.	1978	B/3	+				
2334-17	Wis.	1985	B/2	+				
2338-11	Tex.	1989	B/2	+				
2338-18	Tex.	1989	B/3	+				
2B	W.Va.	1987	B/3		+			
2B33F			B/3		+			
2B20L			B/3		+			
2B24G			B/3		+			
2B34L					+			
A2	Australia	1961	A/5			+	+	
02108	Mo.	1988–1989	A/2			+	+	
03500	Mo.	1988–1989	A/2			+	+	
10849	Mo.	1984–1985	A/2			+	+	
10850	Mo.	1984–1985	A/2			+	+	
11034	Mo.	1985–1986	A/2			+	+	
11126	Mo.	1985–1986	A/2			+	+	
SL11	Mo.	1986–1987	A/2			+	+	
669	Sweden	1959	A/2			+	+	
2008-9	N.Y.	1985	A/2			+	+	
2045-32	Minn.	1985–1986	A/2			+	+	
2139-4	W.Va.	?	A/7			+	+	
2334-8	Wis.	1978	A/7			+	+	
2338-43	Tex.	1989	A/7			+	+	
Long	Md.	1956	A/2	+		+	+	
2008-1	N.Y.	1985–1986	A/1	+		+	+	
2045-25	Minn.	1985–1986	A/1	+		+	+	
2045-33	Minn.	1985–1986	A/1	+		+	+	
3A	W.Va.	1987	A/3	+		+		+
3A20F			A/3	+		+		+
3A20E			A/3	+		+		+
3A28F			A/3	+		+		+

[a] Monoclonal antibody (MAb) reactivity patterns are designated as described by Anderson et al. (2). The letter denotes the group, and the number denotes the reaction pattern within the group.

RESULTS

The complete G gene sequences of the *ts* variant strains were identical to those of their respective parent strains; therefore, we could not identify *ts* variant-specific sequences (5a). We were, however, able to identify sequences specific to the parent strains compared with the available G gene sequences of other RSV isolates. For this comparison, we used group A sequences from Long and A2 strains, which were sequenced by Johnson et al. (12), from the 642, 1734, 5857, 6190, 6256, and 6614 strains, which were sequenced by Cane et al. (4), from the 1935A, 2008-21, 2008-35, and 2040-35 strains, which were sequenced by Sanz et al. (18), and from strains 10849, 10850, 11034, 11126, SL11, 02108, and 03500, which were sequenced in the Centers for Disease Control and Prevention laboratory (5a). For group B strains, we used sequences from strains 18537, 8/60, 1355, 15291, 10010, 4843, and 9320, sequenced by Sullender et al. (20), plus strains 10849, 10850, 11034, 11126, SL11, 02108, and 03500, which were sequenced in the Centers for Disease Control and Prevention laboratory (5a). We focused on two locations in the G gene to differentiate 3A from other strains: one at nucleotide 283 on the mRNA strand, which is T in wild-type strains and C in 3A strains, and one at nucleotides 871 to 873, which is CCA in wild-type strains and TTC in 3A strains (Fig. 1). For 2B strains, we identified a 6-base deletion at nucleotides 486 to 491 in its G gene but not in the G genes of other group B strains (Fig. 2).

On the basis of these differences, we developed one PCR assay specific to wild-type group A strains and one specific to 3A strains. For these PCRs, we used complementary or message sense primers based on nucleotides 269 to 283 of the G protein message (primers AWC and AVC) and virion sense primers based on sequences complementary to those at nucleotides 884 to 871 of the G protein message (primers AWV and AVV), as illustrated in Fig. 1A. AWV is the group A wild-type virion sense primer and AVV is the corresponding vaccine-like primer. The sequence for AWC is 5′-ACACAACCCCAACAT

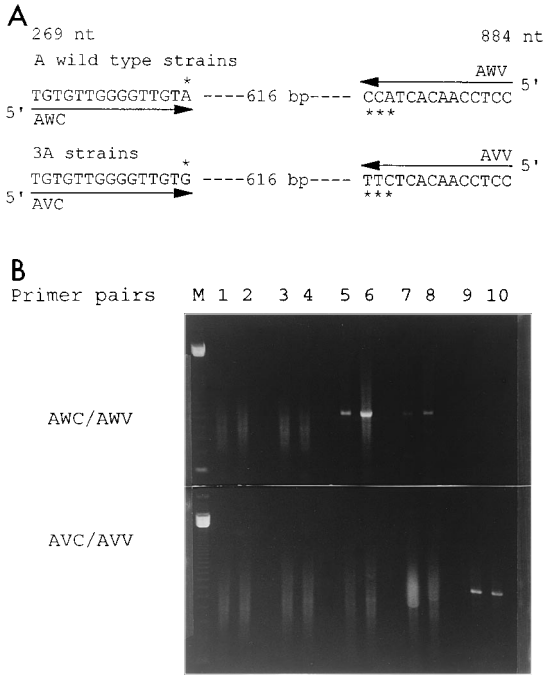

FIG. 1. (A) Sites for primer binding, with the numbers indicating the first nucleotide (nt) at the 5′ end of the primer for group A wild-type (AWC-AWV) and *ts* (AVC-AVV) strains. The line with the arrows indicate the 5′-to-3′ priming directions of the respective primers. Differences between the wild-type and *ts* sequences are highlighted by asterisks. (B) Example of PCR products after amplification with wild-type- and *ts* variant-specific primer pairs, electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. PCR products are from following strains: group B wild-type strains 8/60 and 2045 (lanes 1 and 2); group B parent 2B and *ts* strain 2B33F (lanes 3 and 4); group A wild-type strains A2 and 10849 (lanes 5 and 6); group A wild-type strains Long and 2008 (lanes 7 and 8); and group A parent strain 3A and *ts* strain 3A20F (lanes 9 and 10). Lane M contains DNA molecular weight markers as a 123-bp ladder. Only wild-type group A strains are amplified with primer pair AWC-AWV (lanes 5 to 8 in top panel), and only 3A strains are amplified with primer pair AVC-AVV (lanes 9 and 10 in bottom panel).

and that for AVC is 5′-ACACAACCCCAACAC. The sequence for AWV is 5′-GGAGGTTGTGATGG and that for AVV is 5′-GGAGGTTGTGAGAA. For 2B strains, we developed a PCR assay with the complementary sense primer (BWC) based on wild-type sequences at positions 477 to 491 of the G protein message (Fig. 2) and with the virion sense primer (FV) based on sequences complementary to nucleotides 186 to 163 of the F protein message (FV is not shown in Fig. 2). The F gene is the next gene toward the 5′ end of the RSV genome. The sequence for BWC is 5′-TCCACCAAAAAAACC. The sequence for FV is 5′-GTTATGACACTGGTATACCAACC. These sequences are conserved between groups A and B strains sequenced to date. Primer FV was used to prime complementary or bicistronic read-through RNA (plus sense) to synthesize cDNA for all three of the PCR assays.

To test the ability of the primer pairs to differentiate vaccine strains from wild-type RSV strains, we reacted AWC-AWV, AVC-AVV, and BWC-FV against 18 group A wild-type strains, 9 group B wild-type strains, and the 3A and 2B parent and respective *ts* variant strains (Table 1). Primer pair AWC-AWV gave a PCR product only with the non-3A group A strains, and primer pair AVC-AVV gave a product only with the 3A strains. Neither of the group A primer pairs gave a PCR product with group B strains (Fig. 1 and Table 1).

Primer pair BWC-FV gave a 688-bp product for all group B wild-type strains, a 279-bp product for 2B strains, a 547-bp product for 14 of 18 group A strains, and a 547-bp plus a 688-bp product for the 4 remaining group A wild-type strains and the 3A strains (Table 1 and Fig. 2). Primer BWC proved to be complementary at the 3′ end to several secondary sites in the G gene (Fig. 2). The PCR assay apparently is most efficient when the primer can anneal at its primary site (100% homology), as indicated by the single PCR product of the expected size for group B wild-type strains; a secondary site is also present on these group B wild-type strains, but the PCR product that would result from annealing at this site was not detected (Fig. 2). However, when the primary site is not available or is altered, as with the 2B strains and all group A strains, primer BWC anneals to secondary sites and gives PCR products of different sizes.

DISCUSSION

Since we found few sequence changes unique to the attenuated, *ts* strains, we developed PCR assays specific to the parents, 3A and 2B, and their respective variant strains. In this preliminary evaluation, the assays clearly distinguished the 3A and 2B strains from wild-type RSV strains. However, the ability of these PCR assays to consistently distinguish 3A and 2B strains from other strains will depend on the likelihood that

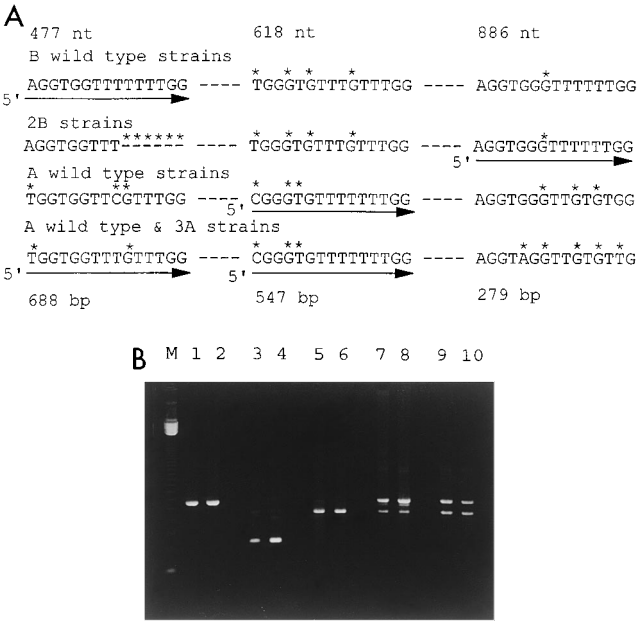

FIG. 2. (A) Sequences (3′ to 5′ in virion RNA sense) for different RSV strains at the three sites on the G gene (numbers indicate the first nucleotide [nt] at the 5′ ends of the primer sequences) where BWC can prime a PCR. For each group of strains, only the site(s) that results in the synthesis of PCR product(s) is underlined. Nucleotide mismatches are indicated by asterisks. For the 2B strains a 6-bp deletion is indicated by a broken line. This deletion precludes priming at nucleotide 477, and the high degree of homology at site 886 (one base pair mismatch) allows priming and gives a 279-bp product. All group A strains have sufficient homology at site 618 (three base pair mismatches toward the 5′ end) to prime and give a product of 547 bp, and some also have sufficient homology at site 477 (two base pair mismatches) to prime and give a 688-bp product. (B) Examples of PCR products amplified from the following RSV strains: group B wild-type strains 8/60 and 2045 (lanes 1 and 2), group B parent 2B and *ts* 2B33F strains (lanes 3 and 4), group A wild-type strains A2 and 10849 (lanes 5 and 6), group A wild-type strains Long and 2008 (lanes 7 and 8), and group A parent strain 3A and *ts* strain 3A20F (lanes 9 and 10). PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. Lane M contains DNA molecular weight markers as a 123-bp ladder.

some cocirculating wild-type strains will give the same pattern of PCR products. None of the wild-type strains we tested gave PCR products suggestive of the 3A or 2B strains. However, several groups have reported that strains isolated from geographically distinct locations and over many years (5, 7) can have very similar G gene sequences. These reports suggest that strains similar to 3A or 2B could still be circulating and might be confused with the *ts* variant strains in these PCR assays. Consequently, additional tests may be needed to confirm the identity of RSV isolates presumptively identified as *ts* like. We have identified a region in the SH gene that has multiple changes specific to one of the group B *ts* strains (5a). This site can be used to confirm the identity of this strain, and other sites on the genome will be evaluated for their ability to specifically identify other *ts* strains. It will also be important to determine if candidate vaccine strains can mutate during replication in patients, give wild-type PCR band patterns, and be falsely identified as wild-type strains. This possibility, however, is unlikely for these two sets of strains since it would require replacement of a 6-bp deletion for the 2B strains and a change in sequences at two primer-annealing sites for the 3A strains.

The PCR assay for the group B strains had unexpected but useful features. We had not anticipated that the PCR primers would anneal at alternate sites in the G gene and give PCR products of different sizes depending on the strain of RSV. Since this PCR assay amplifies sequences from all strains and differentiates strain type by product size, it can be used both to screen for strain type and to test the quality of the PCR template.

Characterizing isolates is probably best done by making use of several different assays. The simplest assay, an enzyme immunoassay to determine monoclonal antibody reaction patterns, can be used to screen isolates and identify those which should be studied further. PCR assays can then be used to further characterize isolates, e.g., as vaccine like or wild type. Finally, if needed, sequence studies of selected regions of the genome, or other studies, can be used to confirm the identity of an isolate. Strain-specific PCR assays can be developed for other RSV strains in a similar manner, and this system can be used to efficiently screen isolates from clinical studies of a variety of live attenuated RSV vaccines.

ACKNOWLEDGMENTS

The oligonucleotides were synthesized by the Biological Core Facility, Centers for Disease Control and Prevention.

H. Zheng is supported by a grant from Lederle-Praxis Biologicals to the Task Force for Child Survival and Development, Emory University, Atlanta, Ga.

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