

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for Diagnosis of Respiratory Syncytial Virus Infection in Adults: Use of a Single-Tube “Hanging Droplet” Nested PCR

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Rapid diagnosis of Respiratory Syncytial virus (RSV) infection is difficult in elderly persons due to the low quantities of virus shed. Therefore, reverse transcription-polymerase chain reaction (RT-PCR) was used to detect viral RNA in respiratory secretions. A single-tube nested RT-PCR that used primers from a conserved F gene sequence was developed using a “hanging droplet” to physically separate outer and inner primer pairs during the first round of the PCR reaction. This was accomplished by placing the inner primers in a 5 μ L droplet on the underside on the reaction tube cap and mixing after the first round of PCR. As few as 0.05 pfu of virus could be detected and gave positive results with RSV strains that represented the major groups and subgroups of RSV grown in tissue culture. The nested PCR was \sim 100-fold more sensitive than standard single primer PCR reactions and equivalent to standard two-tube nested PCR. Viral RNA was detected in nasopharyngeal samples from 12 of 15 culture positive illnesses and in 5 of 17 culture-negative, seropositive illnesses despite specimen volumes less than 1 μ L in some samples. The method was also positive in 14 of 25 elderly volunteers inoculated with a live attenuated RSV vaccine candidate, only one of whom was culture positive. Use of a nested RT-PCR significantly improves the ability to detect RSV in respiratory samples and should improve the ability to rapidly diagnose RSV infection in adults, especially in the elderly.

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INTRODUCTION

Respiratory Syncytial Virus (RSV), a negative strand RNA virus of the genus *Paramyxoviridae*, is the most important cause of serious lower respiratory tract

illness in infants [Kim et al., 1973; Mufson et al., 1973]. Reinfections occur frequently throughout life and may be severe in certain adult populations, including the frail elderly, persons with cardiopulmonary disease, and the immunocompromised [Englund et al., 1988; Falsey et al., 1995; Walsh et al., 1999]. Diagnosis of RSV infection by either culture or antigen detection in nasopharyngeal secretions is not difficult in infants with primary infection since infectious virus is abundant in the upper respiratory tract. However, these tests are insensitive in adults and investigators have relied primarily on serology for diagnosis [Falsey et al., 1992, 1995, 1996; Dowell et al., 1996]. In the elderly, virus can be isolated in less than half of illnesses even when specimens are processed under optimal conditions. Antigen detection is considerably less sensitive than culture, demonstrating a positive result in approximately 10% of cases [Falsey et al., 1996]. Low viral titers in secretions and pre-existing nasal antibody may reduce virus recovery in adults [Hall et al., 1976, 1978; Englund et al., 1996]. In addition, it is frequently not possible to collect nasal washes from frail elderly adults and samples of respiratory secretions are often limited to nasopharyngeal swabs.

Reverse transcription-polymerase chain reaction (RT-PCR) is a highly sensitive method for viral diagnosis and is especially useful for detection of low titer virus or non-viable viral RNA in respiratory secretions. In several published reports RT-PCR has been found to be more sensitive than culture in infants and in immunocompromised adults for the diagnosis of RSV but its use in the elderly has not been reported [vanMilaan et al., 1994; Freymuth et al., 1995; Gilbert

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et al., 1996; Henkel et al., 1997; Mazzulli et al., 1999]. In this paper we report the development and assessment of a novel single-tube nested RT-PCR for detection of RSV in respiratory secretions in elderly persons.

METHODS

Viruses

The A2 strain of RSV was used to optimize reaction conditions for each of the RT-PCR reactions. Prior to fast freezing, the virus was titered on a HEp-2 monolayer under a methycellulose overlay. The titer of the stock virus was 2.7×10^5 plaque forming units (pfu)/milliliter. For development of the PCR, the virus stock was often diluted in nasal secretions from asymptomatic persons to approximate the type of conditions expected with clinical samples. Inhibition of the RT-PCR was not encountered under these conditions. Clinical virus isolates representing four unique group A and four group B RSV genotypes were obtained from Dr. Larry Anderson (Center for Disease Control and Prevention, Atlanta, GA) [Peret et al., 1998]. RSV isolates were grown in HEp-2 cells, titered, frozen, and stored at -70°C . Clinical isolates of influenza A virus, influenza B virus, rhinovirus, parainfluenza 1, 2, and 3 virus, and coronavirus 229E were used as controls for specificity of the RT-PCR reaction.

Clinical samples

Nasopharyngeal swabs (NPS) were obtained from adults, predominantly >60 years of age, during previous respiratory surveillance and RSV vaccine studies [Falsey et al., 1995; Gonzalez et al., 2000]. RSV infections were characterized serologically in acute and convalescent sera by using an enzyme immunoassay with purified RSV envelope glycoproteins. NPS samples from the following types of respiratory illnesses were available: RSV culture-positive illnesses (group 1); RSV culture-negative, seropositive illnesses (group 2); RSV culture-negative, seronegative winter illnesses (group 3); and samples from 30 healthy asymptomatic elderly subjects were collected in late summer prior to the onset of the RSV season (group 4). In addition, NPS samples were obtained from 25 elderly subjects who had been inoculated intranasally with 10^7 pfu of a cold passaged temperature sensitive (cpts) 248/404 RSV vaccine (group 5) [Gonzalez et al., 2000]. Some of the samples taken during the illnesses had been stored in viral transport medium at -70°C for 8 years. The volume of sample from RSV culture or seropositive illnesses (groups 1 and 2) ranged from trace amounts ($<1 \mu\text{L}$) to $250 \mu\text{L}$. When less than $250 \mu\text{L}$ of sample was available, water was added to achieve a final volume of $250 \mu\text{L}$. All samples for PCR from groups 3, 4, and 5 were available in $250 \mu\text{L}$ volumes.

Processing of samples for RT-PCR

Forty micrograms of transfer RNA (Gibco BRL, Gaithersburg, MD) was added to each $250 \mu\text{L}$ sample,

which was then extracted with $750 \mu\text{L}$ of LS Stat (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. The RNA was precipitated with 100% isopropanol for 15 minutes at -70°C , centrifuged, and the pellet washed $2 \times$ with cold 75% ethanol. After drying, the RNA pellet was dissolved in $10.5 \mu\text{L}$ of water for RT-PCR. For the RT step, $10.5 \mu\text{L}$ of RNA and $2 \mu\text{L}$ of outer primer 1 ($2 \mu\text{M}$) were heated to 95°C for 1 minute and then placed on ice for 1 minute. Four microliters of $5 \times$ RT buffer (Promega, Madison, WI), $2 \mu\text{L}$ dNTP (2 mM each), $1 \mu\text{L}$ (9 units) AMV reverse transcriptase (Promega), and $0.5 \mu\text{L}$ RNAsin ($20 \mu\text{g}/\mu\text{L}$) were then added and the mixture incubated at 42°C for 1 hour, followed by heating to 95°C for 3 minutes. Five microliters of the resulting cDNA product was used in the PCR reaction.

Single-tube nested PCR

Nested PCR is generally considered significantly more sensitive than single primer pair PCR reactions but is hampered by problems of contamination when a two-tube method is used. Therefore, we sought to develop a one-tube nested PCR by using primer pairs from the A2 strain F gene sequence. Optimal conditions for the outer and inner primer pairs were first determined separately, and then the maximal potential sensitivity for a nested PCR was defined by using each of the primer pairs in a two-tube method. In anticipation of contamination associated with a two-tube method, outer and inner primer pairs were selected with significantly different melting temperatures (T_m) so that the outer primer round of the PCR could proceed at an annealing temperature significantly greater than the T_m of the inner primers. This method has been used successfully by others and utilizes the concept that the inner primers anneal poorly at the higher temperature of the first round reaction and thus do not interfere with the initial outer primer reaction [Mathis et al., 1997]. The second round of the PCR is then run at a lower annealing temperature to produce the final nested DNA product. The sensitivity of this "differential temperature" single-tube nested PCR was then determined using the A2 virus stock. Finally, a "hanging droplet" single-tube nested PCR utilizing a 42°C annealing temperature for both outer and inner primer reactions was developed. For this method, the outer and inner primers were physically separated during the first round of the PCR reaction.

Outer primer and inner primer pairs, which would result in DNA products of 411 base pairs (bp) and 263 bp, respectively, were selected from conserved sequences of the published RSV A2 strain F gene [Collins et al., 1984]. The sequences for the primers were: Outer primer 1 (nt 731–771 of F mRNA): 5'ATGCAGGTGTAACAACACCTTTAAGCACTTACATGTTAAC 3', Outer primer 2 (nt 1100–1139): 5'GTAATGTAAACTGTTTCATAGTGTGTCACAAAATACTCGATT 3', Inner primer 1(nt 800–819): 5'GATATGCCTATAACAAATGA 3', Inner primer 2 (nt

1046–1063): 5'GATACTGATCCTGCATT 3'. The outer and inner primer pairs had a calculated T_m of $\sim 70^\circ\text{C}$ and $\sim 40^\circ\text{C}$, respectively.

For the single-tube "differential temperature" PCR, various conditions were tested in an attempt to provide an optimal reaction. The optimal magnesium concentration was determined for each primer, and a range of primer concentrations (0.5 to 160 nM) was tested. These experiments suggested that the following reaction concentrations provided optimal results: each of the primers at 10–80 nM, MgCl_2 at 4 mM, dNTP at 200 μM , and 5 μL Taq polymerase (Promega). The first PCR round was 30 cycles (denaturation at 95°C for 45 seconds, annealing at 50, 60, or 72°C for 1 minute, and extension at 72°C for 1 minute). The second PCR round was 40 cycles with an annealing temperature of 42°C .

For the "hanging droplet" nested PCR, the following were placed in the reaction tube: 5 μL of RT product, 5 μL $10 \times$ PCR buffer, 8 μL MgCl_2 (25 mM), 5 μL dNTP (2 mM), 2 μL each outer primer (1 μM), 1 μL Taq polymerase (5 u/ μL), and 22 μL water. The reaction mixture was then covered with 100 μL of oil. Before the tube was closed a 5 μL droplet, containing 2 μL of each inner primer (1 μM), 0.5 μL Taq polymerase, and 0.5 μL water, was placed in the center of the inside of the reaction tube cap. The tube was closed and heated to 95°C for 2 minutes and the first round of the PCR carried out for 30 cycles (95°C for 40 seconds, 42°C for 1 minute, and 72°C for 1 minute). The reaction was brought to 4°C and the "hanging droplet" incorporated into the reaction mixture by inversion and shaking. The tube was centrifuged briefly to reposition the oil above the reaction mixture and a second round of 40 cycles was completed using identical conditions as the first round with a final 10-minute extension at 72°C .

PCR products were separated by electrophoresis on 1.5% agarose gels and identified by staining with SYBR green (Molecular Probes, Eugene, OR). In representative samples, the identity of the final DNA product was confirmed by direct sequencing (Seqwright, Houston, TX).

RESULTS

When the inner and outer primers were first used separately at primer concentrations of 40 nM and at an annealing temperature of 42°C , each primer pair could detect ~ 17 pfu of RSV (Fig. 1). These same primer pairs at 40 nM concentrations were then used in a standard two-tube nested PCR, again using annealing temperatures of 42°C . This PCR was significantly more sensitive and detected ~ 0.17 pfu of RSV (data not shown).

Next, both inner and outer primers were combined in the reaction mixture for a single-tube PCR in an attempt to exploit the differential T_m of the two primer sets. However, despite multiple alterations in primer concentration and annealing temperatures, the sensitivity could not be enhanced beyond that achieved with the single primer pairs (~ 17 pfu). Even at the highest initial annealing temperature of 72°C , we failed to increase the sensitivity of the reaction (data not shown). The failure to demonstrate improved sensitivity was likely due to competition between the primer pairs since the reaction resulted in the formation of hybrid PCR products of intermediate size (i.e., seminested PCR products).

Since the two-tube nested PCR was clearly superior to the standard PCR and to the differential temperature single-tube nested PCR, we attempted to separate physically the primer pairs in the reaction tube so that the outer primer reaction could proceed without interference from the inner primers. This was accomplished by incorporating the inner primers and additional Taq polymerase in a 5 μL "hanging droplet" on the underside of the reaction tube cap. Preliminary experiments found that the droplet neither evaporated nor leaked into the reaction mixture during the first round of the PCR. If the tube was not shaken after the first round the final 263 bp DNA product was not visualized, although the 411 bp outer primer product was seen (data not shown). Similarly, a final inner primer product was not seen if the outer primers were not included and the tube was not shaken. This modi-

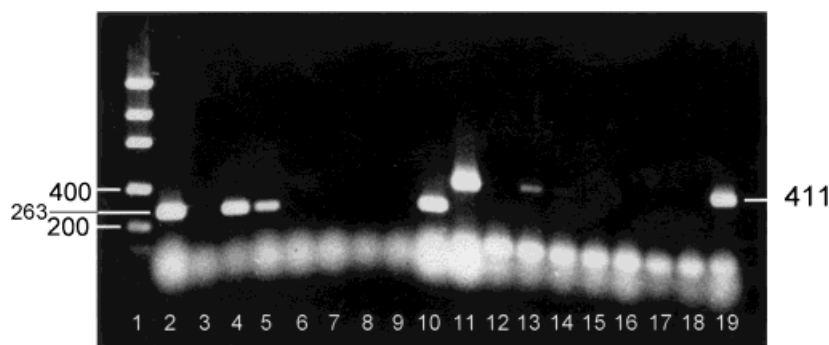


Fig. 1. Results of single primer pair PCR using inner primers (lanes 2–10) and outer primers (lanes 11–19) individually to detect the A2 strain of RSV. DNA products were separated on a 1.5% agarose gel and stained with SYBR green. Positive control is in lanes 2 and 10 and negative control in lane 3. DNA standards are in lane 1. The 263 bp band represents the inner primer product. Virus stock dilutions are from 10-2 through 10-7 (lanes 4–9) and show a positive reaction in

lanes 4 and 5 representing detection of 170 and 17 pfu of virus, respectively. The 411 bp band represents the outer primer product. Positive control is in lane 11 and 19 and negative control in lane 12. Virus stock dilutions are from 10-2 through 10-7 (lanes 13–18) and show a positive reaction in lanes 13 and 14 representing detection of 170 and 17 pfu of virus, respectively.

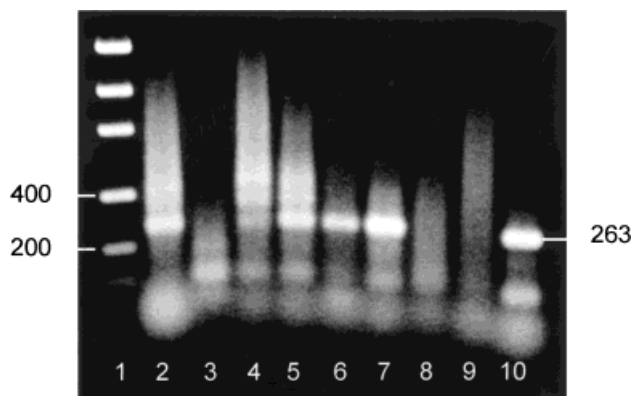


Fig. 2. Results of "hanging droplet" single-tube nested PCR. Positive control is in lanes 2 and 10, negative control in lane 3, and DNA standards are in lane 1. The virus stock dilutions are from 10^{-2} to 10^{-7} dilutions (lanes 4–9) and a band of 263 bp is seen in lanes 4 through 7, detecting 0.17 pfu in lane 7. The 411 bp product and an intermediate (seminested) product are faintly seen in lanes 4 and 5.

fied PCR method, using primer concentrations ranging from 40 to 200 nM, detected ~ 0.05 – 0.17 pfu of RSV in multiple repetitions of the assay, a sensitivity identical to the two-tube nested PCR. A representative result is shown in Figure 2, which was done simultaneously with results in Figure 1. Uninfected HEp-2 and Vero cells were negative as were samples of other respiratory viruses including influenza A & B, rhinovirus, parainfluenza viruses 1, 2, 3 and coronavirus 229E (data not shown). Each of the RSV strains, representing unique genotypes within group A and B RSV, were repeatedly positive by this PCR, and the sensitivity ranged from 0.02–0.5 pfu for each of the virus strains (data not shown).

The "hanging droplet" method was then used to detect viral RNA in clinical samples. Twelve of fifteen samples from culture-positive illnesses were positive. Of 17 culture-negative, seropositive illnesses, 5 (29%) were positive by PCR. In many instances, the volume available was considerably less than 250 μ L. The median volume of sample available from groups 1 and 2 for the "hanging droplet" nested PCR was 15 μ L with a range from < 1 to 250 μ L. Sample volumes for the 3 PCR negative, culture-positive samples were 10, 3, and < 1 μ L. Of 30 RSV culture-negative, seronegative winter illnesses (group 3), one was repeatedly positive. None of 30 NPS specimens collected from asymptomatic elderly persons in the late summer (group 4) were positive. It should be noted that 250 μ L was available for each of the samples from these last two groups.

NPS samples from the cpts 248/404 RSV vaccine trial (group 5) were all available in 250 μ L volumes for analysis. Using samples collected 6 days after virus challenge, the nested PCR detected viral RNA in 14 of 25 (56%) samples, only one of which was culture-positive. Samples from 20 elderly control subjects given placebo were all negative by the PCR.

DISCUSSION

Diagnosis of RSV infection in adults, especially the elderly, is difficult using standard virus culture or antigen detection. In one study of nursing home illnesses, only 18 of 40 (45%) were culture positive despite using optimal specimen and culture techniques [Falsey et al., 1992]. In another report, only one sample from 11 culture positive illnesses in an adult day care setting was positive for RSV antigen by immunofluorescence, and none by enzyme immunoassay [Falsey et al., 1996]. This low yield, in marked contrast to the situation in infants, is not surprising since adults shed significantly lower amounts of virus for shorter periods of time [Hall et al., 1976, 1978; Englund et al., 1996]. This problem is compounded by the extreme lability of RSV even when placed in viral transport media and kept at low temperature [Walsh and Hall, 1989]. The inability to make an early diagnosis of RSV infection in adults has hampered both the accurate study of the epidemiology in this age group and made investigation of the immune response to infection nearly impossible. A highly specific and sensitive RT-PCR should significantly improve this situation.

It is generally accepted that nested PCR is more sensitive and therefore we developed a nested PCR using conserved sequences within the F gene that could detect as little as 0.05–0.17 pfu of the A2 strain of RSV. This was similar to the sensitivity of the two-tube nested PCR. To avoid potential cross contamination that complicates two-tube nested methods, a single-tube reaction exploiting differential primer annealing temperatures based upon markedly disparate T_m was attempted. Despite variations in primer concentration and annealing temperatures, the PCR only detected ~ 17 pfu of RSV. However, by suspending a droplet containing the inner primers in the cap of the reaction tube during the first PCR round, we could detect 0.05–0.17 pfu of virus and avoided competition between primers, which appeared to have inhibited the "differential temperature" single-tube nested PCR. This method is very similar to a published technique in which the inner primers were separated from the initial reaction by placing them in a hanging gel matrix [Yourno, 1992]. Another method of physically separating the primers for the first round of a nested PCR has been described by other investigators using wax, rather than oil, to cover the reaction mixture that contains the outer primers [Whelen et al., 1995]. At completion of the first round of the PCR, the wax solidified when the reaction tube was cooled, thus sealing the first reaction mixture. The tube was then opened and a large volume of reaction mixture containing the inner primers was added. Although successful, opening the tube increases the risk of contamination, a concern we avoided by using the "hanging droplet" to separate the primers. Furthermore, this modification obviated the need for the time consuming and complex manipulation of assay parameters when devising a one-tube nested PCR based upon differential primer T_m . This PCR may have

been improved by using shorter primers. The primers were originally chosen to develop a differential temperature PCR and it is possible that false priming or primer interactions may have occurred due to excessive length and the relatively low T_m used. The result may be the smear seen at the bottom of the gels.

The nested PCR was able to detect RSV RNA in 80% of a small number of culture positive samples and in 29% of culture-negative, seropositive samples despite the availability of extremely small sample volumes. It is also important to note that the volume of NPS sample used for virus culture was 400 μ L in all cases. In addition to the small volumes, it is possible that inhibitors to the RT-PCR reaction are present in some nasal secretion samples.

The nested PCR was also able to detect RSV in a high percentage of elderly persons inoculated with cpts 248/404 RSV [Gonzalez et al., 2000]. We had previously used a published F gene single reaction PCR (with a sensitivity of \sim 10 pfu of virus) in these subjects and found that only 6 of 25 (24%) of day 6 samples were positive, significantly fewer than with the nested PCR [14 of 25 (56%)]. Our results suggest that PCR may be very useful in this age group since only one individual was culture-positive. Although the detected RNA may represent persistence of residual inactivated virus inoculum, and not actual virus replication, it does suggest that this PCR should be useful in the clinical setting of acute respiratory infection in elderly persons.

The single culture-negative, seronegative winter illness that was repeatedly PCR positive may represent a false positive result, although all 30 asymptomatic persons tested in the late summer were negative by the nested PCR. We have previously found that a small number of elderly do not develop an antibody response to culture documented RSV infection, and thus it would be expected that some PCR positive RSV illnesses may also be seronegative [Falsey et al., 1992].

In summary, a single-tube nested PCR for diagnosis of RSV infection was developed using a "hanging droplet" method which could detect \sim 0.05–0.17 pfu of virus and was more sensitive than standard single primer pair PCR. The "hanging droplet" method may also prove useful in the development of single-tube nested PCR for other infectious agents. The improved ability to diagnose RSV infection in elderly adults in a timely fashion will enhance the ability to study the epidemiology and immunology of RSV in this group.

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