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Improved Detection of Rhinoviruses in Nasal and Throat Swabs by Seminested RT-PCR

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A seminested RT-PCR (nRT-PCR) was used to detect picornavirus (PV) RNA in cell cultures inoculated with rhinoviruses (HRVs) and enteroviruses (EVs). PCR tests in which a primary "touchdown" PCR was followed by secondary reactions using PV or HRV specific primers were able to differentiate HRVs of 48 serotypes from EVs. PVnRT-PCR and HRVnRT-PCR were then used to test nasal and throat swabs from adult subjects with naturally acquired respiratory virus infections. The swabs were also analysed for respiratory viruses by cell culture techniques and the rates of PV identification by the two methods were compared. PVnRT-PCR was found to be at least five times more sensitive than cell culture for the detection of PVs in these clinical specimens. Paired acute and convalescent serum samples were tested for complement fixing antibodies to adenovirus, influenza A and B, respiratory syncytial virus, parainfluenza viruses 1, 2, and 3, *Mycoplasma pneumoniae*, and *Chlamydia psittaci*. An enzyme-linked immunosorbent assay (ELISA) was used to detect rises in antibody level to coronavirus types 229E and OC43. The overall rate of pathogen identification in 159 swabs from adult asthmatics increased from 28% when only cell culture and serology were used to 57% when these methods were supplemented by PVnRT-PCR.

KEY WORDS: picornaviruses, URTIs, diagnosis

INTRODUCTION

The common cold is a major cause of morbidity, loss of work, and drug expenditure. Each year the average preschool child has 6–10 colds and the average adult 2–4 colds [Sperber and Hayden, 1988]. Research into the effects of colds on normal individuals and on those with chronic lung disease has been hampered by the considerable difficulties encountered in identifying respiratory pathogens. Human rhinoviruses (HRVs), the major causative agents of the common cold [Couch, 1985], are fastidious for certain cells and culture conditions and the large number of serotypes precludes serology for screening [Hamparian et al., 1987]. Rates of virus identification have rarely been greater than 40%. Minor and colleagues (1974) recovered HRVs from 30% of specimens from young asthmatic children with symptomatic respiratory infections, but were only able to isolate HRVs from 12% of specimens from older patients [Minor et al., 1976]. The rates of total virus identification in these studies, using a variety of diagnostic methods, were 49 and 26%.

Nucleic acid hybridization techniques, particularly those based on the polymerase chain reaction [Saiki et al., 1988], are now being used for rapid detection of viruses and slow-growing organisms [Hayden et al., 1991]. Several short conserved regions in the 5′ untranslated sections of picornavirus (PV) genomes [Duechler et al., 1987; Hughes et al., 1988] have provided sequences for oligonucleotide probes and primers [Bruce et al., 1989; Gama et al., 1988, 1989; Hyypia et al., 1989; Torgersen et al., 1989]. Four of these regions (A–D) are shown in Fig. 1.

Radiolabelled synthetic oligonucleotide probes (17mers) complementary to C and D were used by Bruce and colleagues [1989] to detect the RNA of 57 HRV serotypes and to identify HRV14 infection in nasal washes from 57 experimentally infected volunteers. The probes gave results comparable to conventional virus culture on days two and three post-inoculation, but were more sensitive than culture on subsequent days. Negative results were obtained with unrelated respiratory viruses including reoviruses, influenza viruses, coronavirus 229E and herpes simplex virus.

Oligonucleotides (14–18mers) complementary to C and D were used as reverse transcriptase and backward PCR primers by several groups of workers [Gama et al., 1988, 1989; Hyypia et al., 1989; Torgersen et al., 1989]. All groups used part of the conserved A sequence (16 or 18mers) for the forward PCR primer. Gama and colleagues [1989] obtained positive results for nasal washings from all volunteers experimentally infected with HRV2, HRV9 or HRV14. The reaction products were

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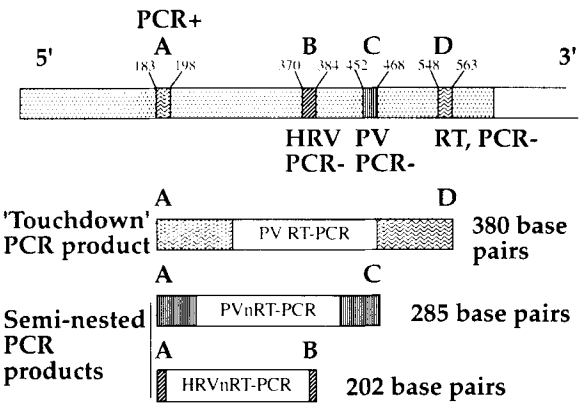

Fig. 1. Four conserved regions in the 5'-untranslated regions of rhinoviruses that have provided sequences for oligonucleotide probes and RT-PCR primers. Numbering shown is for HRV14 [Stanway et al., 1984].

analysed by agarose gel electrophoresis after 30 reaction cycles and further characterised by nucleotide sequencing.

The complementary sequence to region B (16mer) was used by Hyypia et al. [1989] as an HRV probe for Southern blots, who reported results for 29 wild-type PVs isolated from clinical material in LLC or HeLa cells. All except one gave an amplification product of the expected size but only 12 out of 14 products from HRVs hybridized with the probe.

Olive et al. [1990] used sequence D (16mer) for a forward PCR primer with a reverse transcriptase and backward PCR primer (20mer) complementary to a sequence in the downstream VP2-coding region. The reaction products were identified solely by their size as seen in ethidium bromide-stained agarose gels. HRVs could be differentiated from other PVs by this method: PCR products were 530 bp for HRVs and 650 bp for polioviruses, coxsackieviruses, and echoviruses. These workers used PCR directly on nasal swabs but confined their efforts to 41 specimens which had been positive for respiratory viruses by cell culture.

We have devised seminested RT-PCRs (nRT-PCRs) to improve the sensitivity and specificity of this method of PV detection. The 1°PCR uses a primer of sequence A and a primer complementary to sequence D in a "touchdown" reaction cycle to reduce spurious priming [Don et al., 1991]. 2°PCRs using a primer of sequence A together with primers complementary to either B (HRVnRT-PCR) or C (PVnRT-PCR) are then used to differentiate between HRVs and other PVs. We have applied this test directly to nasal and throat swabs from controls and from individuals with naturally acquired colds and compared the rate of PV detection by PVnRT-PCR and by cell culture.

MATERIALS AND METHODS

Study Population

Clinical samples were taken from volunteers in two studies at Leicester University and one at Cardiff University. All subjects were adults: two groups were normal healthy students and the third group was asthmatic. Ethical committee approval and signed informed consent were obtained.

Symptom Assessment

Subjects in Leicester were asked to record symptoms on a 10-day diary card (as described previously, Wiselka et al., 1991). Symptoms were use of handkerchiefs, runny nose, stuffy nose, sneezing, sore throat, hoarseness, red or watery eyes, face-, head-, or earache, malaise, muscle aches, chills, cough, and painful, swollen neck glands. Symptomatic days were those on which two or more symptoms were recorded with at least one symptom moderately severe or worse. The number of symptomatic days and the total symptom score for the ten days were used as a measure of the severity of the cold and those subjects with doubtful colds were excluded from the study group.

Volunteers in Cardiff were self-diagnosed as suffering from colds. They were not selected for this study on the basis of their symptom scores.

Virology

Nasal swabs were placed high in the anterior nares and throat swabs were passed firmly over the pharynx and tonsils. Swabs were placed together in 2.5 ml of virus transport medium containing nutrient broth (10% foetal calf serum, penicillin, streptomycin, and amphotericin B). All specimens were then stored at −70°C.

Volumes (0.2 ml) of nasal and throat swabs were inoculated onto monolayers of Ohio HeLa cells, MRC-5 human lung fibroblasts, C16 cells (a cell line derived from MRC-5 fibroblasts, susceptible to coronavirus), and Madin–Darby canine kidney (MDCK) cells. All cell lines were cultured in roller tubes at 33°C with 5% CO_2 and observed for 14 days. Specimens inoculated onto Ohio HeLa cells were routinely passaged once after 7 days and equivocal specimens were passaged up to three times. HRV infection was diagnosed after observation of characteristic cytopathic effect (CPE). Positive isolations of HRV were confirmed by demonstrating characteristic acid lability at pH3 and inhibition of CPE at 37°C. Influenza viruses were identified by haemadsorption inhibition on MDCK cells.

PVs of known serotype obtained from the MRC Common Cold Unit, Salisbury, U.K. were also cultured. Echoviruses and coxsackie viruses were grown in MRC-5 cells and HRVs in HeLa cells.

Paired acute and convalescent sera were tested for complement fixing antibodies to adenovirus, influenza A and B, respiratory syncytial virus, parainfluenza viruses 1, 2, and 3, *Mycoplasma pneumoniae*, and *Chlamydia psittaci*. An exzyme-linked immunosorbent assay (ELISA) [Kraaijeveld et al., 1980] was used to detect rises in antibody level to coronavirus 229E and a similar ELISA, using antigen prepared from infected suckling mouse brain, was used to detect antibodies to coronavirus OC43.

Nucleic Acid Extraction

Samples (200 μl) of cell culture grown virus or nasal and throat swabs in transport medium were used for nucleic acid extraction. This material was transferred immediately on thawing to 1.5 ml reaction tubes containing 25 μg proteinase K and 1 μg carrier tRNA in 225 μl 2× proteinase K buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS). Appropriate negative controls were always included. After incubation at 37°C for 30 min, the samples were extracted once with phenol/chloroform and twice with chloroform. Nucleic acid was precipitated by the addition of 50 μl of 2 M sodium acetate, pH 5.2, and 1 ml of cold ethanol to each tube. After 10 min on ice, the reaction tubes were centrifuged for 20 min in a microfuge kept at 4°C. The resultant pellet was washed once with 70% ethanol, air dried, and resuspended in 50 μl of cDNA synthesis mixture.

Primers and Probes

Primers for 1°PCR had the following sequences:

RT/PCR− . . . 5′CGGACACCCAAAGTAG3′

PRC+ . . . 5′GCACTTCTGTTTCCCC3′

The sequences of the oligonucleotide probes and 2°PCR primers were

PV probe/PV 2°PCR− . . . 5′GCATTCAGGGGCCGGAG3′

HRV probe/
HRV 2°PCR− . . . 5′GGCAGCCACGCAGGCT3′

PCR+ . . . 5′GCACTTCTGTTTCCCC3′

cDNA Synthesis

The cDNA synthesis mixture used to resuspend the extracted nucleic acids contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, RNAguard ribonuclease inhibitor (Pharmacia, 40U/reaction), M-MLV reverse transcriptase (Gibco-BRL, 200 U/reaction), and RT/PCR− primer (1.0 μM). The reaction tubes were incubated at 37°C for 1 hr and then transferred to an ice bath.

Polymerase Chain Reaction

A 10 μl portion of each reverse transcription reaction was diluted to 100 μl for 1°PCR amplification in reaction tubes containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, Taq polymerase (Cambio, 2.5 U/reaction), BSA (BRL, 10 μg), and primers RT/PCR− and PCR+ (1 μM). A "touchdown" reaction cycle was used (Don et al., 1991): the steps were 92°C for 10 sec, 70°C down to 50°C (2°C intervals) for 20 sec and 72°C for 1 min. There were two cycles for each annealing temperature down to 52°C followed by 10 cycles at 50°C.

Amounts (2 μl) of the 1°PCR products were transferred to new tubes containing 100 μl of 2°PCR reaction mixture. Except for the inclusion of only primers PCR+ and PV 2°PCR− (for PVnRT-PCR) or PCR+ and HRV 2°PCR− (for HRVnRT-PCR), the reagents were the same as those used in the 1°PCR. The reaction cycle was 92°C for 10 sec, 55°C for 20 sec, and 72°C for 1 min. The cycle was repeated 25 times.

The reaction products were separated on 1.5% agarose gels containing ethidium bromide and visualized by fluorescence in ultraviolet light. The 1°PCR products were then transferred to positively charged nylon membranes (Amersham, Hybond-N+) by alkaline blotting.

Hybridization Procedure

Filters were prehybridized for 4 hr in 6 × SSC, 5 × Denhardt's solution, 1% SDS, and 100 μg/ml of single-stranded herring sperm DNA at the calculated melting temperature for the probe to be used. Oligonucleotide probes were 5′ end-labelled using polynucleotide kinase (Sigma) and [γ-^{32}P]ATP (Amersham) as the precursor. Labelled probes were separated from unincorporated ATP on a column of Sephadex G-25 (Pharmacia). A labelled probe was added to the prehybridization mixture containing the filter and allowed to hybridize overnight at the calculated T_m of 61°C. After hybridization, the filters were washed twice in 2 × SSC, 0.1% SDS for 15 min at room temperature and twice in 0.1 × SSC, 0.1% SDS for 15 min at the hybridization temperature. The filters were then autoradiographed on an X-ray film (Kodak X-Omat AR) overnight at −70°C.

RESULTS

RNA was extracted from 48 HRVs and 12 enteroviruses (EVs) of known serotype grown in cell culture. Reverse transcriptase and 1°PCR primers (chosen from the conserved regions A and D shown in Fig. 1) were then used to give 380 bp amplification products from these PVs [Gama et al., 1988, 1989]. After Southern blotting, the 1°PCR products were probed with ^{32}P-labelled oligonucleotide probes at their calculated melting temperatures. The PV probe was a 17mer complementary to region C while the HRV probe was a 16mer complementary to region B [Hyypia et al., 1989].

All the HRVs and Coxsackie viruses gave the expected 1°PCR product as did six of the seven echoviruses. The absence of a reaction product from echovirus 22 was expected [Gama et al., 1989; Hyypia et al., 1989]. The PV probe reacted with all the 1°PCR products except that from HRV41. The HRV probe did not bind to the products from any of the EVs but did bind to 43 of the 48 HRV 1°PCR products. The 1°PCR products from the EVs and HRVs were later reamplified using the HRV probe as the reverse primer. None of the EVs gave a product in this HRV nRT-PCR but all the HRVs, including those which had not given a positive result with the labelled probe, gave the expected 2°PCR product.

The results for clinical samples are summarized in Table I. Nasal and throat swabs from 27 Leicester University volunteers with high symptom scores were chosen for analysis by PVnRT-PCR. No PVs had been cultured from these specimens although two HRVs and one echovirus had been isolated from other subjects in

TABLE I. Total Virus and PV Identification Rates in Adults With and Without Colds

Subjects			Virus identifications				
Group	Number	Presenting condition	% PV by cell culture	% total virus identifications by cell culture and serology	% PV by RT-PCR	% PV by nRT-PCR	% total virus identifications by cell culture, serology and PV nRT-PCR
Normal adults	27	Cold	0	7	26	44	51
Normal adults	27	Cold	4	22	nt[a]	41	59
Normal adults	20	None	0	5	nt	0	5
Asthmatics	159	Cold	8	28	nt	37	57

[a]nt, not tested.

the study. Seven positive swabs were detected in the 1°PCR and the products were analyzed by Southern blotting and hybridisation to labelled probes. All seven reacted with the PV probe but only five with the HRV probe. When small amounts of the 1°PCR products were re-amplified using the PV probe as the reverse primer, five more positive swabs were detected. Thus, 12 out of 27 (44%) nasal and throat swabs which were negative for PV by cell culture were positive when PVnRT-PCR was used. An adenovirus had been isolated from one of the remaining 15 swabs and a further volunteer showed serological evidence of influenza B infection. Thus the use of PVnRT-PCR to detect PVs increased the overall rate of pathogen identification from 7 to 51%.

PVnRT-PCR and HRV nRT-PCR were used to analyse 47 nasal and throat swabs from participants in a Cardiff University study (27 from patients with colds and 20 from healthy controls). The swabs were tested in random order and their identity was not known to the investigator until after the experiments were finished. Swabs that gave positive results were extracted, reverse transcribed, and amplified again to confirm the findings. Eleven of the 27 patient swabs (41%) were positive for PV (six being amplified in both 2°PCRs and thus identified as HRVs) whereas none of the 20 swabs from the healthy controls was positive by either nRT-PCR. Only one of the patient swabs that was positive for PV when tested by PVnRT-PCR was also positive in cell culture. Five of the 16 PV-negative patient swabs had a respiratory pathogen identified by serology. Two were positive for coronavirus, two for influenza A virus and one for adenovirus. The rate of pathogen identification was increased from 22 to 59% by the use of PVnRT-PCR.

PVnRT-PCR and HRV nRT-PCR were also to analyse nasal and throat swabs from subjects who had volunteered for a prospective study of respiratory infections in patients with asthma. Sixty of 159 (37%) swabs taken when the patients had colds were positive for PV when tested by PVnRT-PCR. All of the 1°PCR products were also reamplified using the HRV 2°PCR-primer and were thus identified as HRVs. Thirteen of the 60 nasal and throat swabs that were positive for HRV by HRV nRT-PCR were also positive for HRV in cell culture. There were three coinfections in which HRV was identified by HRV nRT-PCR and another virus was identified by serology. One subject had a coinfection with HCV OC43 and RS virus identified by serology. Overall, the rate of pathogen identification was increased from 28 to 57% by the use of PVnRT-PCR.

Convalescent nasal and throat swabs were taken at least 3 weeks after the acute swabs. Three of 22 convalescent swabs from subjects with HRV-positive acute swabs gave a further positive reaction.

DISCUSSION

In evaluating the results of this study, consideration must first be given to the most commonly voiced criticism of PCR—its particular susceptibility to false positive results due to contamination [Lo et al., 1988]. We attempted to identify any possible sources of contamination and adopt suitable procedures to eliminate them. A separate set of pipettors was used to prepare the mixtures of reagents and aerosol resistant pipette tips (Continental Laboratory Products) were used throughout. Different areas of the laboratory were used to add the 1° and 2°PCR substrates and to run agarose gels containing the PCR products.

Negative control cell cultures were included several times in every assay and never gave any product after extraction, reverse transcription and amplification. No carryover was observed, even when specimens containing large numbers of cell culture-grown virus particles were processed alongside these negative controls. When nasal and throat swabs from control subjects without cold symptoms were tested together with swabs from patients with colds, none of these control samples gave a positive result in the nRT-PCR assays.

A simple RNA extraction method is essential if many specimens are to be processed simultaneously for RT-PCR. Gama and colleagues [1988, 1989] used a method which required seven solvent extractions after the addition of carrier tRNA and vanadyl ribonucleoside complexes. We found that it was unnecessary to add ribonuclease inhibitors to our specimens or reactions if proteinase K in SDS-containing buffer were added to the clinical material immediately after thawing. Following enzyme digestion, we used three solvent extractions (one with phenol/chloroform and two with chloroform) before ethanol/acetate precipitation of the nucleic acids.

The PCRs for rhinovirus detection described by other groups of workers [Gama et al., 1988, 1989; Hyypia et al., 1989; Torgersen et al., 1989] had 30–40 cycles

with annealing temperatures of 40° or 55°C. When these amplification conditions were used in this study, the dominant products were frequently various alternative fragments less than 380 bp long. "Touchdown" PCR was described by Don and colleagues [1991] as a method of preventing such spurious priming during gene amplification. We used a 1°PCR in which the annealing temperature was decreased 2°C every second cycle from 70°C to a touchdown at 50°C at which temperature there followed 10 more cycles. The production of spurious products was inhibited and it was then possible to use a nested primer in a 2°PCR to confirm the specificity of the reaction and to increase the sensitivity of the test.

The oligonucleotide probes used in these experiments were not able to distinguish properly between EVs and HRVs since the HRV probe had incomplete reactivity with the serotypes tested. This incomplete reactivity with HRV serotypes agrees with that observed by Hyypia and colleagues [1989] using less stringent conditions in which the probe reacted with only 12 out of 14 clinical isolates of HRV.

Surprisingly, the HRV 1b 1°PCR product did not hydridize with the HRV probe although a previously published sequence for nucleotides 359–374 of HRV 1b shows perfect complementarity to this probe [Torgersen et al., 1989]. Variation may exist in the 5′ untranslated region for isolates of the same HRV serotype as it does for poliovirus serotypes [Minor and Dunn, 1988].

The problem of restricted reactivity with the HRV serotypes from cell cultures was overcome by using the same oligonucleotide as a 2°PCR primer with an annealing temperature of 55°C. All the HRV 1°PCR products, including those that failed to hybridize with the probe, gave a 2°PCR product, whereas none of the EVs was amplified. Sommer and Tautz [1989] found that primers with a length between 17 and 20 nucleotides needed at least three homologous nucleotides at their 3′-end for successful priming but the remainder of the primer needed surprisingly little homology with the target sequence.

Although all 60 of the PVs identified by PVnRT-PCR in the swabs from asthmatics could be further typed as HRVs by HRVnRT-PCR, five of the 11 1°PCR products from the Cardiff subjects with colds were only reamplified with the PV and not with the HRV 2°PCR primer. MRC-5 and Ohio HeLa cell cultures did not yield any virus from these swabs, which were all taken within a 9-day period. The patients may have been infected with one of several enteroviruses that have been associated with mild illness of the upper respiratory tract. These include coxsackie viruses A2, 10, 21, 24, and B2 and 5; and echoviruses 1, 11, 19, 20, and 22. It is also possible that the virus or viruses responsible were HRV(s) that were not recognized by the HRV 2°PCR− primer.

This study has shown that PVnRT-PCR is at least five times more sensitive than cell culture for the detection of PVs in nasal and throat swabs. PVnRT-PCR is also considerably faster since the whole process can be completed in less than two working days. Amplification directly from clinical materials means that cell culture is unnecessary; nRT-PCR is then no more expensive than the traditional method of virus isolation. Although all the cell culture-grown HRVs tested could be differentiated from other PVs using HRVnRT-PCR, it remains possible that this is not true for all HRVs. It is important that both PV and HRV primers are used in 2°PCRs for HRVs until a sufficiently large number of HRVs from clinical material have been identified by both this method and by cell culture.

Restriction enzyme analysis of HRV PCR products [Torgersen et al., 1989] should enable us to determine whether or not the virus found in convalescent nasal and throat swabs is identical to that found in the corresponding acute specimens. In this way, we will gain information about the persistence of HRV infections.

The fact that many specimens which were negative for PVs by cell culture were positive when tested by PVnRT-PCR has significant implications for future work on the epidemiology and potential therapy of respiratory tract infections.

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