Use of synthetic oligonucleotide probes to detect rhinovirus RNA

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Summary. Current methods of detecting a human rhinovirus (HRV) infection are either based on isolation of virus in appropriate susceptible cell lines, which is time-consuming and requires considerable expertise, or are dependent on knowing the serotype. The extistence of over 100 immunologically distinct serotypes makes serotype specific assays, such as ELISA, unsuitable for general diagnostic assays. In this study a general rhinovirus assay is described which utilises synthetic oligonucleotides as probes in a filter hybridization assay. The probes are designed to bind to short but highly conserved regions of the rhinovirus genome. Indeed, the probes successfully detected all 57 rhinovirus serotypes tested. Furthermore, the test was used to demonstrate rhinovirus infection in clinical samples from 57 volunteers, inoculated with HRV, collected on six consecutive days. Clinical samples were taken prior to inoculation and on days 2–7 after inoculation. The filter hybridization assay gave results comparable to virus culture on days 2 and 3 post-inoculation, but was more sensitive on subsequent days.

Introduction

Human rhinoviruses (HRV) are the major causative agents of the common cold, a frequent, although usually minor, infection in man [5]. The high incidence of infections can be explained, at least in part, by the existence of over 100 immunologically distinct HRV serotypes, several of which can co-circulate within a community [6]. In immunocompromised individuals and in children with a history of bronchitis or allergy HRV infection can be very much more serious [2]. Advances are being made in antiviral chemotherapy for respiratory infections [1] but as the drugs are quite specific in their action, the type of virus causing the infection needs to be determined. A number of respiratory viruses, such as respiratory syncytial virus (RSV), influenza, parainfluenza, and adenoviruses, can now be detected rapidly by either immunofluorescence or ELISA. An ELISA has recently been developed to detect HRV directly in nasal washings but the test was found to be generally serotype specific [3]. Because

of the large number of HRV serotypes, such assays are not useful in the general diagnosis of HRV infection. Thus, cultivation of virus in cell culture remains the only general method available to diagnose rhinovirus infection.

Recently the complete nucleotide sequence of the genomes of five rhinoviruses has been determined, HRV 1B [7], HRV 2 [10], HRV 9 [9], HRV 14 [11], and HRV 89 [4]. Comparison of the available sequence data indicated a high degree of homology in the 5' non-coding region of the genome of rhinoviruses (about 90% between any pair of HRVs). This region is also highly homologous to the corresponding region of enteroviruses such as CA21 and poliovirus [8]. We therefore felt that a cDNA probe from the 5' non-coding end of the rhinovirus genome could potentially be useful as a "general" rhinovirus probe. However, in a comparative study of a number of cDNA probes prepared from the 5' non-coding region of HRVs 14, 9, and 1B we found that, while these probes are useful in examining the genomic relationships between the different rhinovirus serotypes, they do not detect all rhinovirus serotypes with equal efficiency. Therefore such probes are not suitable as diagnostic probes [Al-Nakib, unpubl. data].

Analysis of the nucleotide sequence data from the 5' non-coding region of HRVs and other picornaviruses revealed the presence of short regions of the genome which are totally conserved. These conserved regions vary in length from seven to 22 nucleotides and are spread throughout the 5' non-coding region. We chose two of the longer conserved regions, 18 and 22 bases, and synthesised complementary oligonucleotides. This study shows that such regions are indeed highly conserved among the rhinovirus genus and that oligonucleotide probes can successfully be used to detect rhinoviruses in clinical samples taken from infected volunteers.

Materials and methods

Preparation of viral RNA

All viruses used were obtained from the Common Cold Unit reference stocks. Stock HRVs were grown in Ohio HeLa cells as described early [12]. Virus was harvested when cytopathic effect was approximately 80% and the virus released from the cells by 3 cycles of freeze/thawing. The suspension was cleared of cell debris and the virus pelleted (110k g, 2h at 4 °C). The pellet was taken up in TE buffer (10 mM Tris pH 7.4; 1 mM EDTA), treated with proteinase K (0.2 mg/ml) for 60 min and extracted with phenol:chloroform:isoamyl alcohol (50:50:2). Sodium acetate (pH 6.0) was added to the aqueous phase to a final concentration of 200 mM and 2.5 volumes of ethanol were added. The RNA was allowed to precipitate at –20 °C and pelleted by centrifugation (13,000 rpm, 10 min) in an Eppendorf centrifuge. The precipitated RNA was taken up in TE buffer and the amount of RNA in the sample estimated by measuring the optical density at 260 nm and 280 nm. If the ratio of these readings was below 1.8 the sample was re-extracted with phenol/chloroform.

Preparation of viral samples for filter hybridization

Viral suspensions were either treated with SSC/formaldehyde directly after proteinase K treatment or mixed with SSC/formaldehyde after the RNA had been extracted with phenol/

chloroform. Samples were mixed with an equal volume of a 3:2 mixture of 20 × SSC—37% formaldehyde and incubated at 60 °C for 15 min.

The samples were then applied to nitrocellulose membranes (63 × 288 mm, obtained from Schleicher and Schuell) previously soaked in 10 × SSC with the aid of a manifold. The filters were dried and baked under vacuum at 80 °C for 2 h. Prehybridization was performed in Hyb-S (2 × SSC, 5 × Denharts reagent, 200 μg/ml sonicated salmon sperm DNA, 1.25 mg/ml yeast RNA).

Synthetic oligonucleotide probe preparation

Two synthetic deoxyribonucleic acid probes were synthesised on a Biosearch 8650 automatic DNA synthesiser using cyanoethyl phospho aniodate chemistry. These we designated JWAER'1 (GAGGCCGGGGACTTACG) which is complementary to no. 452–468 of the nucleotide sequence of HRV14 and JWAER' 2 (GATGAAACCCACAGGCA) which is complementary to base no. 548–564 of HRV14. 5'-end labelling was carried out using T_4 phosphonucleotide kinase (PNK) and γ–^{32}P. The reaction was carried out in a total volume of 100 μl containing 10 μl 10 × PNK buffer (0.5 M Tris-HCl, pH 7.6, 0.1 M $MgCl_2$, 0.01 M DTT, 0.002 M EDTA), 4 μl (600 ng) oligonucleotide, 10 μl γ–^{32}P ATP (10 mCi/ml, 5,000 Ci/mmol, Amersham) and 10 μl PNK (BRL, 2 U/μl) at 37 °C for 1 h. The oligonucleotides were then separated from the γ–^{32}P ATP on a 20 cm column of sephadex G25. Both synthetic olignucleotides could be labelled and separated simultaneously. The specific activity of the probes was approximately 4–6 × 10^8 cpm/μg.

Hybridization

Filters were hybridized in 50 ml Hyb-S containing 10 ng/ml probe for 16 h at 47 °C (Tm-5 °C). The Tm values of these probes were calculated using an approximate formula; Tm (°C) = 4 (G + C) + 2 (A + T), where G, C, A, T represent number of each nucleotide contained in the oligonucleotide probe [13]. The incubation temperature was calculated for the probe with the lowest Tm. Filters were then washed in 2 × SSC at 47 °C for 2 × 5 min and at 20 °C 3 × 30 min. The filters were air dried and exposed to X-ray film (Fujifilm RX) at –70 °C for up to seven days using an X-ray cassette with intensifying screens.

Human specimens

57 volunteers inoculated intranasally with HRV-14 and three inoculated with coronavirus 229E were used in this study. Nasal washings were collected prior to inoculation and on days 2–7 after virus challenge by instilling 5 ml of a balanced salt solution into each nostril. The expelled fluid was either mixed with an equal volume of nutrient broth and stored at –70 °C prior to virus isolation in Ohio HeLa cells, or mixed with vanadyl ribonucleoside complex (VRC) final concentration 50 mM and stored at –20 °C prior to virus detection by filter hybridization test. Nasal wash viral RNA was prepared for hybridization with proteinase K and SSC/formaldehyde as described above.

The volunteer experiments were undertaken with prior approval from the Harrow District Ethical Committee.

Results

The detection of various rhinovirus serotypes using synthetic oligonucleotide probes

The synthetic probes (JWAER'1 and 2) were hybridized to the RNA extracted from a panel of rhinovirus serotypes as shown in Fig. 1. All 57 HRV tested and the animal rhinoviruses, calf rhinovirus SD1 and bovine rhinovirus EC11,
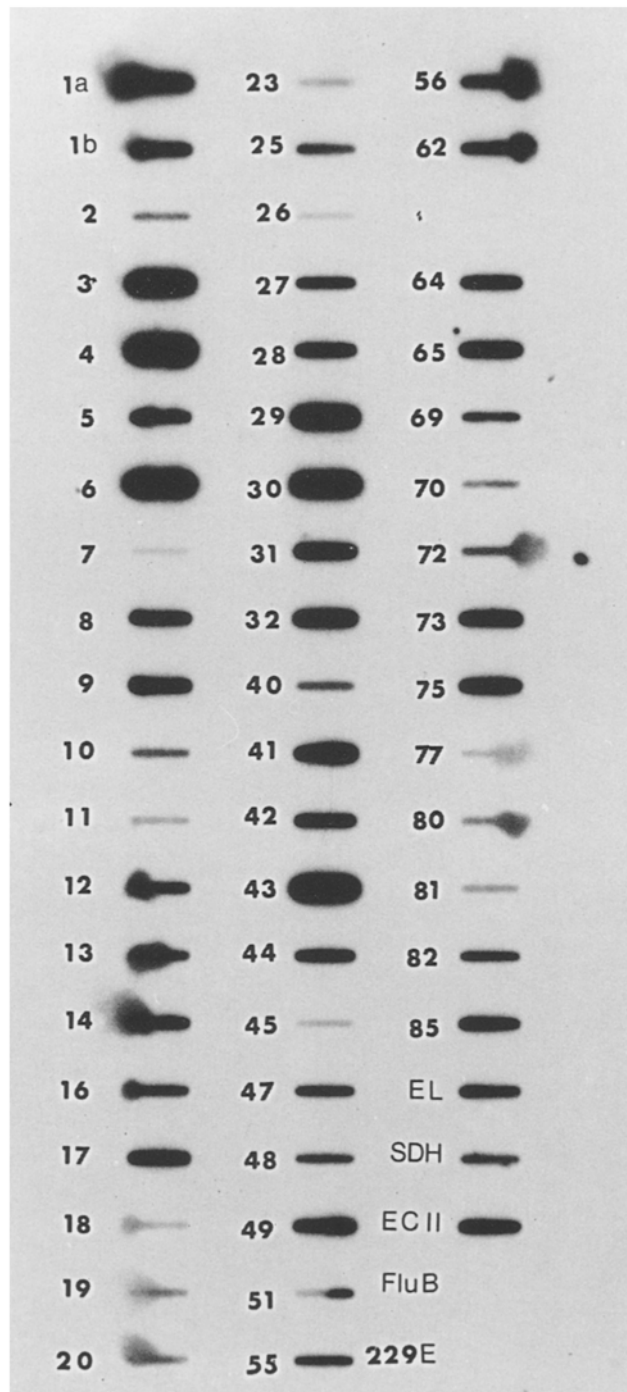
Fig. 1. Detection of RNA from 57 rhinovirus serotypes using synthetic oligonucleotide probes against conserved sequences in the 5′ non-coding region. $250\,\mu l$ tissue culture supernatant was taken for each virus indicated, treated as described in the Methods and applied to the filter. The film was exposed to the filter for 48 h
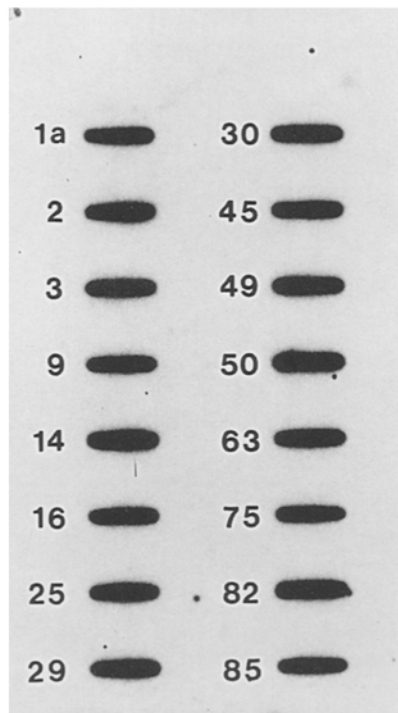
Fig. 2. Constant amounts of RNA (100 ng) from different rhinovirus serotypes detected with synthetic oligonucleotide probes. Film exposed for four days

were clearly detected although the intensity of the signal varied considerably. It was shown that this was due to variations in the amount of RNA in the samples. Figure 2 shows the results when equal amounts of RNA were applied to the filter for several different HRV serotypes and it can be seen that the ratio of the intensity of the signal to the amount of RNA was constant. The probes also react with a wide range of enteroviruses including several serotypes of Coxsackieviruses A and B, echo viruses and all three serotypes of poliovirus (unpublished data). However, none of the other viruses tested such as reoviruses 1 and 3, influenza A, parainfluenza 3 or herpes simplex virus (unpublished data), influenza B or coronavirus 229E gave any hybridization signals.

Detection of human rhinovirus in nasal washings

Figure 3 shows examples of the results obtained with nasal washings collected from three different volunteers on consecutive days following virus challenge. The filters were made in triplicate and a sample considered positive if the signal obtained was clearly different from any background seen in the control samples. For example, in Fig. 3, day 2 for volunteer 1 was found positive on all three filters whereas day 3 was not. Also on day 7 of volunteer 3, although possibly weakly positive on the filter shown, the sample was considered negative because replicate filters were negative. Samples from a total of 57 volunteers who were
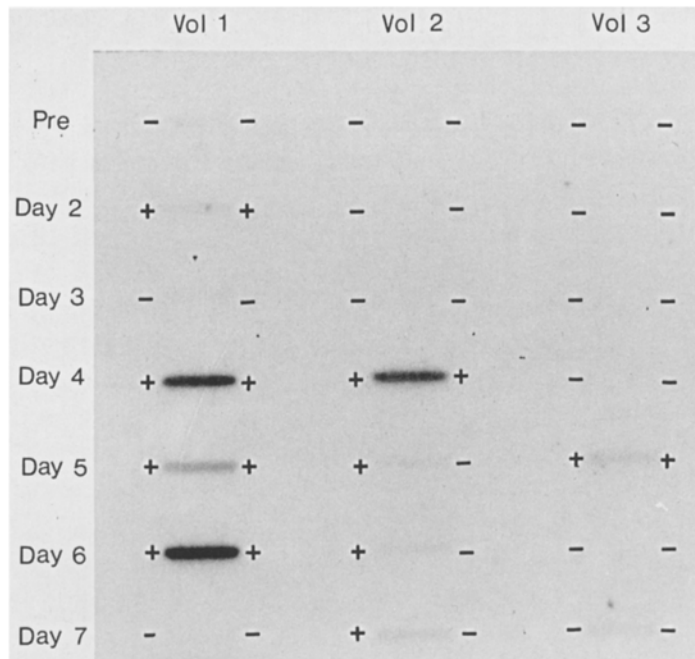
Fig. 3. Detection of RNA in nasal washings obtained from three volunteers (*Vol*) infected with rhinovirus 14. Washings were taken before inoculation (*Pre*) and on days 2–7 after inoculation. 200 µl of nasal washing was prepared and applied to the filter as described in the Methods. The film was exposed for seven days. + or − on the left of the slot indicates a positive or negative result for the filter hybridization assay (from triplicate filters). + or − on the right of the slot indicates the result obtained by culture
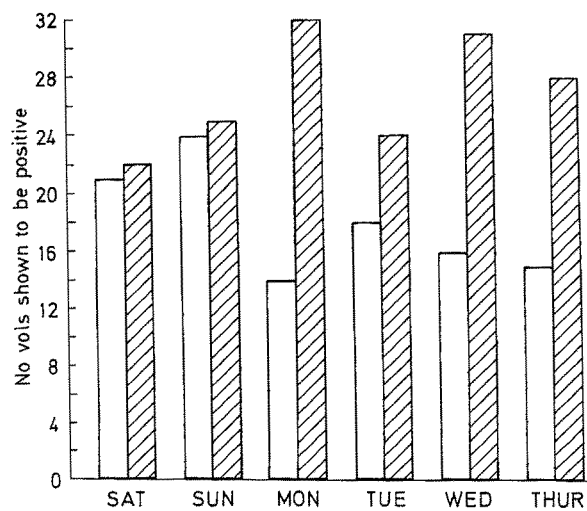
Fig. 4. Number of volunteers found to have rhinovirus present in their nasal washings on each day, as detected by the filter hybridization assay ▨ or by virus isolation ▢. Samples were taken on day 2 post inoculation (*SAT*) through to day 7 post inoculation (*THUR*)

infected with HRV 14 and three volunteers infected with coronavirus 229E were tested. On no occasion was a coronavirus sample positive with the rhinovirus oligonucleotide probes although they were shown to be strongly positive with a probe for 229E [pers. comm. from Dr. S. Myint of our laboratory].

Of the 57 HRV inoculated volunteers, 54 were shown to be positive (positive on at least one day post inoculation) by hybridization assay as compared with 41 by virus culture. Twenty-four volunteers were symptomatic and of these 22 were positive by hybridization and 21 positive by tissue culture. The hybridization assay was usually positive over a longer period than virus isolation by culture as shown in Fig. 4. The difference in numbers of volunteers detected on days 4, 6, and 7 by hybridization as compared to culture were significantly higher with P values of <0.001, <0.001, and <0.01, respectively. Only one sample was found to be positive by culture but not by hybridization.

Discussion

This study demonstrates that synthetic oligonucleotide probes designed to bind to highly conserved regions of the rhinovirus genome will detect all of the 57 rhinovirus serotypes tested. We predict that these regions are conserved throughout the rhinovirus genus and that these probes will therefore be useful as "general" rhinovirus diagnostic probes. Indeed, using filter hybridization, we have also demonstrated that the probes can detect HRV RNA directly in clinical samples. In a study involving 57 volunteers inoculated with HRV 14, the probe detected as many rhinovirus positive samples as conventional virus cultures during the first two days of virus shedding. However, later in the course of infection, days 4–7, the probe detected more positive samples than culture. Overall the hybridization assay found 54 rhinovirus positive volunteers as compared with 41 by tissue culture. The two methods detected approximately equal numbers of positives among symptomatic volunteers, 22 by hybridization and 21 by tissue culture. However, hybridization detection more evidence of infection among asymptomatic volunteers than culture. This may be due to several factors. Very little is known about the particle number to infectious dose ratio. It is known that non-infectious particles are formed at some stages of an infection. If these were to contain the appropriate region of the genome (5' non-coding region) the hybridization assay would detect these non-infectious particles whereas culture methods would not. Also at later stages of infection, many of the virus particles may be antibody bound, again preventing detection by culture but not by hybridization. Further experiments would be required to determine the reason for the differences between the two methods.

The hybridization assay was specific for rhinoviruses since none of the volunteers who were infected with coronavirus who shed virus were positive for rhinovirus. These samples included two symptomatic and one asymptomatic coronavirus infections containing a normal range of coronavirus titres ($\leqslant 10^3$ $TCID_{50}$). Also much higher amounts of virus (10^7 $TCID_{50}$) grown in tissue

culture were found to be negative. The volunteers' own pre-inoculation nasal wash was also used as a control.

Furthermore, all volunteers who were rhinovirus RNA positive by hybridization and from whom a rhinovirus was not cultured, showed rising titres of specific neutralizing antibody and were therefore infected. One sample was found to be positive by culture but not by hybridization but this was only found on blind passage (not normally carried out).

In the present test the probes were labelled with ^{32} phosphorus. However, it is envisaged that in the future it would be possible to use biotinylated synthetic oligonucleotides. Such a modification could decrease still further the time required and make it more suitable for general diagnostic use. Viruses could then be typed if required either by using cDNA probes prepared from the 3' end of the genome [Al-Nakib, unpubl. data] or by ELISA [3].

Since these conserved regions of the rhinovirus genome are also found in enteroviruses, we feel that these probes could also be used to detect enteroviruses in appropriate clinical material.

Acknowledgements

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