Detection of rhinovirus RNA in nasal epithelial cells by in situ hybridization

Christine Bruce[1], Patricia Chadwick[1] and Widad Al-Nakib[1,2]

[1]MRC Common Cold Unit, Salisbury, U.K. and [2]Department of
Microbiology, Faculty of Medicine, University of Kuwait, Safat, Kuwait

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Summary

This paper describes the development and evaluation of in situ hybridization
(ISH) for the detection of rhinovirus in cells obtained from nasal washings of
volunteers infected with human rhinovirus 14 (HRV-14).

Twenty-five (66%) and 27 (71%) of 38 volunteers inoculated with HRV-14 had
evidence of infection by virus isolation and ISH, respectively, on at least one of 4
days investigated after virus challenge. In contrast, only 14 of 38 (37%) volunteers
had significant antibody rises as detected by the neutralization test.

Of the 38 volunteers inoculated with HRV-14, only 13 (34%) had symptoms
of colds. Of these, 12 (92%) and 10 (77%) were positive by virus isolation
or ISH, respectively, on at least one day. Six (46%) had significant antibody
rises by neutralization. Similarly, of the 38 volunteers challenged, 22 (58%) were
asymptomatic and of these 10 (45.5%) and 12 (54.5%) were positive by virus
isolation and ISH, respectively, on at least one day. Only 8 (36.4%) of these
asymptomatic volunteers showed significant antibody rises by neutralization.

There were significant associations between the detection of rhinoviruses by ISH
and virus isolation on the third day ($P<0.025$) after virus challenge in the group
as a whole and in the symptomatic group.

These results show that generally rhinovirus detection by ISH compares well
with virus isolation and both tests are clearly more sensitive than the neutralization
test in detecting evidence of infection. It is concluded that ISH is an interesting
new technique that may play an important role in the study of rhinovirus infection
and pathogenesis.

Rhinoviruses; cDNA; Probes; Picornaviruses; In situ hybridization; Respiratory
viral infections

Introduction

Human rhinoviruses (HRV) are the major causative agents of the common cold (Couch, 1984). Until recently, the presence of rhinovirus in the nasal secretions of an individual presenting with symptoms of the common cold, could only be demonstrated by cultivation in a sensitive cell or organ culture (Al-Nakib and Tyrrell, 1988). Recently, however, cDNA probes have been successfully used to detect the presence of viral RNA in filter hybridization assays, although viruses present at low concentrations ($<10^3$ TCID$_{50}$/ml) may not be detected (Al-Nakib et al., 1986). In situ hybridization has recently been applied in the rapid diagnosis of a number of virus infections such as cytomegalovirus (CMV) in liver graft cells (Naoumov et al., 1988), herpes simplex virus (HSV) in brain cells (Forghani et al., 1985), human papillomavirus in cervical tissues (Burns et al., 1987); and adenoviruses in cells obtained from nasopharyngeal secretions (Gomes et al., 1985). This technique is useful in that viral nucleic acid sequences can be located within individual cells using complementary probes labelled with an isotope such as ^{32}-phosphorus or non-isotopic label such as biotin. We report on the application of in situ hybridization for the detection of rhinoviruses directly in cells obtained from nasal washings of human volunteers infected with a rhinovirus. This technique may have important application both for the detection of infection by demonstrating virus RNA in single cells and also for the study of the pathogenesis of this virus in the respiratory tract.

Methods

Specimen collection and processing for ISH

Nasal wash samples from volunteers were collected as recently described (Al-Nakib and Tyrrell, 1988). Half the volume of each nasal wash was used to isolate rhinoviruses while the other half was processed for rhinovirus detection by ISH.

Mucus from the samples being processed for ISH was removed using a Percoll density centrifugation step. Thus, samples were mixed with 45% Percoll (in PBS) in a ratio of 2:1 (15% Percoll final concentration) and then layered onto a 20% Percoll cushion. Samples were spun for 5 min at $500 \times g$ in a bench centrifuge. This resulted in the mucus remaining at the top of the gradient while the cells had pelleted. The cells were resuspended in PBS and aliquoted out onto 12-well multitest microscope slides (Flow) which had previously been coated with poly-l-lysine. After air drying, the cells were fixed with freshly prepared periodate-lysine-paraformaldehyde [PLP; three parts buffered lysine (0.1 M lysine, 0.005 M Na$_2$ HPO$_4$), one part 8% paraformaldehyde, 0.01 M sodium periodate]. The samples were fixed for 20 min at room temperature, washed four times with sterile water and dehydrated through a graded series of ethanol washes (30, 60, 80 and 100%, respectively) for 5 min in each. The slides were then air dried and stored at $-70°C$ until required. Control slides were prepared by infecting

confluent layers of Ohio HeLa cells grown in Lab Tek (Flow Laboratories) eight chamber glass slides with HRV-14. When approximately 50% of the cells showed cytopathic effect (CPE), the slides were washed three times with PBS and fixed with PLP as described for the clinical samples.

Post-fixation procedure

Slides stored at −70°C were brought to room temperature and washed twice with PBS, incubated with 0.02 M HCl for 10 min followed by 0.01% Triton X-100 in PBS for 90 s. They were then washed three times with PBS, incubated with proteinase K (1 μg/ml) for 18 min at 37°C and washed with PBS containing 2 mg/ml glycine. The slides were then dehydrated through ethanol as described above.

Hybridization

Hybridization was carried out in a hybridization mixture containing 5 × SSC, 5% Denhardt's reagent, 50 mM sodium phosphate, pH 6.5, 0.25 mg/ml denatured salmon sperm DNA, 40% formamide and 10% dextran sulphate.

^{32}P-labelled probe was prepared by primer extension using a single-stranded M13 template of HRV-14 cDNA of some 800 nucleotides from the extreme 5′ non-coding region of the genome as described previously (Al-Nakib et al., 1987). The probe was labelled with high specific activity-^{32}P-dATP (3000 μCi/mmol; Amersham International). The specific activity of the probe was approximately 4–6 × 10^{6} cpm/μg. The radioactively labelled strand was separated from the template by heat denaturation at 90°C for 5 min immediately before use. Twenty microlitres of the probe in the hybridization mixture was applied to each well of the slides including those of the controls. The slides were incubated at 95°C for 8 min and then at 42°C for 16 h in humidified boxes. After hybridization, the slides were washed three times for 15 min in 2 × SSC at room temperature and then overnight in 2 × SSC with gentle agitation. Slides were then rinsed in H$_2$O twice, air dried and dipped in a K 5 nuclear emulsion (Ilford Scientific) diluted 1:1 with H$_2$O and prewarmed to 45°C. The emulsion was allowed to dry on the slides which were then left to expose at 4°C for 5–10 days before being developed. The cells were then counter-stained with Giemsa and examined under light microscopy.

Volunteers

Volunteers of both sexes aged between 18–50 years were recruited and housed in isolation in groups of 2 or 3 according to previously described procedures (Beare and Reed, 1977).

The volunteers took part in a trial to assess the efficacy of steam inhalation in the treatment of colds induced by human rhinovirus 14 (HRV-14). Details of these trials have recently been described (Tyrrell et al., 1989). Approval of these

trials was given by the Harrow District Ethical Committee.

Briefly, after an observation period of 48 h, volunteers were challenged twice, 1 h apart, with 1000 $TCID_{50}$ of HRV-14, by instilling 0.5 ml of the virus inoculum in each nostril. Symptoms were assessed and scored as previously described (Beare and Reed, 1977; Tyrrell et al., 1989). Only mild, moderate or severe colds were considered significant.

Nasal washings for virus isolation and for ISH were collected on the day before and on each day after virus challenge for 5 days.

Only volunteers with complete data for all the parameters being assessed (e.g. clinical scores, virus isolations and ISH results on all days) were included in the analysis.

Cells from nasal washing samples were also collected over the same period of time from 5 volunteers who were challenged with a coronavirus 229E-like virus and from whose nasal washings coronavirus 229E-like virus was isolated in C16 cells (Phillpotts, 1983). These samples were also found to contain coronavirus RNA by a recently described hybridization method (Myint et al., 1989). Furthermore, they were processed for rhinovirus RNA detection by ISH in the same manner as those samples obtained from rhinovirus challenged volunteers and hence were used as controls.

Virus isolation and detection of antibody rises

Rhinoviruses were isolated in Ohio Hela cells as described previously (Al-Nakib et al., 1987). Similarly, significant rises ($\geq$ 4-fold) in antibody titre between a sample taken prior to virus challenge and one collected 2 to 3 weeks later, were detected using neutralization assays as described previously (Al-Nakib and Tyrrell, 1988).

Statistical analysis

The statistical analysis was carried out using the SPSSX statistical package running on the VAX 8810 machine. Chi-square (χ^2) or Fisher's exact test were used to test the association between virus isolation, ISH and antibody rises.

Results

Development of assay

The optimal assay conditions were initially established using confluent mono-layers of Ohio HeLa cells, grown in Lab Tek 8 chamber glass slides and infected with HRV-14. Various different fixatives such as Carnoy's (6 parts absolute alcohol, 1 part acetic acid, 3 parts chloroform) and PLPG (periodate-lysine-paraformaldehyde-glutarate, 0.5% paraformaldehyde, 1% glutarate in buffered lysine [see PLP] and 0.01 M sodium periodate) were initially evaluated. How-

ever, PLP gave the most consistent results and was particularly useful since it also maintained cell morphology. Eighteen minutes incubation with proteinase K produced the best results, whereas incubation of less than 15 min or more than 20 min were not optimal and often resulted in fewer cells being positive.

Preparation of clinical samples

Cells were collected by centrifugation of the nasal wash (at 6000 rpm in an Eppendorf centrifuge for 5 min) and the resulting pellet was resuspended in PBS and spotted out onto multitest slides. In samples which had no mucus, this method was adequate. However, if mucus was present, which it frequently was, it was pelleted down with the cells and later transferred to the multitest slide. The presence of the mucus prevented the cells from properly adhering to the slide surface and hence were lost following subsequent processing. The use of a Percoll gradient to separate the mucus from the cells, therefore, greatly improved cell retention although still some 10–40% of cells were lost during the procedure.

The number of cells found in different nasal wash samples varied considerably $(0–10^3$ cells). However, the majority of cells observed in nasal washings were mononuclear. The number of epithelial cells in each sample was generally very low (0–50 cells) and averaged about 10 cells per slide. More epithelial cells, however, were obtained by using a nasal scrape although this was not suitable for routine daily sampling as was carried out in this study. Fig. 1a and b shows typical rhinovirus RNA positive and negative cells as detected by ISH.
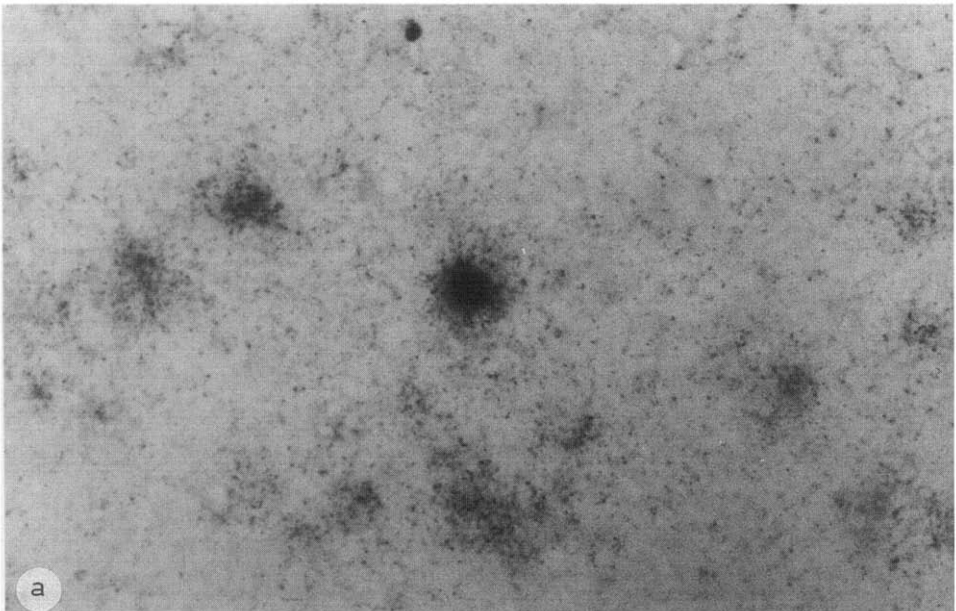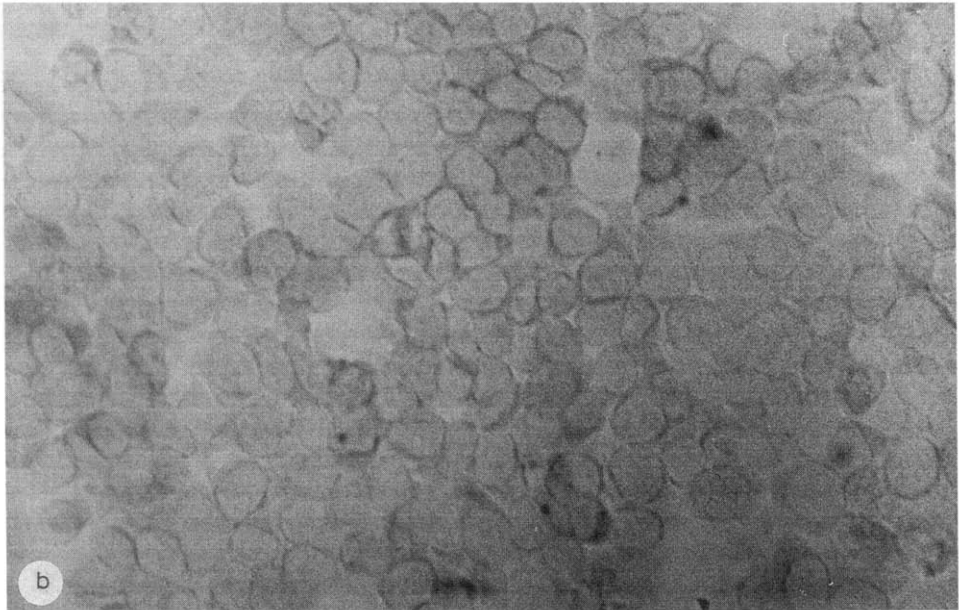

Fig. 1.

Fig. 1. Rhinovirus RNA detection by ISH using ^{32}P-labelled rhinovirus cDNA probes; (a) infected cells (b) uninfected cells.

Detection of rhinovirus infection by virus isolation and in situ hybridization

Thirty-eight volunteers had complete data for all the parameters being assessed and therefore were included in the present analysis. As can be seen from Table 1, 25 (66%) and 27 (71%) of volunteers inoculated with HRV-14 had evidence of infection by virus isolation and by ISH, respectively, on at least one of the four days investigated. Only 14 of 38 (37%) volunteers had significant antibody rises as detected by the neutralisation test. Of the 38 volunteers analysed, 13 (34%) had significant colds. Of these 12 (92%) and 10 (77%) were positive by virus isolation or ISH, on at least one day, respectively. Six (46%) had significant antibody rises (Table 1). Similarly, of the 38 volunteers challenged, 22 (58%)

TABLE 1

Frequency of positive results for different tests in different groups of volunteers

Test	Number (%) of volunteers		
	Whole group (n=38)	Symptomatic (n=13)	Asymptomatic (n=22)
Virus isolation*	25 (66)	12 (92)	10 (45.5)
ISH *	27 (71)	10 (77)	12 (54.5)
Ab rises	14 (37)	6 (46)	8 (36.4)

* Positive on at least one day.

were asymptomatic and of these 10 (45.5%) and 12 (54.5%) were positive by virus isolation and ISH, on at least one day, respectively (Table 1). Only eight (36.4%) of these asymptomatic volunteers showed significant antibody increases by neutralization. These results clearly show that both virus isolation and ISH were more sensitive than serology in providing evidence of infection.

Fig. 2, shows the percentage of volunteers showing a positive test on different days after virus challenge when the whole group of volunteers was considered. As shown in the figure there generally was no significant association between the proportion of volunteers who excreted virus and those who were positive for HRV-14 RNA by ISH, except on day 3 after virus challenge, ($P=0.02$).

Fig. 3 compares the proportion of volunteers who excreted virus with those who were positive for viral RNA by ISH on different days after virus challenge in the group of volunteers who developed symptoms of colds. As shown in the figure, generally more volunteers who developed colds were found positive by virus isolation than by ISH in the first four days after virus challenge and there was a significant association ($P=0.025$) between HRV-14 RNA detection by ISH and the detection of virus by isolation on day 3. However, on day 5 after virus challenge, 54% of volunteers showed a positive test by ISH as compared with only 38% by virus isolation ($P=0.08$).

Fig. 4 compares the results of both tests on different days after virus challenge when the asymptomatic group of volunteers was considered. Generally, there was no significant association ($P=>0.05$) in the proportion of volunteers who were positive when the results of the two tests were compared.

All serial nasal samples obtained from volunteers infected with coronavirus 229E-like virus were found negative for rhinovirus RNA by ISH when processed in exactly the same manner as those from volunteers challenged with a rhinovirus.
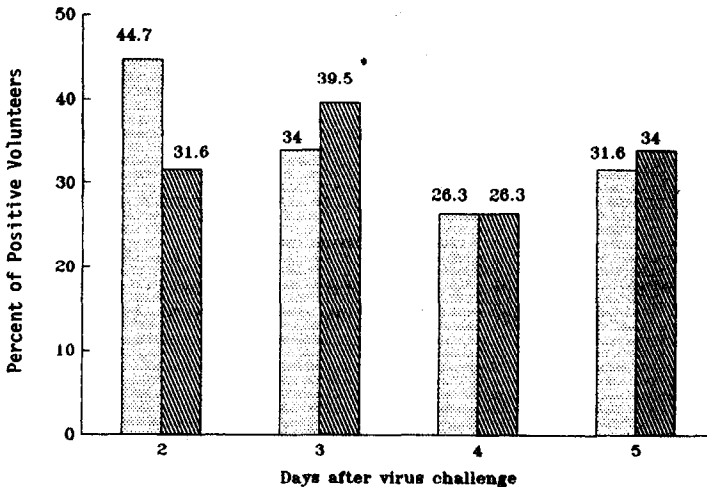

Fig. 2. Frequency of rhinovirus positive results by virus isolation ▨ and ISH ▩ on different days after virus challenge.
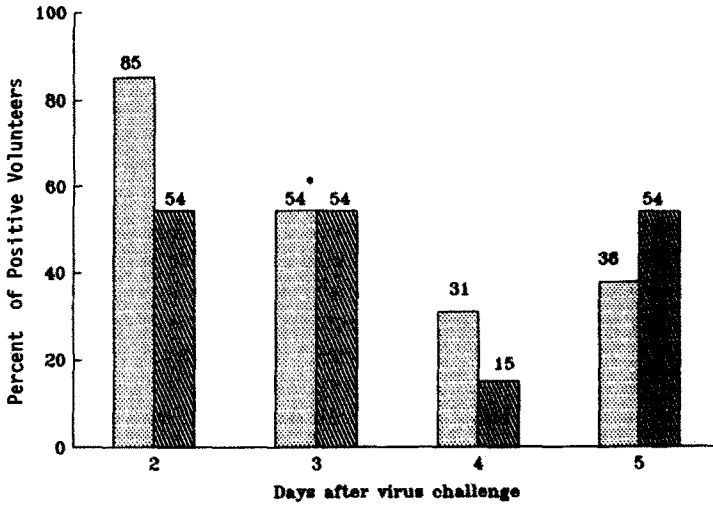
Fig. 3. Frequency of rhinovirus positive results by virus isolation ▨ and ISH ▧ on different days after virus challenge in the symptomatic group of volunteers.

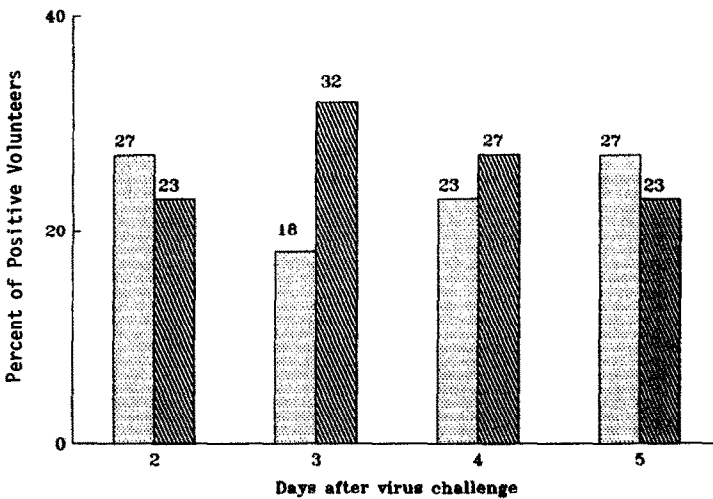Fig. 4. Frequency of rhinovirus positive results by virus isolation ▨ and ISH ▧ on different days after virus challenge in the asymptomatic group of volunteers.

Discussion

This study shows for the first time that rhinovirus RNA can be detected by in situ hybridization directly in cells obtained from nasal washings of volunteers who were challenged with a rhinovirus. Generally, both ISH and virus isolation detected evidence of infection in a high proportion (>66%) of these volunteers

although significant association between the two tests could only be established on day 3 after virus challenge in the group as a whole and in the symptomatic group (P=0.020 and 0.025, respectively).

No attempt was made to establish the sensitivity or specificity of ISH in relation to virus isolation since clearly the two tests measure two different parameters that may not necessarily be both present in the same sample. Thus, for virus isolation to score a positive result it requires a 'viable' virus to be present in a nasal wash sample on a particular day after infection. Furthermore, this virus must replicate successfully in a sensitive cell culture system and produce a recognizable CPE. In contrast, ISH detects specific viral RNA sequences in cells present in the nasal washings irrespective whether 'viable' virus is present or not. Indeed, Gomes et al. (1985) also found that some cells present in nasopharyngeal secretions of adenovirus infected individuals may show adenovirus DNA and antigen by ISH or immunofluorescence, respectively, although virus could not be conclusively isolated.

Nevertheless, the data generally demonstrate that viral RNA may be detected more frequently than virus by isolation in samples obtained from asymptomatic than symptomatic infected individual. In contrast, viable virus was more frequently detected from symptomatic volunteers than RNA. This is not completely unexpected since in symptomatic individuals, infection generally tends to be more severe as a result of more extensive virus replication.

In this study, [32]-phosphorus rather than biotin was used to label the cDNA probe since our early evaluation of biotin labelled probes by ISH showed that these probes may not be sufficiently sensitive to detect rhinovirus RNA consistently in nasopharyngeal cells (unpublished data). Indeed, our experience is similar to that of Kandolf et al. (1987) for the detection of enteroviral genomes by ISH in myocardial cells. They too found that high sensitivity could only be achieved when using radiolabelled cloned cDNA probe. Similarly, Jiang et al. (1989) also showed that to detect and quantitate hepatitis A virus in infected cells, it was essential to use ^{32}P-labelled probes. This not only improved the sensitivity but also the intensity of hybridization.

The ISH procedure described in this study is clearly not rapid and requires some 16 h for hybridization and 5–10 days for optimal intensity of signals to develop in the emulsion. Interestingly, Jiang et al. (1989) also found that to detect HAV RNA optimally, one to two weeks were required to obtain optimal results. One explanation they provided was that a high copy number of progeny viral genome is present relatively early in cells. It is difficult to speculate in this study on the copy number of rhinovirus genomes that may be present in nasal cells following infection in vivo in man.

The specificity of our cDNA rhinovirus probes has been extensively established with regards to reaction with nucleic acids obtained from other viruses and human cells (Al-Nakib et al., 1986, 1987; Forsyth et al., 1989). Furthermore, in this study, all serial nasal washing samples obtained from volunteers infected with coronavirus 229E-like virus and which contained coronavirus as demonstrated by virus isolation and RNA hybridization were consistently negative for rhinovirus

RNA by ISH. In addition, all pre-challenge nasal wash samples from our volunteers were negative for rhinovirus by virus isolation and rhinovirus RNA by ISH. Generally, however, there were very few cells in the pre-challenge samples.

The ISH procedure described would clearly not be suitable for routine diagnosis of rhinovirus infection for a number of reasons. For example, it is not rapid and requires at the present time ^{32}P-labelled probes and therefore does not offer any particular advantage over virus isolation or detection of viral RNA in nasal washings by the use of ^{32}P-labelled synthetic oligonucleotide probes (Bruce et al., 1989a). However, this procedure may complement other methods and can be useful for the study of rhinovirus pathogenicity. Indeed, the whole process of rhinovirus infection of the nasal epithelium and the mechanism by which it produces disease in man is still not entirely clear (Sperber and Hayden, 1988). Furthermore, the role of this virus in the development of lower respiratory tract infection which may be serious in immunocompromised patients (Krilov et al., 1986) or in triggering episodes of bronchitis and asthma (Gregg, 1983) require more precise and detailed investigations. We consider, therefore, that ISH will play an important role in the study of the disease process caused by rhinoviruses in the respiratory tract.

It is considered that further modification of the present ISH test will make the procedure more attractive for wider application. For example, we have recently shown that the use of synthetic oligonucleotide probes corresponding to short but highly conserved regions from the 5' end of the rhinovirus genome could detect all rhinoviruses investigated (Bruce et al., 1989a). Indeed, the same probes also detected all other picornaviruses investigated (Bruce et al., 1989b). Clearly, further research to develop a 'sensitive' non-isotopic ISH for rhinovirus and enterovirus RNA detection, should, indeed, provide a very important procedure to study both rhinovirus and enterovirus infection and pathogenesis.

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