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Detection of Human Coronavirus 229E in Nasal Washings Using RNA:RNA Hybridisation

S. Myint, S. Siddell, and D. Tyrrell

Institute of Virology, University of Würzburg, Federal Republic of Germany (S.M., S.S.); MRC Common Cold Unit, Salisbury, England (D.T.)

A method is described for the detection of human coronavirus 229E (HCV 229E) in nasal washings using RNA:RNA filter hybridisation. Volunteers were inoculated with HCV 229E, and daily nasal washings were collected. These washings were then examined for the presence of viral RNA using a single-stranded RNA probe. Nucleic acid hybridisation is shown to be a sensitive technique for the diagnosis of HCV 229E infections.

KEY WORDS: coronavirus diagnosis, Riboprobes, nucleic acids

INTRODUCTION

Coronaviruses are a group of positive-strand RNA viruses that cause a wide spectrum of disease in mammals and birds [Siddell et al., 1983]. Human coronaviruses are thought to cause about 15% of all common colds [Monto, 1982] and have also been associated with lower respiratory tract infection [Isaacs et al., 1983; McIntosh et al., 1974]. Other disease associations have been suggested but are less well documented [MacNaughton and Davies, 1981; Riski and Hovi, 1980]. Part of the difficulty in defining the role of HCV in disease is the difficulty in detecting the virus. Currently this is dependent on culture of the virus, in either cell monolayers or organ culture, which has the disadvantage of being a lengthy procedure requiring specialist skills. Immunofluorescence has been used [McIntosh et al., 1978] but has not been shown to be reliably sensitive.

Human coronaviruses can be divided into four serological groups, of which the OC38/43 and 229E groups cause the overwhelming majority of coronavirus-associated colds. In this paper we describe a specific and sensitive test to detect one of these major groups, HCV 229E, in nasal washings.

MATERIALS AND METHODS

Materials

T7 RNA polymerase was supplied by Pharmacia. Boehringer Mannheim supplied the restriction enzymes. ^{32}P-labelled nucleotides were purchased from Amersham International. Promega/Biotec supplied the plasmid pGEM-1 and RQ1 DNase. Vanadyl–ribonucleoside complex was purchased from Bethesda Research Laboratories. All other chemicals were supplied by Sigma.

cDNA Cloning and Subcloning

The isolation of HCV-specific cDNA clones will be described in detail elsewhere (Myint et al., submitted). Briefly, using a method based on that of Gubler and Hoffmann [1983], cDNAs were generated from HCV 229E RNA isolated from infected C16 cells [Phillpotts, 1983]. One cDNA, which contained the entire open reading frame of the nucleocapsid gene, was inserted into the polylinker region of the "Riboprobe vector," pGEM-1. This plasmid, pSMGF1, has promotor sequences for SP6 and T7 RNA polymerases flanking the multiple cloning site, and thus single-stranded, HCV-specific RNA transcripts can be generated.

Probe Preparation

RNA probes were transcribed and labelled with ^{32}P using the following reaction: 4 μl 5× transcription buffer (0.2M Tris HCl, pH 7.5, 30 mM $MgCl_2$, 50 mM NaCl, 10 mM spermidine), 2 μl 100 mM DTT, 0.8 μl RNasin (25u/μl), 1 μl 2.5 mM ATP, 1 μl 2.5 mM GTP, 1 μl 2.5 mM UTP, 2.2 μl 100 μM CTP, 2 μl (1 μg) HindIII linearised pSMGF1 DNA, 5 μl ^{32}P-CTP (10 μCi/μl), 1 μl T7 polymerase (10 u/μl). This was incubated at 37°C for 1 hr. Then 1 μl of RQ1 DNase (1 μg/μl) was added and the reaction incubated again at 37°C. After 15 min the reaction was stopped and deproteinised by phenol extraction. The aqueous phase was then precipitated overnight at −20°C by the addition of 10 μl of 7.5 M ammonium acetate and 75 μl ethanol. After centrifugation, the RNA precipitate was resuspended in 100 μl TE (10 mM Tris HCl, pH 7.5, 1 mM EDTA) buffer.

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Address reprint requests to Dr. Steven Myint, Department of Medical Microbiology, University College, London WC1E 6JJ, England.

Probe Characterisation

The RNA probe has been characterised regarding its sensitivity and specificity. Details of this characterisation will be described elsewhere (Myint et al., submitted). Hybridisation to known quantities of HCV 229E RNA showed that less than 1 ng of virus-specific RNA from C16 cells infected with HCV 229E could be detected. Hybridisation to the RNA of 42 common cold viruses showed that only the HCV 229E group was detected.

Nasal Washings

Nasal washings were collected from seven volunteers. Details of the method of collection and design of trials at the Common Cold Unit have been described by Beare and Reed [1977]. Nasal washings were collected prior to challenge with HCV 229E at a titre of 100 $TCID_{50}$/ml and on the second to the sixth day thereafter. Washings were collected in two aliquots. The first 1 ml of washings was collected into an empty pot, and the rest was collected directly into 500 μl of a 200 mM stock solution of vanadyl–ribonucleoside complex (VRC). The VRC concentration was adjusted to 20 mM end-concentration once the final volume of nasal washing from each volunteer was known. Nasal washings were then stored in an equal volume of nutrient broth at −70°C until required.

Slot-Blot Filter Hybridisation

At the end of the trial, nasal washings were thawed out and subjected to protein digestion in the following reaction: 500 μl nasal washings, 60 μl proteinase K ×10 buffer (0.1 M Tris HCl, pH 7.8, 0.05 M Na EDTA, 5% SDS), and 10 μl proteinase K (20 mg/ml, stock). The reaction was allowed to proceed at 37°C for 1 hr, and then the proteins were extracted with phenol. One hundred microlitres of 3 M sodium acetate was added to 450 μl of the aqueous phase, and the nucleic acid was precipitated at −70°C for 30 min. After centrifugation at 13,000*g* for 10 min, the precipitate was resuspended in 100 μl TE buffer and mixed with 100 μl of 6.15 M formaldehyde/10× SSC (1× SSC is 0.15 M NaCl, 0.01 M sodium acetate, pH 7.0). The material was then applied directly to a nitrocellulose filter using a Schleicher and Schuell slot-blotting manifold. A positive control, 5 ng of poly A-selected RNA from HCV 229E-infected C16 cells, and a negative control, 50 ng of poly A-selected RNA from uninfected C16 cells, were also applied. After baking at 80°C for 2 hr the filters were incubated in hybridisation buffer (50% formamide, 50 mM sodium phosphate, pH 6.5, 5× SSC, 0.1% SDS, 0.05% Ficoll, 0.05% PVP, 200 μg/ml denatured herring sperm DNA) at 56°C for at least 4 hr. The probe was added, and hybridisation was allowed to proceed at the same temperature for 16 hr. The nitrocellulose filter was washed three times in 0.1× SSC/0.1% SDS at 65°C, each wash being 20 min. Autoradiography was for 6 hr at −70°C. Positive signals were identified visually and by densitometry.

Virus Titration

Virus in nasal washings was titrated by an end-point dilution method in flat-bottomed microtitre wells. Then 5×10^4 C16 cells were inoculated into each well of a microtitre plate and allowed to attach at 37°C for 2 hr. Six 10-fold dilutions of 100 μl nasal washing that had been stored without VRC were made in C16 growth medium. Each dilution was inoculated into four wells of a row of a microtitre tray, the last two rows being used as cell controls. After 24 hr the medium was replaced with fresh C16 maintenance medium, and again at 5 days. After 10 days, the plates were fixed in formol-saline for 4 hr and stained with crystal violet. The $TCID_{50}$ titre was estimated using the formula of Reed and Muench.

ELISA Tests for Specific IgG Antibodies

The methods used have been described by Callow [1985]. Specific IgG was measured in sera collected prior to virus challenge and in sera collected 2–3 weeks after challenge.

Clinical Score

Volunteers were assessed daily by a clinician who ascribed a clinical score on the basis of systemic and local symptoms and local signs. This score, along with the clinician's judgement, was used to grade the clinical illness into one of five categories: no cold, doubtful cold, mild cold, moderate cold, or severe cold (further details have been given by Beare and Reed [1977]).

RESULTS

The results of virus titration and probing of nasal washings from seven volunteers are presented in Table I. The ELISA data are given as supportive evidence of infection. A ratio of 1.5 or greater is taken to indicate infection.

Figure 1 shows a typical autoradiograph of washings from three volunteers, only one of whom suffered a cold. Three of the seven volunteers suffered a cold, and all three volunteers had detectable coronavirus RNA in their nasal washings. None of the asymptomatic volunteers had detectable viral RNA in their nasal washings. No virus was cultivated from these patients.

Table II shows a comparison of the sensitivity and specificity of virus isolation and the hybridisation method. There were no false positives or false negatives. However, there was serological evidence of infection in three volunteers who did not shed virus.

DISCUSSION

The results we have obtained show that the detection of HCV 229E infection by nucleic acid hybridisation is a reliable and specific method. It is rapid, it does not depend on having cultures of susceptible cells available, and it does not require trained personnel to rec-

TABLE I. Virus Isolation, RNA Hybridisation, and Specific IgG Analysis of Nasal Washings From Volunteers Challenged With HCV 229E

	A		B		C		D		E		F		G	
	RNA probe	Viral titre[a]	RNA probe	Viral titre	RNA probe	Viral titre	RNA probe	Viral titre	RNA probe	Viral titre	RNA probe	Viral titre	RNA probe	Viral titre
Day														
0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	+	10^3	–	–	+	10^1	–	–	–	–	–	–
3	–	–	+	$10^{3.5}$	–	–	–	–	–	–	–	–	+	10^3
4	–	–	+	$10^{3.5}$	–	–	–	–	–	–	–	–	+	10^3
5	–	–	+	10^2	–	–	–	–	–	–	–	–	+	10^3
6	–	–	+	10^1	–	–	–	–	–	–	–	–	+	$10^{2.5}$
7	–	–	–	–	–	–	–	–	–	–	–	–	+	10^1
Clinical score[b]	0.5 (no cold)		60.5 (moderate cold)		9.0 (no cold)		34.0 (mild cold)		0.5 (no cold)		4.0 (no cold)		10.5 (mild cold)	
IgG ELISA ratio[c]	0.7		4.9		1.6		1.3		2.5		9.3		14.4	

[a]Viral titres are expressed as $TCID_{50}$/ml.
[b]The clinical score is a semiobjective means of determining the severity of clinical illness [for details, see Beare and Reed, 1977].
[c]See Callow [1985].

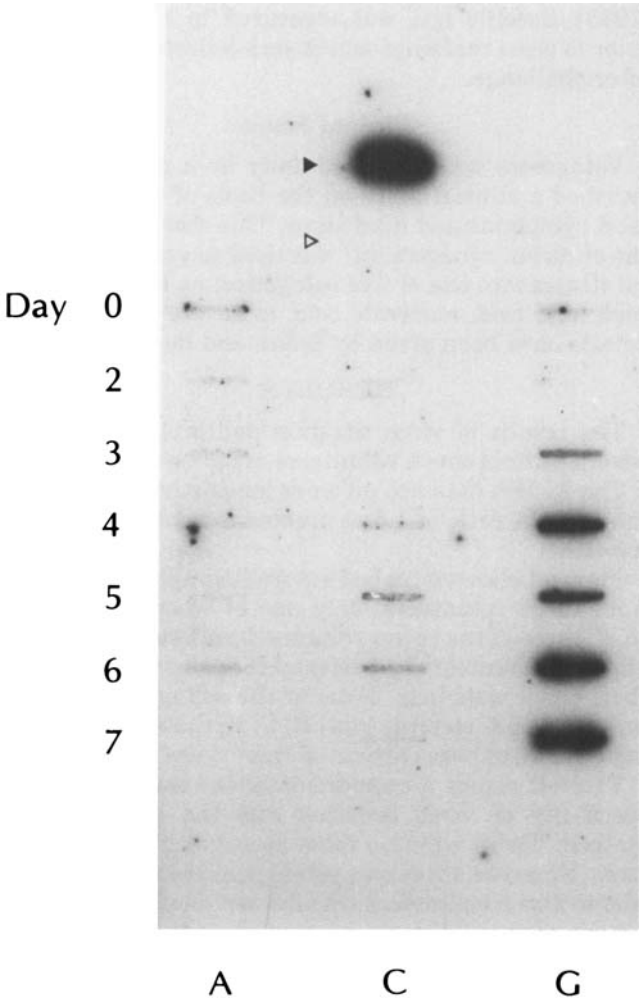

Fig. 1. Hybridisation analysis of HCV 229E RNA in nasal washings from three volunteers (volunteers A, C, and G in Table I). Open arrowhead: 50 ng of poly A^+ RNA from uninfected C16 cells was immobilised on the nitrocellulose filter; closed arrowhead: 5 ng of poly A^+ RNA from HCV 229E-infected C16 cells were immobilised.

TABLE II. Comparison Between Virus Isolation and Nucleic Acid Hybridisation

		Hybridisation result	
Virus culture		Positive	Negative
Positive	11	11	0
Negative	38	0	38

ognise the rather uncharacteristic cytopathic effect of HCV infection.

The results also indicate that the method is as sensitive as the procedures for virus titration used in this study. However, it is probable that virus titration may not be as sensitive as other isolation procedures, such as adaption to tissue culture by blind passage. Indeed, the results of the IgG immunoassay we performed suggest that three of the seven volunteers were infected, although we were not able to isolate virus. On the other hand, we have not yet tried to optimize fully the specific radioactivity of the RNA probe, nor have we systematically investigated the optimal hybridisation conditions. We believe the sensitivity of the hybridisation method can also be significantly increased.

Despite these limitations it is clear that this nucleic acid hybridisation method is applicable to the diagnosis of coronavirus infections in the clinical setting. We intend to evaluate this method further in volunteers and in field trials, and we are sure it will prove to be a useful epidemiological tool in such studies, particularly as a large number of specimens can be simultaneously examined. It is also our intention to modify the test, in particular, by adaption to a nonradioactive labeling system. It could then be used as the primary test for the diagnosis of HCV 229E infections. Indeed, it might be the only detection method applicable in certain situations, such as in detecting virus bound to an antibody or drug.

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REFERENCES

Beare AS, Reed SE (1977): The study of antiviral compounds in volunteers. In Oxford JS (ed): "Chemoprophylaxis and Virus Infections of the Respiratory Tract." Cleveland, OH: CRC Press, pp, 28–55.

Callow KA (1985): Effect of specific humoral immunity and some non-specific factors and resistance of volunteers to repiratory coronavirus infection. Journal of Hygiene 95:173–189.

Gubler U, Hoffmann BJ (1983): A simple and very efficient method for generating cDNA libraries. Gene 25:263–269.

Isaacs D, Flowers D, Clarke JR, Valman B, MacNaughton MR (1983): Epidemiology of coronavirus respiratory infections. Archives of Disease in Childhood 38:500–503.

MacNaughton MR, Davies HA (1981): Human enteric coronaviruses. Brief review. Archives of Virology 70:301–313.

McIntosh K, Chad RK, Krause HE, Wasil R, Mosega HE, Mufson MA (1974): Coronavirus infection in acute lower respiratory tract disease of infants. Journal of Infectious Diseases 130:502–507.

McIntosh K, McQuillin J, Reed SE, Gardner PS (1978): Diagnosis of human coronavirus infection by immunofluorescence: Method and application to respiratory disease in hospitalised children. Journal of Medical Virology 2:341–346.

Monto AS (1982): Coronaviruses. In Evans AS (ed): "Viral Infections of Humans." New York: Plenum Medical Book Company, pp 151–165.

Phillpotts RJ (1983): Clones of MRC-C cells may be superior to the parent line for the culture of 229E-like strains of human respiratory coronavirus. Journal of Virological Methods 6:267–269.

Riski H, Hovi T (1980): Coronavirus infections of man associated with diseases other than the common cold. Journal of Medical Virology 6:259–265.

Siddell SG, Anderson R, Cavanagh D, Fujiwara K, Klenk HD, MacNaughton MR, Pensaert M, Stohlman SA, Sturman L, van der Zeijst BAM (1983): Coronaviridae—Third report of the coronavirus study group. Intervirology 20:181–189.