Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Molecular and Cellular Probes (1994) **8**, 357–364

Evaluation of nested polymerase chain methods for the detection of human coronaviruses 229E and OC43

S. Myint,[1]* S. Johnston,[2] G. Sanderson[2] and H. Simpson[3]

Departments of [3]Child Health and [2]Microbiology, University of Leicester and [1]Department of Medicine, University of Southampton, UK

(Received 8 March 1994, Accepted 3 May 1994)

Currently, the diagnosis of human respiratory coronavirus infection is either slow or insensitive. This paper describes nested polymerase chain reaction assays for the detection of human coronaviruses OC43 and 229E. The specificity and sensitivity of the assays have been determined and they have been applied to the detection of the viruses in nasal aspirates. These assays are more rapid and sensitive than cell culture and may replace the latter as the diagnostic method of choice.

KEYWORDS: coronavirus, respiratory tract, diagnosis, polymerase chain reaction.

INTRODUCTION

Human coronaviruses are the second most frequently isolatable agents from individuals with common colds. There are two major serotypic groups represented by human coronaviruses 229E and OC43. Apart from common colds, they have also been found to commonly cause upper respiratory infections antecedent to the onset of acute wheezing in children.[1] It is in this latter context of serious disease that rapid, sensitive diagnosis would be most useful in clinical practice when antiviral therapy becomes available. Previously, the detection of human coronavirus 229E has relied, mainly, on cell culture or serology. The former requires specialised cells and may take longer than a week before a cytopathic effect is discernible, the latter method similarly takes time because of the requirement for paired sera. A nucleic acid hybridisation method based on Northern blotting has also been developed but this still requires 48–72 h to elapse before the most sensitive result is obtained.[2] Diagnosis of OC43 has depended, primarily, on examination of paired sera for a four-fold antibody rise and organ or mouse brain culture; both of these latter techniques require specialist expertise. A rapid immunofluorescence method has been developed but is, relatively, insensitive.[3] The polymerase chain reaction (PCR) affords a rapid and more sensitive diagnostic method for both viruses. In this study we have evaluated the use of 'nested' PCR assays for the detection of human coronaviruses 229E and OC43.

MATERIALS AND METHODS

Viruses

The 229E and LP-strain viruses were isolates from inoculated volunteers that had been subsequently passaged and grown in C16 cell culture. OC43 was grown in mouse-brain culture. Other viruses (influenza A, parainfluenza, adenovirus, respiratory syncytial virus and rhinovirus) were laboratory isolates from patients with clinical illness.

* For correspondence at: Department of Microbiology, University of Leicester, Leicester LE1 9HN, UK.

Table 1. HCV 229E oligonucleotide primer sequences

Outer primers	
Sense	GGTACTCCTAAGCCTTCTCG
Antisense	TGCACTAGGGTTAATGAAGAGG
Inner primers	
Sense	TTTGGAAGTGCAGGTGTTGTGG
Antisense	GACTATCAAACAGCATAGCAGC

Table 2. HCV OC43 *oligonucleotide primer sequences*

Outer primers	
Sense	AGGAAGGTCTGCTCCTAATTCC
Antisense	TGCAAAGATGGGGAACTGTGGG
Inner primers	
Sense	GTTCTGGCAAAACTTGGCAAGG
Antisense	TTATTGGGGCTCCTCTTCTGGC

Clinical samples

Nasal aspirates were collected from children with recurrent wheezing following an antecedent upper respiratory tract illness as part of community based studies into childhood asthma (papers in preparation). Aspirates were collected into 1 ml of sterile ice-cold phosphate-buffered saline containing 100 U ml^{-1} recombinant RNAasin (Promega). After a brief period (2–6 weeks) of storage at −70°C these samples were inoculated into cell culture. Matched paired sera were collected from the same children. Thirty samples were used to evaluate the PCR in this study: 15 samples were ELISA or culture positive for coronaviruses, 15 were negative; of this latter group, 10 were culture-positive for other viruses (rhinovirus, adenovirus, respiratory syncytial virus, parainfluenza or influenza virus) and five were negative.

Coronavirus culture

Nasal aspirates were inoculated into C16 cells in roller culture tubes at 33°C. Cytopathic effect (CPE) was sought daily for 2 weeks. Facilities were not available for the routine isolation of OC43.

Oligonucleotide primers

Primers were synthesised on an Applied Biosystems model 391 DNA synthesizer and used without further purification. 'Nested' primers were designed from the nucleotide sequence of the LP strain of human coronavirus 229E[4] and of OC43[5] using commercially-available software (Primer Detective v1·0, Clontech, Palo Alto, USA). The sequences of these primers in a 5′ to 3′ orientation are given in Tables 1 and 2.

Predicted 229E primer products are 370 and 116 base pairs from the first and second round reactions respectively. OC43 primer products are 450 and 100 base pairs from first and second amplification rounds, respectively.

RNA extraction

Nucleic acid was extracted under RNase-free conditions using an acid guanidium thiocyanate-phenol-chloroform extraction procedure.[6] All samples were resuspended in 100 µl Tris-EDTA buffer with the addition of 10 U μl^{-1} recombinant RNase inhibitor (Promega).

RT-PCR

cDNA synthesis with the downstream outer primer was carried out in the following reaction mix (20 µl total volume): 2 µl 10 × RT buffer (500 mM Tris-HCl pH 8·3, 750 mM KCl, 30 mM $MgCl_2$), 4 × 0·4 µl 10 mM dNTPs, 0·5 µl 0·1 M dithiothreitol, 5 µl (100–200 pmoles) RNA suspension, 2 × 1 µl (100 pmol) primer, 4·9 µl water. This mix was subjected to 65°C for 5 min and then quenched on ice. cDNA synthesis was then carried out with 5 µl (200 U μl^{-1}) MoMLV reverse transcriptase at 37°C. Enzyme activity was then stopped by heating to 95°C for 5 min.

cDNA was then amplified in the following (50 µl total volume): 5 µl RT-mix, 5 µl 10 × PCR buffer (Perkin-Elmer Cetus), 6 µl $MgCl_2$ (25 mM) 4 × 0·2 µl dNTPs, 2 × 0·5 µl (50 pmol) primers, 31·7 µl water. After heating to 95°C for 2–5 minutes, 0·5 µl (1 U) Stoffel *Taq* polymerase (Perkin-Elmer Cetus) was added. Cycling parameters were set on a Perkin-Elmer 480 machine as 94°C for 60 s, 62°C for 90 s and 72°C for 120 s for 25 cycles. Second round amplification was in the following mix: 5 µl first round mix, 5 µl 10 × PCR Buffer, 6 µl $MgCl_2$ (25 mM), 4 × 0·2 µl dNTPs, 2 × 1 µl (50 pmol) primer, 31·7 µl water. After denaturation at 95°C for 2–5 min, 0·5 µl Stoffel *Taq* polymerase was added. This second round consisted of 25 cycles of 94°C for 60 s, 66°C (for 229E) or 67°C (for OC43) for 60 s and 72°C for 100 s. All reaction tubes had a 50 µl sterile paraffin oil overlay. Amplification products were visualised in 2% agarose gels with ethidium bromide staining.

Positive controls were a plasmid, pSMFG1, which contains the entire nucleocapsid gene for 229E,[4] or tissue culture-derived virus (229E or suckling mouse brain-grown virus (OC43).

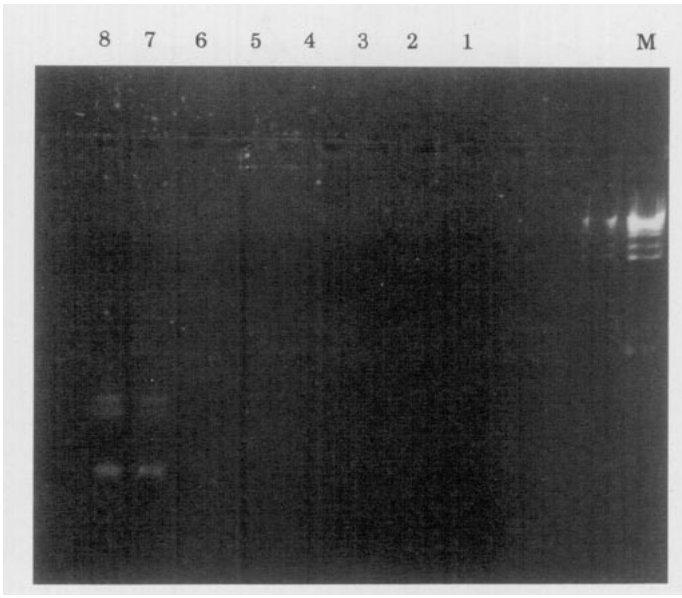

Fig. 1. The specificity of 229E assay. Lane M, 1-kb molecular weight ladder (Gibco). Lanes 1–8: influenza A, adenovirus, respiratory syncytial virus, rhinovirus, prainfluenza virus type 3, OC43, LP and 229E respectively.

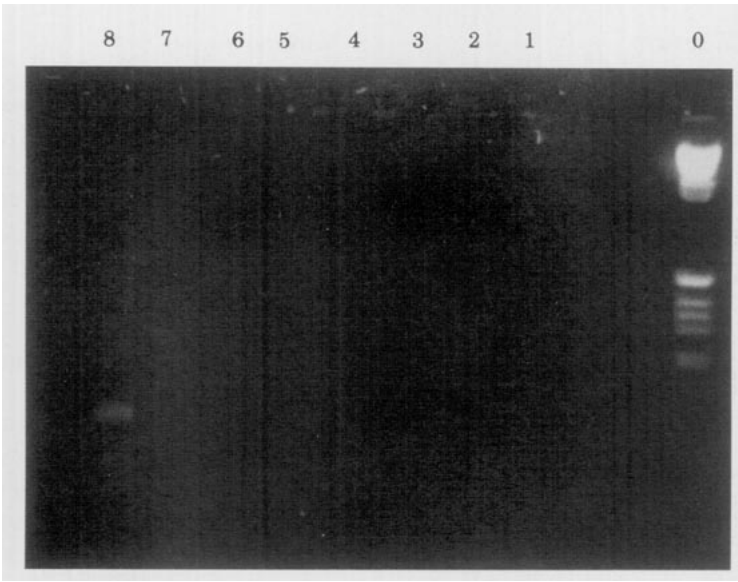

Fig. 2. The specificity of OC43 assay. Lane M, 1-kb molecular weight ladder (Gibco). Lanes 1–8: parainfluenza type 3, respiratory syncytial virus, rhinovirus, influenza A, adenovirus, 229E, LP and OC43 respectively.

Negative controls were water and C16 tissue culture extracted DNA; these were used in all experiments.

All amplifications were carried out using optimal conditions to minimise contamination, including physical separation of pre- and post-PCR reactions. All positive reactions were confirmed by repeating the PCR 1–2 weeks later after a period when coronavirus PCR had not been attempted in the same laboratory.

Specificity of PCR assay

Coronaviruses 229E, LP, OC43, rhinovirus (unknown serotype), parainfluenza type 3, respiratory syncytial virus, adenovirus, and influenza A virus were subjected to both 229E and OC43 assays under identical conditions. The infectious load of OC43 was unknown but the infectious titre of the other viruses used was prepared as 10^3–10^4 $TCID_{50}\,ml^{-1}$.

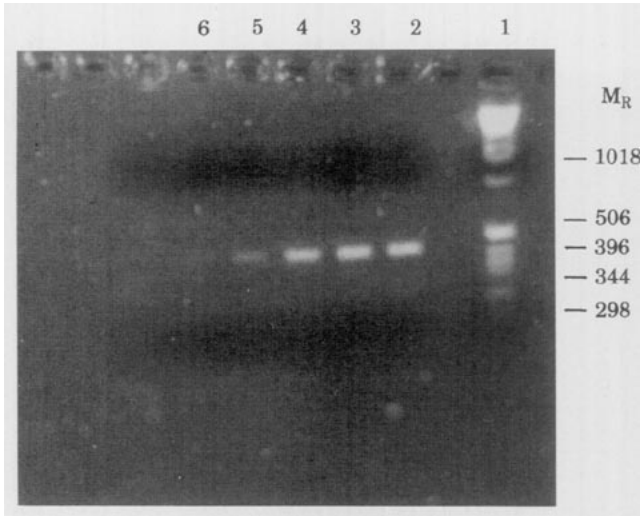

Fig. 3. *Sensitivity of 229E assay. Lane 1, molecular weight ladder as shown, lanes 2–6 1 ng, 100 pg, 1 pg and 0·1 pg of* pSMFG1 plasmid respectively.

Sensitivity of 229E PCR assay

Ten-fold dilutions of a 1 ng μl^{-1} stock solution of pSMFG1 were made and each dilution used as template for the RT-PCR.

ELISA

ELISA methods for OC43 and 229E were carried out under the conditions previously described from the MRC Common Cold Unit, Salisbury.[7]

Other viruses

PCR diagnosis for rhinoviruses,[8] *parainfluenza (unpublished data), respiratory syncytial virus (unpublished data), influenza*[9] *and adenoviruses (unpublished data) was undertaken on specimens not yielding a positive diagnosis by coronavirus PCR.*

RESULTS

Specificity

The specificity of the tests is shown in Figs 1 and 2. As anticipated the 229E assay was specific for 229E and the closely-related LP strain. The OC43 assay did not detect LP virus.

Sensitivity

Use of the outer primers alone was able to detect 1 pg of pSMFG1 (Fig. 3). The nested assay was 5–10 time more sensitive (not shown). This is about 1000-fold more sensitive than the 'Riboprobe' previously described, which was able to detect 1—10 $TCID_{50}$ ml^{-1} of virus in spiked nasal washings.[10]

Application to clinical specimens

First round amplification products were analysed in 2% agarose gels but only plasmid positive controls and samples 2, 3 and 7 yielded visible primer products. Gel electrophoresis of second round products is shown in Fig. 4. Table 3 summarises the ELISA, *cell culture and PCR data for 20 specimens. Table 4* shows a comparison between PCR and the combined use of the two more traditional methods.

DISCUSSION

We have chosen primers from the nucleocapsid gene of the viruses as these might be expected to be conserved. The 'nested' approach and the design of primers that would yield a small primer product were used to increase both sensitivity and specificity; although the increase in sensitivity over just using the internal primers for 35 cycles apears to be marginal (Fig. 5).

Both the OC43 and 229E methods are specific and

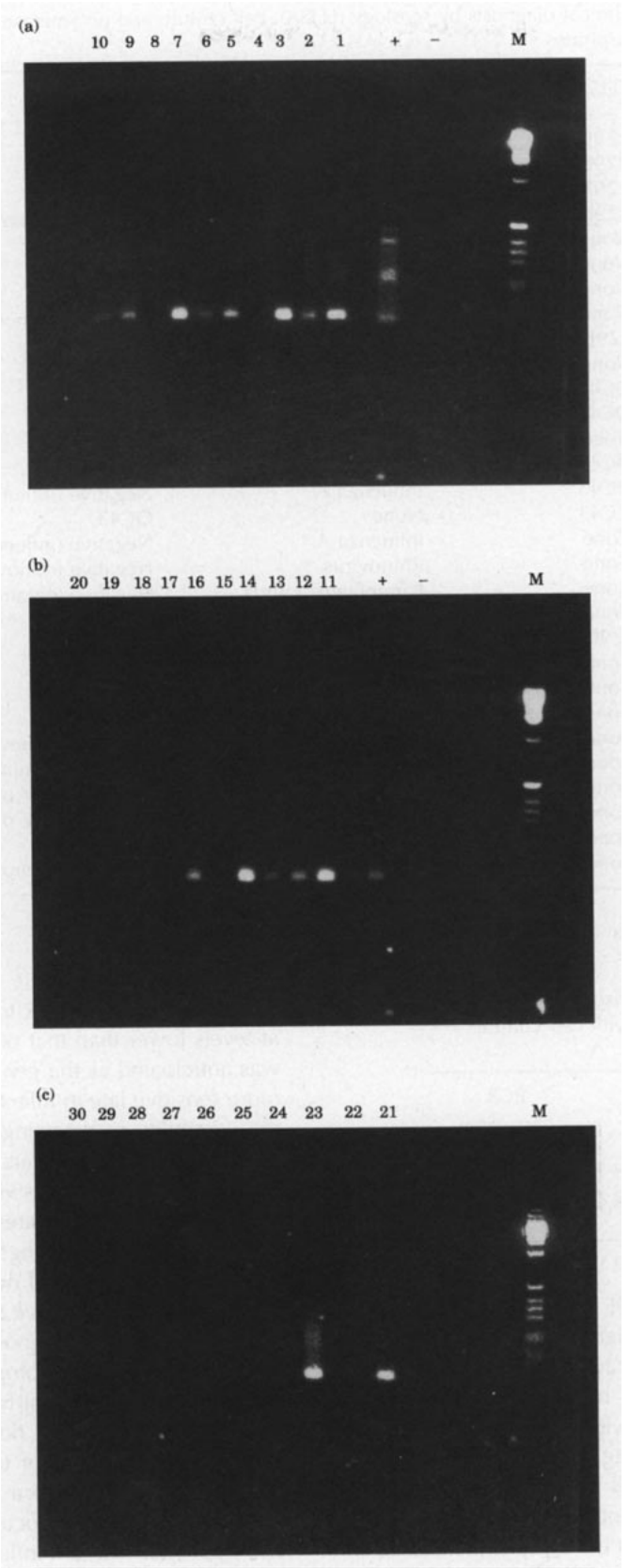

Fig. 4. PCR amplification of clinical material. The numbers on the horizontal axis refer to the specimen number in Table 2. In (a), the positive control was the LP strain of virus, in (b), mouse brain-derived OC43, in (c), 1 pg pSMFG1.

Table 3. Analysis of clinical diagnosis by serology (ELISA), cell culture and polymerase chain reaction amplification of nasal aspirates

Sample number	*ELISA result*	*Culture result**	*PCR result*
1	229E	None	229E
2	229E	229E	229E
3	229E	229E	229E
4	229E	None	Negative (rhinovirus)
5	None	229E	229E
6	None	None	229E
7	None	229E	229E
8	None	Rhinovirus	Negative (rhinovirus)
9	229E	229E	229E
10	None	229E	229E
11	OC43	None	OC43
12	OC43	None	OC43
13	None	None	OC43
14	OC43	None	OC43
15	OC43	Influenza A	Negative (influenza A virus positive)
16	OC43	None	OC43
17	None	Influenza A	Negative (influenza A virus positive)
18	None	Rhinovirus	Negative (rhinovirus positive)
19	None	Parainfluenza virus	Negative (parainfluenza virus positive)
20	None	None	None
21	229E	229E	229E
22	None	None	None
23	None	None	229E
24	None	None	None
25	None	Rhinovirus	Negative (rhinovirus positive)
26	None	Parainfluenza virus	Negative (parainfluenza virus positive)
27	None	RSV	Negative (RSV positive)
28	None	RSV	Negative (RSV positive)
29	None	RSV	Negative
30	None	Adenovirus	Negative (adenovirus positive)

* Not done for OC43.
RSV = Respiratory syncytial virus.

Table 4. Comparison of PCR for 229E and OC43 with cell culture and/or ELISA

		PCR	
		+	−
Cell culture	+	13	2
ELISA	−	2	13

sensitive assays. The 229E also detects the closely related LP strain and might be expected to yield positive results with other 229E-like coronaviruses as the nucleocapsid gene is relatively conserved. The specificity of the assay with respect to adenovirus was only determined using an RNA extraction procedure, not one designed to give a good yield of DNA; this is considered not to reduce the validity of our claim for specificity for the assay as the extraction procedure is an integral part of the assay. Other extraction procedures have also been tried but do not result in a better yield of RNA (unpublished data).

The ability of the PCR to detect coronavirus RNA at levels lower than that of infectivity in cell culture was anticipated as the gene probe method was also more sensitive late in infection: gene detection offers the possibility of detecting antibody-bound virus or virus that is non-infectious because of other factors. Moreover, non-infectious virus particles appear to be common in clinical isolates.

The results of detecting virus genome in clinical material, from this small number of samples, shows that PCR is more sensitive than either cell culture or ELISA alone for the diagnosis of human coronavirus infections, but a direct comparison might suggest that a combination of the last two methods affords more sensitive diagnosis. This does not take into account the lack of specificity of the ELISA methods used. Specimen 4 was due to a rhinovirus infection (this was confirmed on reinoculation into cell culture), and specimen 15 an influenza virus. We do not believe them to be dual infections as in the case of the false-positive 229E there was also failure to isolate the virus in cell culture though this does not entirely

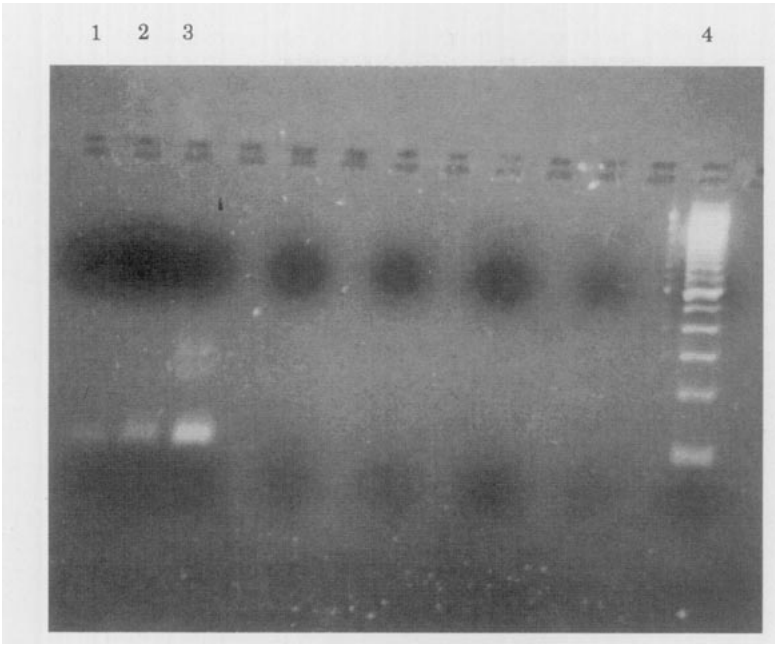

Fig. 5. Nested vs single round RT PCR. Lane 1, inner primers for 35 cycles; lane 2, inner primers for 40 cycles; lane 3, nested primers; lane 4, 100-bp molecular weight ladder (Gibco).

rule out the possibility. These ELISA results may be due to the crudeness of the antigen extract used. If these spurious ELISA results are ignored, it can be seen that there were no false negatives by the PCR method. Indeed, PCR is more sensitive in that it yielded one more positive result (specimen 14) which was collected from the sibling of another subject with OC43 infection.

The assays could be performed in less than a working day and appeared to suffer from few non-specific priming artefacts in clinical specimens although increasing the amount of template tended to increase the likelihood of this phenomenon. Five of the second-round amplification 229E products were transferred to nylon membranes by Southern blotting and probed with a ^{32}P-labelled Riboprobe using previously published methods.[2] These were all positive (data not shown) and because of this and the absolute specificity of the assay, probing of prime products for confirmation purposes was felt unnecessary. An identical experiment with OC43 but using an internal ^{32}P-end-labelled internal oligonucleotide probe gave the same results and conclusion (data not shown).

Diagnostic tests for human coronaviruses are not routinely used for two important reasons: colds are considered trivial illnesses which spontaneously resolve without sequelae, and the current diagnostic methods are not without problems. With the increasing evidence that these viruses are important precipitants of wheezing attacks in asthmatic individuals, there is now a good medical rationale for attempting antiviral therapy. In such cases PCR offers a rapid, sensitive and specific test which would allow therapeutic intervention.

PCR is currently an expensive method compared to cell culture and ELISA but offers several distinct advantages. For 229E, it is more rapid and has greater sensitivity than culture and greater specificity than ELISA. This should be confirmed as more samples are analysed during ongoing clinical studies. In the case of OC43, primary isolation is difficult and ELISA tests utilise crude antigen and might be expected to lack absolute specificity. Developments in the PCR methodology, such as the adaptation of these methods to an ELISA format with multiplex primers for several respiratory viruses, will greatly enhance their use both for routine diagnosis and epidemiological surveys.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Foundation for the study of infant deaths and the National Asthma Campaign. We thank Jo Moffitt, Min Shi and John Hays for excellent technical assistance. We thank Dr Philip Pattemore for help with the collection of clinical material in this study.

REFERENCES

1. McIntosh, K., Ellis, E. F., Hoffmann, L. S., Lybass, T. G., Eller, J. J. & Fulginiti, V. A. (1973). The association of

viral and bacterial respiratory infections with exacerbations of wheezing in young asthmatic children. *Journal of Paediatrics* **82**, 579–90.
2. Myint, S., Siddell, S. G. & Tyrrell, D. A. J. (1990). The detection of human coronavirus 229E in nasal washings using RNA:RNA hybridisation. *Journal of Medical Virology* **29**, 70–3.
3. McIntosh, K., McQuillin, J., Reed, S. E. & Gardner, P. S. (1978). Diagnosis of human coronavirus infection by immunofluorescence: method and application to respiratory disease in hospitalised children. *Journal of Medical Virology* **2**, 341–6.
4. Myint, S., Harmsen, D., Raabe, T. & Siddell, S. G. (1990). Characterisation of a nucleic acid probe for the detection of human coronavirus 229E. *Journal of Medical Virology* **31**, 165–72.
5. Kamahora, T., Soe, L. H. & Lai, M. M. (1989). Sequence analysis of nucleocapsid gene and leader RNA of human coronavirus OC43. *Cirus Research* **12**, 1–9.
6. Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid-guanidium thiocyanate phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–9.
7. Kraajeveld, G. A., Reed, S. E. & MacNaughton, M. R. (1980). Enzyme-linked immunosorbent assay for coronaviruses HCV 229E and MHV 3. *Journal of General Virology* **49**, 83–9.
8. Balfour-Lynn, I. N., Valman, H. B., Stanway, G. & Khan, M. (1992). Use of the polymerase chain reaction to detect rhinovirus in wheezy infants. *Archives of Disease in Childhood* **67**, 760.
9. Pisareva, M., Bechtereva, T., Plyusnin, A., Dobretsova, A. & Kisselev, O. (1992). PCR-amplification of influenza A virus specific sequences. *Archives of Virology* **125**, 313–8.
10. Myint, S. (1989). The development of a nucleic acid hybridisation assay for the detection of human coronavirus 229E. MD thesis, University of London.