

Comparison of immunofluorescence with monoclonal antibodies and RT-PCR for the detection of human coronaviruses 229E and OC43 in cell culture

Jacques Sizun¹, Nathalie Arbour, Pierre J. Talbot*

Laboratory of Neuroimmunovirology, Armand-Frappier Institute, University of Quebec, 531 Boulevard des Prairies, Laval Que. H7V 1B7, Canada

Received 2 September 1997; accepted 19 December 1997

Abstract

Human coronaviruses, with two known serogroups named 229E and OC43, cause up to one third of common colds and may be associated with serious diseases such as nosocomial respiratory infections, enterocolitis, pericarditis and neurological disorders. Reliable methods of detection in clinical samples are needed for a better understanding of their role in pathology. As a first step in the design of such diagnostic procedures, the sensitivities and specificities of two viral diagnostic assays were compared in an experimental cell culture model: an indirect immuno-fluorescence assay using monoclonal antibodies and reverse transcriptase-polymerase chain reaction amplification of viral RNA from infected cells. Immunofluorescence detected human coronaviruses in cells infected at a MOI as low as 10^{-2} (log TCID₅₀/ml = 4.25 for HCV-229E and 2.0 for HCV-OC43; log PFU/ml = 4.83 for HCV-229E and 1.84 for HCV-OC43) versus 10^{-3} (HCV-OC43) or 10^{-4} (HCV-229E) for reverse transcriptase-polymerase chain reaction amplification (log TCID₅₀/ml = 1.75 for HCV-229E and 1.5 for HCV-OC43; log PFU/ml = 2.3 for HCV-229E and 1.34 for HCV-OC43). There were no false positive signals with other human respiratory pathogens: influenza virus, respiratory syncytial virus and adenovirus. Moreover, each assay was coronavirus serogroup-specific. These results demonstrate the potential usefulness of immunofluorescence with monoclonal antibodies and reverse transcriptase-polymerase chain reaction RNA amplification for the rapid detection of human coronaviruses in infected cell cultures. Both methods could be applied to clinical specimens for the diagnosis of human infections. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Coronavirus; 229E; OC43; Immunofluorescence; RT-PCR; Diagnostic

* Corresponding author. Tel.: +1 514 6875010 ext. 4406; fax: +1 514 6865531; e-mail: Pierre.Talbot@iaf.quebec.ca

¹ Present address. Department of Pediatrics, University Hospital, 29609 Brest, France.

1. Introduction

Human coronaviruses (HCV) are a group of enveloped positive-stranded RNA viruses represented by two known serogroups, designated OC43 and 229E when they were first isolated (Hamre and Procknow, 1966; McIntosh et al., 1967). These ubiquitous but not well studied viruses have been associated with ~30% of common colds in humans (Myint, 1995). Experimentally, both the 229E and OC43 prototype strains can infect human astrocytes and microglia in primary cultures (Bonavia et al., 1997) and their RNA was detected in a significant proportion of human autopsy brain samples (Stewart et al., 1992; Arbour and Talbot, unpublished results), which is consistent with their neurotropic potential. In clinical studies, various reports have associated human coronaviruses with nosocomial respiratory infections in premature newborns, as diagnosed by indirect immunofluorescence (Sizun et al., 1995), and enterocolitis in neonates with evidence of human coronaviruses shown by electron microscopy (Chany et al., 1982). Serodiagnosed human coronaviruses were also associated with exacerbations of asthma (Nicholson et al., 1993), and pericarditis (Riski and Hovi, 1980). Therefore, these viruses might be involved in diseases far more serious than mild upper respiratory tract infections.

Further epidemiological studies to evaluate the pathological potential of human coronaviruses will require reliable and rapid methods for their detection in clinical samples such as nasal or tracheal aspirates, cerebrospinal fluids or other tissues. Various methods have been described for the detection of human coronaviruses. Virus isolation in cell culture is difficult, time-consuming and effective with adapted strains in specialised laboratories. Serological methods, mainly based on ELISA, were used for epidemiological studies but do not allow rapid virus identification (Kraaijeveld et al., 1980; McNaughton et al., 1983; Gill et al., 1994). Immunofluorescence with rabbit antisera was developed for the rapid detection of human coronaviruses in nasopharyngeal secretions (McIntosh et al., 1978). However, specificity of this technique was disputed because of cross-re-

action between the two human coronaviruses strains, OC43 and 229E (Myint, 1995) and sensitivity was difficult to ascertain since no human coronaviruses antigen was detected in a prospective evaluation of 106 children hospitalized for respiratory tract infections. Detection of nucleic acids by reverse transcription-polymerase chain reaction (RT-PCR) was used in nasal aspirates (Myint et al., 1994). Nested PCR was apparently as sensitive as a combination of viral isolation in cell culture and serology.

The aim of the current study was to compare the sensitivity and specificity of two assays, immunofluorescence (IF) with monoclonal antibodies (mAbs) and RT-PCR, for the rapid detection of human coronaviruses in an experimental cell culture model as a first step in the design of rapid diagnostic procedures in clinical samples.

2. Materials and methods

2.1. Viruses and cells

Susceptible cell lines (L132 from human embryonic lung and HRT-18 from a human rectal tumor) and the 229E and OC43 strains of human coronaviruses were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown at 37°C in a humidified atmosphere containing 5% (v/v) CO₂ in Earle's minimal essential medium (EMEM): Hank's M199 (1:1, v/v), supplemented with 0.13% (w/v) sodium bicarbonate and 50 µg/ml gentamicin (Canadian Life Technologies, Burlington, Ont.) and fetal bovine serum (FBS) (5% (v/v) for L132 and 10% (v/v) for HRT-18 cells). Viruses were plaque-purified twice and grown on either L132 cells (HCV-229E) or HRT-18 cells (HCV-OC43), as described previously (Jouvenne et al., 1992; Mounir and Talbot, 1992). The third passage of HCV-229E in the susceptible L132 cell line, with a titer of 5.5×10^5 TCID₅₀/ml (2.1×10^6 PFU/ml) and the fourth passage of HCV-OC43 in the susceptible HRT-18 cell line, with a titer of 5.15×10^5 TCID₅₀/ml (3.6×10^5 PFU/ml), from laboratory stocks kept at -90°C, were used for all experiments.

Human respiratory syncytial virus (RSV) (Long strain, sub-group A), human adenovirus and human influenza virus (A/USSR strain) viruses were obtained from Drs Michel Trudel, Serge Dea and Jit Arora, Armand-Frappier Institute, respectively.

2.2. Infection of cells

Approximately 2×10^6 cells in 2 ml EMEM: Hank's (1:1, v/v), with 0.13% (w/v) sodium bicarbonate and 5% (v/v) FBS were mixed in a 25 cm² tissue culture flask (Corning, Cambridge, MA) with 1 ml of virus stock diluted to give a multiplicity of infection (MOI) equal to 1 and successive logarithmic dilutions. Incubation took place in a humidified chamber incubator at 33°C and 5% (v/v) CO₂ and lasted 24 h for HCV-229E and 48 h for HCV-OC43 (previously determined optimal times). At the end of the incubation period, cell-free supernatant was removed. Cell monolayers were washed twice in phosphate buffered saline (PBS), dissociated with 0.25% (w/v) trypsin (Canadian Life Technologies) for L132 cells and 0.05% (w/v) trypsin plus 0.53 mM EDTA (Canadian Life Technologies) for HRT-18 cells, and washed in PBS (7 min, 1200 rpm, 340 × *g* in a Beckman GS-6R centrifuge). Cells were resuspended in 3 ml PBS.

A total of 25 μl of the infected cell suspension was deposited onto each well of a 12-well glass slide (Flow, ICN Biomedical, Mississauga, Ont.), dried in a laminar flow hood and fixed in acetone at –20°C for 30 min. Slides, supernatants and residual cell suspensions were used immediately or stored at –70°C. Negative controls consisted of non-infected cells.

2.3. Indirect immunofluorescence assay

Virus-specific mAbs produced in our laboratory by standard hybridoma technology were used in an indirect immunofluorescence (IF) test on cells infected at various MOIs with either HCV-229E or HCV-OC43. We used a mouse IgG1 mAb (5–11H.6) for HCV-229E detection and a mouse IgG1 mAb (1–10C.1) for HCV-OC43 detection. These two mAbs were shown by Western im-

munoblotting and radioimmunoprecipitation to recognise the surface *S* glycoprotein of HCV-229E and HCV-OC43, respectively (data not shown).

Primary antibody at a 1/100 dilution was added to thawed slides and incubated for 1 h at 37°C. This was followed by three washes in PBS and addition of the fluorescein-conjugated mouse-specific goat secondary antibody (Cappel, Durham, NC) and incubation for 30 min at 37°C. After three washes in PBS, slides were stained with Evans Blue (0.003% (w/v) in distilled water) and mounted with glycerol:PBS (9:1). Slides were observed immediately with a Leica fluorescence microscope or stored at –20°C for later observation. A monolayer was scored negative if it contained no cells with typical fluorescence in ten fields containing at least 30 cells each. An isotype control antibody consisted of a mAb against an unrelated plant virus (Robbins et al., 1994).

2.4. Immunoperoxidase assay for infectious virus

Infectious virus titers of samples used for evaluation of diagnostic techniques were measured using an immunoperoxidase method. Susceptible cells (L132 or HRT-18) at 70% confluence were inoculated with logarithmic dilutions of samples in a 96-well Linbro plate (Flow, McLean, VA). After 4–5 days of incubation in a humidified chamber at 33°C and 5% (v/v) CO₂, the cells were washed with PBS and fixed with 0.3% (v/v) hydrogen peroxide (Sigma) in methanol for 30 min. After washing with PBS, they were incubated for 2 h at 33°C and 5% (v/v) CO₂ with an appropriate antiviral antibody (5–11H.6 for HCV-229E and 1–10C.1 for HCV-OC43, with control reactions using the 229E-specific mAb on OC43-infected cells and the OC43-specific mAb on 229E-infected cells). The cells were then washed five times with PBS and horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (KPL, Gaithersburg, Maryland) were added and incubated for 2 h at 37°C without CO₂. Bound antibodies were detected by incubation with 0.025% (w/v) 3,3'-diaminobenzidine-tetrahydrochloride (Bio-Rad, Richmond, CA) and 0.01% (v/v) hydrogen peroxide (Sigma) in PBS. The chromogenic reaction

Table 1
Primers for RT-PCR amplification of HCV RNA

HCV	Primers
229E ^a	5'-TCTGCCAAGAGTCTTGCTCG-3' (E7, sense) 5'-AGCATAGCAGCTGTTGACGG-3' (E9, anti-sense)
OC43 ^b	5'-CCCAAGCAAAGCTGCTACCTCTCAG-3' (O1, sense) 5'-GTAGACTCCGTC AATATCGGTGCC-3' (O3, antisense)

^a Amplified region corresponding to nucleotides 819–1054 of the nucleocapsid protein gene (Schreiber et al., 1989; Bonavia et al., 1997).

^b Amplified region corresponding to nucleotides 215–520 of the nucleocapsid protein gene (Kamahora et al., 1989; Stewart et al., 1992).

was stopped with deionized water. Infectious virus titers were calculated by the Karber method (Payment and Trudel, 1989). Negative and positive controls consisted of non-infected cells and cells infected with the appropriate virus stock, respectively.

2.5. RT-PCR

To obtain total cellular RNA from infected and controls cells, 20 μ l of the cell suspension was

mixed with 80 μ l of PBS, spun down at $5900 \times g$ for 10 min (Eppendorf centrifuge, Model 5415C) and resuspended in 150 μ l of 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma). The solution was boiled for 5 min, cellular debris eliminated by centrifugation for 2 min at $12000 \times g$ and the supernatant used immediately or stored at -90°C (Bonavia et al., 1997). The oligonucleotide primer pairs used for RT-PCR are described in Table 1. The RT-PCR protocol was used as described previously (Stewart et al., 1992).

3. Results

3.1. Sensitivity of virus detection

Using IF, we were able to detect human coronaviruses antigens in viral preparations that showed viral titers of either 4.25 (HCV-229E) or 2.0 (HCV-OC43) log TCID₅₀/ml, as monitored by an immunoperoxidase assay that detects infectious virions in cells infected with the sample to be tested (Table 2). This corresponds to 4.83 and 1.84 log PFU/ml, respectively. On the other hand, RT-PCR was more sensitive for both viruses than IF since it allowed the detection of human coronaviruses in preparations containing as low as 1.75 or 1.5 log TCID₅₀/ml of HCV-229E or HCV-

Table 2
Detection of human coronaviruses in cell culture by IF and RT-PCR

HCV	MOI ^a	Infectious viral titer (log TICD ₅₀ /ml)	IF mAb 5–11H.6	IF mAb 1–10C.1	RT-PCR primers E7–E9	RT-PCR primers O1–O3
229E	1	4.0	+	–	+	–
	10 ⁻¹	4.75	+	–	+	–
	10 ⁻²	4.25	+	–	+	–
	10 ⁻³	2.75	–	–	+	–
	10 ⁻⁴	1.75	–	–	+	–
	10 ⁻⁵	<0.5	–	–	–	–
OC43	10 ⁻¹	3.0	–	+	–	+
	10 ⁻²	2.0	–	+	–	+
	10 ⁻³	1.5	–	–	–	+
	10 ⁻⁴	<0.5	–	–	–	–
	10 ⁻⁵	<0.5	–	–	–	–

^a Susceptible cells were infected at the indicated MOI to yield virus preparations with the indicated infectious viral titers measured by an immunoperoxidase assay. These viral preparations were used for IF using mAbs 5–10H.6 (229E-specific) or 1–10C.1 (OC43-specific) or RT-PCR with primer pairs E7–E9 (229E-specific) or O1–O3 (OC43-specific).

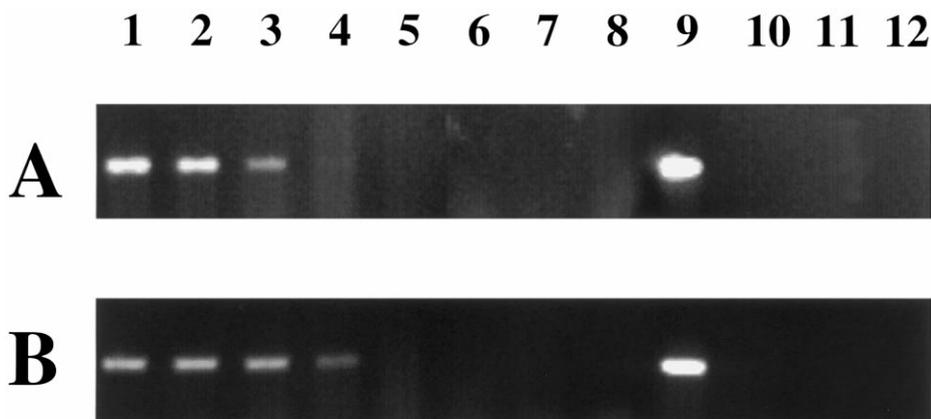


Fig. 1. Results of RT-PCR detection of human coronaviruses. Panel A: RT-PCR for HCV-OC43: lanes 1–5, HRT-18 cells infected with logarithmic dilutions of HCV-OC43 ($\log \text{TCID}_{50}/\text{ml} = 3.0, 2.0, 1.5, <0.5, <0.5$, respectively); lanes 6–8, cells infected with either human respiratory syncytial virus, influenza virus or adenovirus, respectively; lane 9, positive control with HCV-OC43 RNA; lane 10, L-132 cells infected with HCV-229E; lanes 11 and 12, negative controls (RT and PCR, respectively). Panel B: RT-PCR for HCV-229E; lanes 1–5, L-132 cells infected with logarithmic dilutions of HCV-229E ($\log \text{TCID}_{50}/\text{ml} = 4.75, 4.25, 2.75, 1.75, <0.5$, respectively); lanes 6–8, cells infected with either human respiratory syncytial virus, influenza virus or adenovirus, respectively; lane 9: positive control with HCV-229E RNA; lane 10: HRT-18 cells infected with HCV-OC43, lanes 11 and 12, negative controls (RT and PCR, respectively).

OC43, respectively (Fig. 1). This corresponds to 2.3 and 1.34 \log PFU/ml, respectively. Importantly, the RT-PCR assay did not detect input virions, since RT-PCR performed on RNA extracted from cells inoculated with virus at 4°C (where there is no viral penetration into cells) were negative (data not shown). However, it is not possible to evaluate the sensitivity of the RT-PCR assay in terms of the amount of viral RNA since its proportion in total cellular RNA is not known.

3.2. Specificity of virus detection

The mAbs showed a characteristic cytoplasmic fluorescence in human coronaviruses-infected cells (Fig. 2), but not in non-infected cells (data not shown). Control respiratory human viruses (influenza virus, adenovirus and respiratory syncytial virus) yielded negative results by IF (data not shown). Each mAb was specific for one human coronavirus serogroup since no fluorescence was observed when the 229E-specific mAb was used on OC43-infected cells or when the OC43-specific mAb was used on 229E-infected cells (Fig. 2).

There was a good correlation between results obtained by IF and by RT-PCR.

4. Discussion

Two methods for the detection of human coronaviruses strains 229E and OC43 were compared: indirect immunofluorescence with virus-specific mAbs and RT-PCR. A cell culture experimental model was used rather than clinical samples since assays undertaken on human samples do not allow precise analysis of the sensitivities and specificities of human coronaviruses detection methods. This is because the number of infectious virus particles in coronavirus-containing secretions is unknown. Moreover, reference methods such as the culture of clinical samples are difficult to carry out. Finally, possible double viral infections may adversely affect studies of specificity (Valenti et al., 1982; Wilson et al., 1989).

Indirect immunofluorescence is used routinely for the diagnosis of viral respiratory infections and we use it routinely to evaluate human coronavirus infections. The results of this study

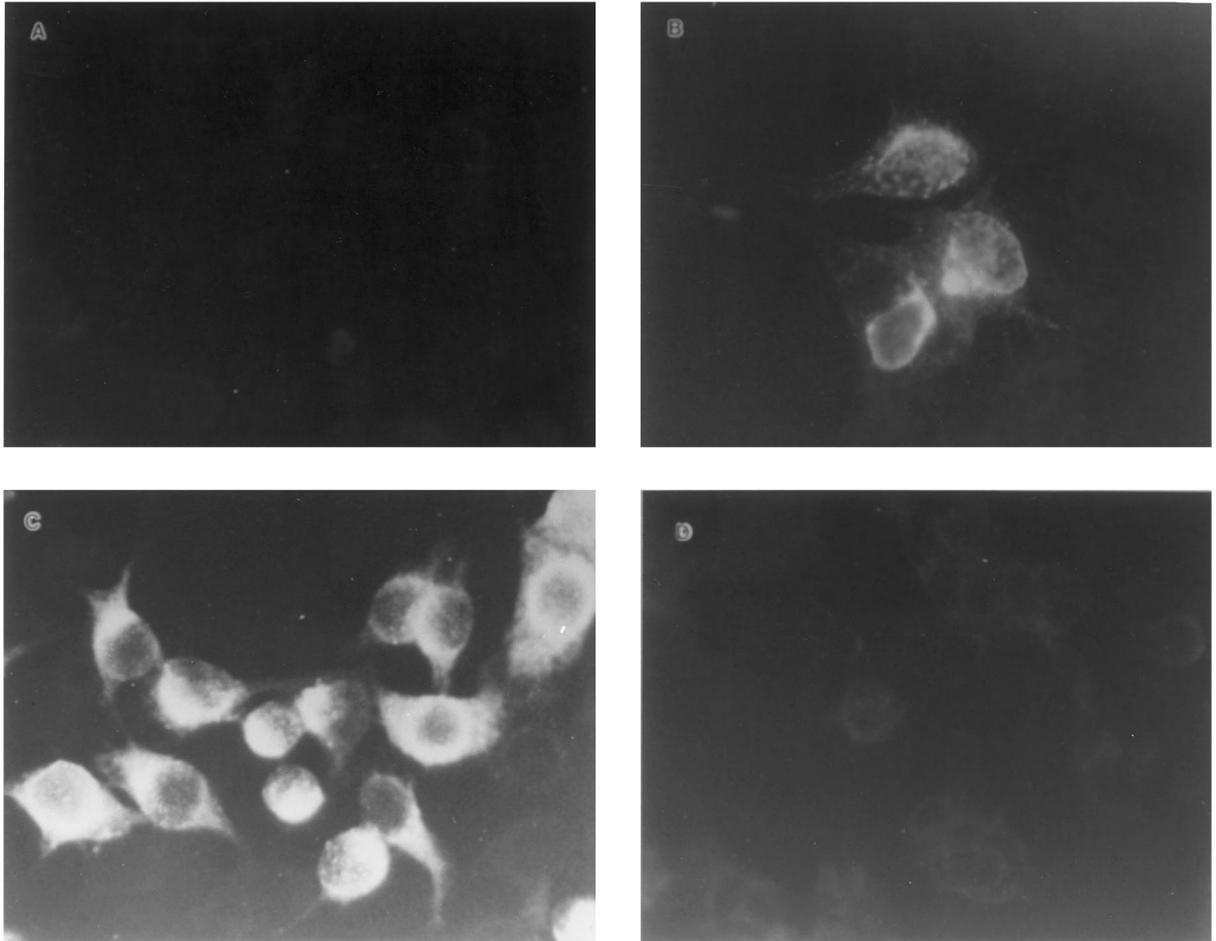


Fig. 2. Detection of human coronaviral antigens by indirect immunofluorescence on infected cells. Panels A and B: HRT-18 cells infected with HCV-OC43; Panels C and D: L132 cells infected with HCV-229E. Immunodetection was achieved with mAbs to either HCV-229E (5–11H.6: panels A and C), or HCV-OC43 (1–10C.1: panels B and D). Magnification: $\times 1200$.

confirm the appropriate sensitivity of this technique. Indeed, IF allowed the detection of human coronaviruses in cell suspensions with infectious viral titers of 4.25 log TCID₅₀/ml for HCV-229E and 2.0 log TCID₅₀/ml for HCV-OC43. No false positive signals were observed with human influenza virus, human respiratory syncytial virus and human adenovirus. Importantly, each mAb was human coronaviruses serogroup-specific. Polyclonal antibodies used in a previous study did not show such serogroup specificity since OC43-infected cells yielded a positive signal when the 229E-specific antibody was used (McIntosh et al.,

1978). Epidemiological studies have shown that patients infected with the 229E strain of human coronaviruses rarely have circulating antibodies against the OC43 strain (Kapikian et al., 1969). Therefore, it would be interesting to distinguish between HCV-229E and HCV-OC43 during a clinical study and the serogroup-specific tests we describe would be useful for that purpose.

The RT-PCR technique was as specific and more sensitive than IF since it allowed the detection of human coronaviruses in cell suspensions with viral titers as low as 1.75 log TCID₅₀/ml for HCV-229E and 1.5 log TCID₅₀/ml for HCV-

OC43. In the only other published report on the detection of human coronaviruses by RT-PCR, the sensitivity of a nested PCR technique was compared in clinical samples to two techniques, ELISA and cell culture, the latter being only available for HCV-229E (Myint et al., 1994). The PCR technique appeared more sensitive than either cell culture or ELISA, but less than the two associated techniques. Specificity in clinical use was difficult to ascertain because of possible dual infections (human coronaviruses with either rhinovirus or influenza virus). These investigations used the classical single-step RNA extraction of Chomczynski and Sacchi (1989), which requires 3–4 h to execute, and they undertook two PCR amplification rounds. It would be difficult to obtain results within 1 day using this protocol. A simple cells lysis with DEPC-treated water and elimination of proteins and cell walls allowed us to perform RT-PCR for several samples very rapidly. The technique we used would be more appropriate for treating a large number of human samples containing few cells. We have already reported the use of the RT-PCR technique with clinical samples (Stewart et al., 1992; Bonavia et al., 1997).

Interestingly, the immunoperoxidase assay we used to validate the quantification of virus to be detected by IF and RT-PCR appeared more sensitive than these two techniques, most likely because of the amplification afforded by another round of virus replication in susceptible cells. However, such an assay is not suitable for clinical applications since the results are only obtained after several days and require viral infection, which works well with cell culture-adapted prototype strains but may not be possible with field virus isolates.

The results of the current study demonstrate the potential usefulness of mAb-based IF and RT-PCR for the detection of human coronaviruses. Interestingly, it was recently emphasized that nucleic acid amplification techniques for the diagnosis of respiratory tract infections in the clinical laboratory are warranted when rapid conventional methods are not available, which is the case for coronaviruses, as it is for rhinoviruses and enteroviruses (Ieven and Goossens, 1997).

The results of the current study show the feasibility of such a technique for the rapid detection of human coronaviruses.

Both IF and RT-PCR techniques presented in the current study showed good sensitivity and specificity for the two known human coronaviruses serogroups

Acknowledgements

This work was supported by operating grant MT-9203 from the Medical Research Council of Canada to PJT, who also acknowledges senior scholarship support from the Fonds de la recherche en santé du Québec.

References

- Bonavia, A., Arbour, N., Wee Yong, V., Talbot, P.J., 1997. Infection of primary cultures of human neural cells by human coronaviruses 229E and OC43. *J. Virol.* 71, 800–806.
- Chany, C., Moscovici, O., Lebon, P., Rousset, S., 1982. Association of Coronavirus with neonatal necrotizing enterocolitis. *Pediatrics* 69, 209–214.
- Chomczynski, P., Sacchi, N., 1989. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Gill, E.P., Dominguez, E.A., Greenberg, S.B., Atmar, R.L., Hogue, B.G., Baxter, B.D., Couch, R.B., 1994. Development and application of an enzyme immunoassay for Coronavirus OC43 antibody in acute respiratory illness. *J. Clin. Microbiol.* 32, 2372–2376.
- Hamre, D., Procknow, J.J., 1966. A new virus isolated from the human respiratory tract. *Proc. Soc. Exp. Biol.* 121, 190–193.
- Ieven, M., Goossens, H., 1997. Relevance of nucleic acid amplification techniques for diagnosis of respiratory tract infections in the clinical laboratory. *Clin. Microbiol. Rev.* 10, 242–256.
- Jouvenne, P., Mounir, S., Stewart, J.N., Richardson, C.D., Talbot, P.J., 1992. Sequence analysis of human coronavirus 229E mRNAs 4 and 5: Evidence for polymorphism and homology with myelin basic protein. *Virus Res.* 22, 125–141.
- Kamahora, T., Soe, L.H., Lai, M.M.C., 1989. Sequence analysis of the nucleocapsid gene and leader RNA of human coronavirus OC43. *Virus Res.* 12, 1–9.
- Kapikian, A.Z., James, H.D., Kelly, S.J., Dees, J.H., Turner, H.C., McIntosh, K., Kim, H.W., Parrott, R.H., Vincent, M.M., Chanock, R.M., 1969. Isolation from man of

- 'Avian infectious bronchitis virus-like' viruses (coronaviruses) similar to 229E virus, with some epidemiological observations. *J. Infect. Dis.* 119, 282–290.
- Kraaijeveld, C.A., Reed, S.E., McNaughton, M.R., 1980. Enzyme-linked immunosorbent assay for detection of antibody in volunteers experimentally infected with human coronavirus strain 229E. *J. Clin. Microbiol.* 12, 493–497.
- McIntosh, K., Dees, J.H., Becker, W.B., Kapikian, A.Z., Chanock, R.M., 1967. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proc. Natl. Acad. Sci. USA* 57, 933–940.
- 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 hospitalized children. *J. Med. Virol.* 2, 341–346.
- McNaughton, M.R., Flowers, D., Isaacs, D., 1983. Diagnosis of human coronavirus infections in children using enzyme-linked immunosorbent assay. *J. Med. Virol.* 11, 319–325.
- Mounir, S., Talbot, P.J., 1992. Sequence analysis of the membrane protein gene of human coronavirus OC43 and evidence for *O*-glycosylation. *J. Gen. Virol.* 73, 2731–2736.
- Myint, S.H., 1995. Human coronavirus infections. In: Siddell, S.G. (Ed.), *The Coronaviridae*. Plenum, New York, pp. 389–401.
- Myint, S., Johnston, S., Sanderson, G., Simpson, H., 1994. Evaluation of nested polymerase chain methods for the detection of human coronaviruses 229E and OC43. *Mol. Cell. Probes* 8, 357–364.
- Nicholson, K.G., Kent, J., Ireland, D.C., 1993. Respiratory viruses and exacerbations of asthma in adults. *Br. Med. J.* 307, 982–986.
- Payment, P., Trudel, M., 1989. Isolement et identification des virus. *Manuel de Techniques Virologiques*. Presses de Université du Québec, Québec, pp. 21–44.
- Riski, H., Hovi, T., 1980. Coronavirus infections in man associated with diseases other than the common cold. *J. Med. Virol.* 6, 259–265.
- Robbins, M.A., Witsenboer, H., Michelmores, R.W., Fortin, M.G., 1994. Genetic mapping of turnip mosaic virus resistance in *Lactuca sativa*. *Theor. Appl. Genet.* 89, 583–589.
- Schreiber, S.S., Kamahora, T., Lai, M.M.C., 1989. Sequence analysis of the nucleocapsid protein gene of human coronavirus 229E. *Virology* 169, 142–151.
- Sizon, J., Soupre, D., Legrand, M.C., Giroux, J.D., Rubio, S., Chastel, C., Alix, D., de Parscau, L., 1995. Neonatal nosocomial respiratory infection with coronavirus: A prospective study in a neonatal intensive care unit. *Acta Paediatr.* 84, 617–620.
- Stewart, J.N., Mounir, S., Talbot, P.J., 1992. Human coronavirus gene expression in the brains of multiple sclerosis patients. *Virology* 191, 502–505.
- Valenti, W.M., Clarke, T.A., Hall, C.B., Menegus, M.A., Shapiro, D.L., 1982. Concurrent outbreaks of rhinovirus and respiratory syncytial virus in an intensive care nursery: Epidemiology and associated risk factors. *J. Pediatr.* 100, 722–726.
- Wilson, C.W., Stevenson, D.K., Arvin, A.M., 1989. A concurrent epidemic of respiratory syncytial virus and parainfluenza virus type 3 in a newborn nursery. *Pediatr. Infect. Dis. J.* 8, 24–29.