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Direct diagnosis of human respiratory coronaviruses 229E and OC43 by the polymerase chain reaction

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Abstract

An RT-PCR-hybridization was developed that amplified genetic material from the M protein gene of HCoV-229E and HCoV-OC43. The analytic sensitivity of these original primers were compared with primers defined in the N gene and described previously. The results show that 0.05 $TCID_{50}$ of HCoV-229E and 0.01 $TCID_{50}$ of HCoV-OC43 can be detected by this molecular method using the original method. Detection of HCoV-229E and HCoV-OC43 in clinical specimens is possible using this method: 348 respiratory specimens (202 sputum and 146 nasal aspirates) were tested with this RT-PCR-hybridization and 12 human coronavirus are detected (3%). The method could provide a useful tool for demonstrating the role of human coronavirus in infections of the respiratory tract.

Keywords: Gene M; HcoV-229E; HcoV-OC43; Molecular method; Respiratory coronavirus

1. Introduction

Human coronaviruses were described initially in patients infected with rhinitis. They belong to a group of viruses that concern human and different animal species. They are implicated in diseases involving the respiratory tract, the digestive system, and the central nervous system (Vabret et al., 1998). Apart from rhinitis, human coronavirus is associated with more severe pulmonary infection (Myint, 1994). As for other respiratory viruses (influenza viruses, rhinoviruses), they are associated with bronchitic hyperactivity even in non-atopic patients (Trigg et al., 1996; Freymuth et al., 1999). Epidemiological inquiries have led to the conclusion that these viruses circulate widely in seasonal oubreaks.

Coronaviruses are enveloped viruses, pleiomorphic with a long (30 kb) RNA molecule. The human strains are divided into two distinct antigenic groups which are both represented by a prototype virus, HCoV-229E and HCoV-OC43.

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Few detection methods of coronavirus are available at present. Consequently, these viruses are sought rarely in diagnostic laboratories, and the associated clinical symptoms associated are not defined. Some molecular detection methods were described recently for screening for human coronavirus: PCR amplification, simple or nested, with primers defined in the N protein gene (Myint et al., 1994; Stewart et al., 1995).

In this paper, two methods are described for the routine detection of two groups of human coronavirus. They consist of a PCR, where primers are defined in the M protein gene, followed by molecular hybridization using nonradioisotopic probes. The alignment of the nucleotidic sequence of the M genes of the 229E and OC43 shows a homology of 43%, insufficient to define a common system of detection (sequences extracted from Genbank™, software GCG™).

Those detection systems have been developed on both the prototype strains HCoV-229E and HCoV-OC43, and are compared to the other method using primers defined in the N protein gene as published (Myint et al., 1994; Stewart et al., 1995; Sizun et al., 1998).

2. Materials and methods

2.1. Viruses and cells

The two cell line-adapted strains of prototypes human coronavirus (229E and OC43) were obtained from ATCC, Rockville, MD.

HCoV-229E was propagated by inoculation into a human embryonic lung diploid fibroblast cell strain (MRC5) at 2–3 days old and incubated for 48 h at 35 C in Eagle's basal medium (MBE, GibcoBRL) supplemented with 0.6% sodium bicarbonate, 2% fetal bovine serum and antibiotics (penicillin 150 U/ml, and gentamycin 50 mcg/ml). The cytopathic effect produced by HCoV-229E in MRC5 is not characteristic and appears as an extensive lysis. The identification of coronavirus is carried out by using an indirect immunofluorescent test with a monoclonal antibody (mouse IgG1 mAb, 5-11H.6, obtained from Talbot, P.J. Canada). Strain HCoV-229E was amplified by several passages in cell cultures so as to obtain a viral suspension with a titer of 5×10^4 $TCID_{50}$/ml. Laboratory stocks of HCoV-229E were kept at − 80 °C, and used for further experiments.

HCoV-OC43 was propagated by inoculation into a 1-day old human rectal tumor cell strain (HRT18) and incubated for 48 or 72 h at 35 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium (GibcoBRL) supplemented with 2% fetal bovine serum and antibiotics. The isolation of HCoV-OC43 required the presence of trypsin (porcine pancreatic trysin, Sigma T0134, 1.5 μg/ml) to cleave the hemagglutinin–esterase protein. The identification of HCoV-OC43 was carried out by an indirect immunofluorescent test using a monoclonal antibody (mouse IgG1 mAb1-1OC.1, obtained from Talbot, P.J Canada.). After a few passages, a viral suspension with a titer of 5×10^5 $TCID_{50}$/ml was obtained. The laboratory stocks of this viral suspension was kept at − 80 °C and used for all experiments.

Infectious virus titers of samples used for evaluation of diagnostic techniques were measured using the immunofluorescent method described previously. Susceptible cells (MRC5 or HRT18) were inoculated with logarithmic dilutions of cell culture supernatant in a 48-well plate. After 2 or 3 days of incubation, the number of infected wells were determinated by the immunofluorescence test. Infectious titers were calculated by the Karber method. Each one of the logarithmic dilutions was stored for 60 min in order to extract RNA. For the control of specificity, strains of human respiratory syncytial virus, sub-group A and B, human adenovirus type 2, influenza virus A (H3N2) and B, herpes simplex virus, cytomegalovirus strain Ad169, rhinovirus type 31, parainfluenza virus type 2 and 3 were isolated in cell culture.

2.2. RT-PCR-EIA

For RT-PCR, 500 μl of each one of the tenfold dilutions of the viral suspension were mixed with 500 μl of RNAzol™ B (Bioprobe, France), and RNA was extracted by a guanidium isothiocyanate procedure as recommended by the manufacturer. RNA was precipitated from the extract

with cold isopropanol and purified by washing with 70% cold ethanol. The extracted RNA was resuspended in 50 μl of distilled water treated with diethylpyrocarbonate (DEPC) and 1 μl of RNasin® Ribonuclease Inhibitor (Promega, Madison, WI).

The RT-PCR used our original primers and probes defined in the M gene of HCoV-229E and HCoV-OC43, and the other primers and probes defined previously in the N gene of these two viruses (Stewart et al., 1995; Myint et al., 1994; Jouvenne et al., 1990) (Tables 1 and 2).

RT-PCR was carried out in 50 μl of a reaction mixture containing 5 μl of extracted RNA, 5 μl of 2 mM dNTPs, 4 μl of cDNA primer at 10 μM, 5 μl of $10 \times MgCl_2$ 15 mM (GeneAmp® Perkin Elmer), 10 U (0.5 μl) of RNasine (Promega, Madison, WI), 8 U (1 μl) of avian myeloblastosis virus reverse transcriptase (Promega), 2.5 U (0.25 μl) of Taq polymerase (Perkin–Elmer Cetus), and 25 μl of sterile water.

The final mixture was overlaid with mineral oil, and the RT-PCR was carried out in an Omnigene thermocycler (Hybaid): first 45 min at 48 °C, then 5 min at 94 °C, then 45 cycles: denaturation, 94 °C, 30 s; annealing, at a variable temperature (Tables 1 and 2) corresponding to the primers of reaction, 45 s; extension, 72 °C, 30 s; final extension 72 °C, 10 min. Each RT-PCR test included water controls that were treated identically to the virus samples throughout.

PCR amplification products were detected by agarose gel electrophoresis and by a DNA Enzyme Immunoassay (GEN-ETI-K DEIA, Sorin). This test is based on the hybridization of amplified DNA with a single stranded DNA, 5′-biotinylated probe, coated on the wall of a microtiter plate with a streptavidin-biotin bond. The hybrid of the probe and DNA was detected by using an anti-ds-DNA monoclonal antibody and by the addition of an enzyme tracer (anti-mouse IgG conjugated to horseradish peroxidase). The optimal concentration of the probe required for the test was 0.1 ng/μl for all the probes used. The assay was carried out as recommended by the manufacturer, and an index value was defined as OD sample value/OD cut-off value.

A positive RT-PCR-EIA was defined by a DNA fragment visualized at the right position on agarose gel associated with a positive hybridization (index value >1). Since many studies show that DNA Enzyme Immunoassay (DEIA) is superior to gel electrophoresis for detection of PCR amplicons, and as it has been shown by experience gained with different probes used in this system that absorbance values greater than the cut-off indicate that specific hybridization has taken place, we consider as positive a negative gel electrophoresis associated with a hybridization index >1 (Freymuth et al., 1995; Garcia et al., 1995; Levy et al., 1996; Cantaloube et al., 1997). Fig. 1 shows that DNA Enzyme Immunoassay

Table 1
Primers and probes for RT-PCR-EIA of HCoV-229E

Primers/probes	Gene	Positions	Sequence 5′–3′	Annealing θ (°C)
Sens[a]	N	762–782	CGTACTCCTAAGCCTTCTCG	55
Antisens[a]	N	1198–1219	TCGACTAGGGTTAAGAAGAGG	55
Probe[a]	N	692–716	(b)[d]TTTGGAAGTGCAGGTGTTGTGGCCAAGGTGTT	50
E1[b]	N	497–521	AGGCGCAAGAATTCAGAACCAGAG	60
E3[b]	N	782–806	AGCAGGACTCTGATTACGAGAAAG	60
Probe E2[b]	N	692–716	(b)[d]ATGAAGGCAGTTGCTGCGGCTCTT	50
MD1[c]	M	78–98	TGGCCCCATTAAAAATGTGT	60
MD3[c]	M	631–651	CCTGAACACCTGAAGCCAAT	60
Probe MD2[c]	<	421–449	(b)[d]CCGTATCAACACTCGTTATGTGGGTGA	50

[a] Myint et al., 1994; size of predicted amplified products: 460 pb.
[b] Stewart et al., 1995; size of predicted amplified products: 308 pb.
[c] Size of predicted amplified products: 574 pb.
[d] Biotin.

Table 2
Primers and probes for RT-PCR-EIA of HCoV-OC43

Primers/probes	Gene	Positions	Sequence 5′–3′	Annealing θ (°C)
sens[a]	N	655–677	AGGAAGGTCTGCTCCTAATTC	58
antisens[a]	N	1003–1025	TGCAAAGATGGGGAACTGTGGG	58
probe[a]	N	800–822	(b)[d]GTTCTGGCAAAACTTGGCAAGG	5-
O1[b]	N	215–239	CCCAAGCAAACTGCTACCTCTCAG	60
O3[b]	N	498–522	GTAGACTCCGTCAATATCGGTGCC	60
probe O2[b]	N	418–442	(b)[d]GATGGCAACCAGCGTCAACTGCTG	50
MF1[c]	M	215–235	GGCTTATGTGGCCCCTTACT	58
MF3[c]	M	530–549	GGCAAATCTGCCCAAGAATA	58
probe MF2[c]	M	361–388	(b)[d]TATTAGAACTGGAAGTTTTTGGAGTTT	50

[a] Myint et al., 1994; size of predicted amplified products: 367 pb.
[b] Stewart et al., 1995; size of predicted amplified products: 280 pb.
[c] Size of predicted amplified products: 334 pb.
[d] Biotin.

(GEN-ETI-K DEIA, Sorin) increases the sensitivity of the detection of the HCoV RNA. After extraction of tenfold dilutions of a HCoV-OC43 viral suspension, the results of RT-PCR using primers defined in M gene (MF1, MF2) show that the viral suspension and first dilution (10^{-1}) are positive by gel detection and that the hybridization index is greater than 1. For the 10^{-2} dilution, no band was detected on the agarose gel when the hybridization index was positive and equal to four. The positivity of this detection was confirmed by the vizualization of a band at the right position (169 pb) resulting from the hemi-nested RT-PCR using primers MF1, MF2, and MF3.

The analytic sensitivity of the RT-PCR-EIA molecular method was determined by considering that the highest positive dilution represents the limit of detection. Since the infectious titer of the viral suspension is known, it is possible to deduce the correlation between the limit of molecular detection and the infectious titer ($TCID_{50}$).

2.3. Patients samples

From October 1998 to February 1999, 202 respiratory specimens (sputum) were taken from adult patients who suffered from an acute illness of the lower respiratory tract. A total of 146 nasal aspirates were collected from children who suffered from an acute attack of asthma.

All these respiratory specimens (sputum and nasal aspirates) were resuspended in 2 ml of viral transport medium and frozen at -80 C.

Nucleic acids were extracted by RNAzol B™ (Bioprobe, France) and the RT-PCR using primers defined in gene M and described previously were carried out from these frozen samples. Positive and negative control were included and treated in the same way as the virus sample.

3. Results

3.1. Detection of human coronaviruses 229E and OC43 by RT-PCR and hybridization

PCR amplification assays were carried out on the extracted RNA of the two prototype strains using the different primers defined in N and M genes (Tables 1 and 2) generate unique fragments having the expected molecular level and visible on agarose gel under ultraviolet light. For HCoV-229E, bands were located at 308 and 460 pb in N gene for assays using primers defined by Stewart et al. (1995) and by Myint et al. (1994), and at 574 pb in M gene for assays using our original primers. For HCoV-OC43, bands are located at 280 and 367 pb in N gene for assays using primers defined by Stewart et al. (1995) and by Myint et al. (1994) and at 334 pb in M gene for assays using original primers.

These PCR amplification products hybridize specifically with the corresponding probes in the hybridization test with an index value greater than 1.

3.2. Analytic sensitivity of RT-PCR hybridization

To assess the sensitivity of the detection of HCoV by RT-PCR hybridization, the infectious titers ($TCID_{50}$) of tissue culture-grown viruses in MRC5 cells for HCoV-229E and in HRT18 for HCoV-OC43 were determined. Two dilution series were made from each viral suspension. Nucleic acid was extracted from each dilution for cDNA synthesis and PCR. Thus, the end point of detection of infectious virus could be directly compared with the end point of viral detection by RT-PCR-hybridization. The results are summarized in Table 3.

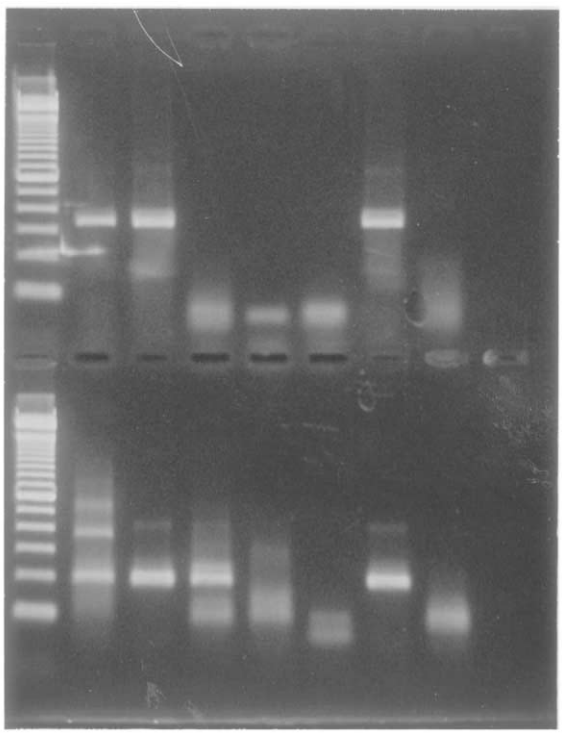

Fig. 1. Ethidium bromide staining of a 2% agarose gel showing tenfold dilutions of a HcoV-OC43 suspension positive or negative for RT-PCR HcoV-OC43, gene M (334 pb) and heminested-RT-PCR HcoV-OC43, gene M (169 pb), and the correspondent hybridization index. Lane 1 and 9, molecular weight marker (100 pb); lane 2: pure viral suspension RT-PCR; lane 3, dilution 10^{-1} RT-PCR; lane 4, dilution 10^{-2} RT-PCR; lane 5, dilution 10^{-3} RT-PCR; lane 6, dilution 10^{-4} RT-PCR; lane 7, RT-PCR positive control; lane 8, RT-PCR negative control; lane 10, pure viral suspension 1/2 nested RT-PCR; lane 11, dilution 10^{-1} 1/2 nested-RT-PCR; lane 12, dilution 10^{-2} 1/2 nested RT-PCR; lane 13, dilution 10^{-3} 1/2 nested-RT-PCR; lane 14, dilution 10^{-4} 1/2 nested RT-PCR; lane 15, 1/2 nested RT-PCR positive control; lane 16, 1/2 nested-RT-PCR negative control.

For HCoV-229E, RT-PCR-hybridization detected 20 and 0.05 $TCID_{50}$ using the primers defined in N gene by Myint et al. (1994) and Stewart et al. (1995) respectively, and 0.05 $TCID_{50}$ with our primer set defined in the M gene. For HCoV-OC43, RT-PCR-hybridization with the primers defined in N gene by Myint et al. (1994) and Stewart et al. (1995) are not sensitive. They did not detect less than 500 $TCID_{50}$ while our prime set defined in M gene detected 0.01 $TCID_{50}$. Thus, comparing the end points, RT-PCR-hybridization in M gene is 40-times more sensitive for HCoV-229E and 100 times more sensitive for HCoV-OC43 than the viral isolation technique.

3.3. Clinical sensitivity of the RT-PCR-hybridization in M gene

From October 1998 to February 1999, a total of 348 respiratory specimens were collected from adult patients suffering from an acute lower respiratory tract illness and from children with an acute attack of asthma. All the specimens were analysed for HCoV-229E and OC43 by RT-PCR-hybridization using primers and probes defined in the M gene. As shown in Table 4, HCoV were detected in six of 202 sputum (3%) from adult patients (three HCoV-229E and three HCoV-OC43), and six of 146 nasal aspirates (4%) of children (two HCoV-229E and four HCoV-OC43)

4. Discussion

The diagnosis of a coronavirus respiratory infection is difficult. In the first place, beside prototype strains, very few wild strains grow in culture. The reference detection technique is electron mi-

Table 3
Value of the limit of molecular detection for each system of detection of human coronaviruses 229E and OC43 by RT-PCR using different primers and probes defined in N and M genes

HCV	Gene	Primers/probes references	Value of the limit of detection
229E	N	Myint et al., 1994	20 $TCID_{50}$/ml
	N	Stewart et al., 1995	0.05 $TCID_{50}$/ml
	M	–	0.05 $TCID_{50}$/ml
OC43	N	Myint et al., 1994	500 $TCID_{50}$/ml
	N	Stewart et al., 1995	>500 $TCID_{50}$/ml
	M	–	0.01 $TCID_{50}$/ml

croscopy. Electron microscopy is not a sensitive technique, and it requires an experienced technician. In respiratory samples, images of coronavirus are very hard to differentiate from other cellular structures. Search of intracellular viral antigen by direct immunofluorescence on respiratory cells is disappointing, and many diagnostic laboratories do not undertake this test. In our laboratory, only one marketed antibody (PIV-11646, Argène France) was used between 1996 and 1999 for the systemic search of coronavirus 229E in nasal aspirates sampled from hospitalised children, and bronchoalveolar liquids (BAL) from hospitalised adults. Among the 7120 tests done, only six samples (five bronchoalveolar liquid and one nasal aspirate) were found positive for HCoV-229E (data not shown). The sensitivity of this antibody has not been defined in viral diagnosis and it is possible that the epitopes recognized are only slightly or not expressed by the cells infected by the wild-type HCoV-229E. Furthermore, the specificity of that monoclonal antibody is not defined.

In order to validate the results of immunofluorescence and to devise a diagnostic method for respiratory samples, we have developed a detection system by RT-PCR-hybridization, sensitive and specific. The use of nested PCR, similar to that published in the literature has been discarded because of the high comtamination risk that it represents when many samples are tested. Each amplified PCR product is submitted to a molecular hybridization that uses a specific probe recognising HCoV-229E or HCoV-OC43, thus on one hand allowing a control of its specificity, and on the other hand an increase of its sensitivity. The test is simple and does not require the use of radioactive materials.

In the literature, the primers allowing the amplification of human coronavirus were chosen mainly in the gene of the N nucleocapside protein. Two reasons justify this choice: this protein is a priori well conserved and the correspondent RNAm is present in large amounts in the infected cell (Van Der Most and Spaan, 1995). We decided to compare two of these primers with an original system defined in the M protein gene. Of all the protein components of the virion, the M protein is the most abundant. It is a transmembranous protein with a N-terminal hydrophilic ectodomain and three hydrophobic regions containing three transmembrane helixes (Rottier, 1995). The nucleotidic sequence of its gene is a priori conserved. The results obtained show that in the prototype strains and in the detection, these primers permit

Table 4
Detection of HCoV-229E and HCoV-OC43 by RT-PCR-hybridization using original primers defined in the M gene in 146 nasal aspirates from children suffered from an acute attack of asthma and 202 respiratory specimens of adults with acute illness of low respiratory tract

	Nasal aspirates ($N=146$)	Sputum ($N=202$)
Detection of HCV-229E	3	2
Detection of HCV-OC43	3	4
Number of co-infection 229E and OC43	0	1
Total of positive specimens	6 (4%)	5 (3%)

a very sensitive detection assay on a scale of 0.05 $TCID_{50}$ for HCoV-229E and 0.01 $TCID_{50}$ for HCoV-OC43. These methods, therefore, allow the detection of less than one infectious particle. The phenomenon can be explained by the synthesis in cell culture of many defective particles. These particles contain identifiable genetic material, but do not have the capacity to infect other cells.

One inconvenient aspect of this method is that it requires a different detection system for the two types of coronavirus. The low percentage of homology of the nucleotidic sequence between the N and M protein genes does not permit a common detection system. Only one recent publication by Stephensen et al. (1999) suggests a detection of the polymerase gene (ORF1b) and the development of a coronavirus consensus PCR. This approach is interesting as it can be used to detect a new coronavirus, or at least a variant of the prototype strains, although the sensitivity of this method is still to be defined.

The use of classical diagnostic methods provides diagnosis in only 40% of the samples received when there is a suspicion of viral respiratory infection (Freymuth et al., 1987). The development of molecular methods to detect viruses that are not identified by these classical detection protocols (especially coronavirus and rhinovirus) seems useful (Ieven and Goossens, 1997).

The results obtained by using the RT-PCR-hybridization in the M protein gene on different respiratory samples can validate the use of this technique. These are concordant with the recent results of Nokso-Koivisto et al. (2000). These researchers were looking for 229E and OC43 coronavirus in many samples (1474 nasal aspirates and 391 medium ear sample) that came from a prospective cohort of 2-month old children, followed over a period of 24 months and sampled during each infectious episode. The method used was a RT-PCR duplex HCoV-229E and HCoV-OC43, followed by a molecular hybridization on microplates. The primers are localised in the gene of the N nucleocapside protein and are different from those used in this study. The results show that the coronavirus was found in 2.6% of the samples (46 in total, 21 HCoV-OC43 and 25 HCoV-229E), of which half were obtained from children presenting other infectious diseases (Nokso-Koivisto et al., 2000): bronchitis, pneumonia, quinsy, laryngitis, conjunctivitis, and exanthema. In order to compare the sensitivity of the different molecular methods described for the detection of coronaviruses 229E and OC43, it is advisable to apply in parallel many respiratory specimens since the percentage of positive samples found in the studies is low, around 3%. It is also necessary to correlate those results with the clinical signs presented by the patients, and to study controls without respiratory clinical signs.

In conclusion, even if the isolation by culture and the search for intracellular viral antigens remain the 'gold standard' of detection techniques of respiratory classical viruses (respiratory syncytial virus, and influenza virus), the use of molecular methods for viruses, such as coronavirus, seems justified in a diagnostic approach in order to determine the clinical implications and the importance of those viruses in respiratory pathology. The molecular technique of detection defined in this study has numerous advantages: it is simple to carry out, its analytical sensitivity on the prototype strains is high and the localisation of the primers in the M protein gene is useful.

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References

Cantaloube, J.F., Charrel, R.N., Attoui, H., Biagini, P., De Micco, P., De Lamballerie, X., 1997. Evaluation of four PCR sustems amplifying different genomic regions for molecular diagnosis of GB virus C infections. J. Virol. Methods 64, 131–135.

Freymuth, F., Quibriac, M., Petitjean, J., Daon, F., Amiel, M.L., 1987. Les virus responsables d'infections respiratoires en pédiatrie. Bilan de 3480 aspirations nasales réalisées chez l'enfant sur une période de six ans. Ann. Pédiatr. 34, 493–501.

Freymuth, F., Eugène, G., Vabret, A., Petitjean, J., Gennetay, E., Brouard, J., Duhamel, J.F., Guillois, B., 1995. Detec-

tion of Respiratory Syncytial Virus by reverse transcription-PCR and hybridization with a DNA enzyme immunoassay. J. Clin. Microbiol. 33 (12), 3352–3355.

Freymuth, F., Vabret, A., Brouard, J., Toutain, F., Verdon, R., Petitjean, J., Gouarin, S., Duhamel, J.F., Guillois, B., 1999. Detection of viral, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* in exacerberations of asthma in children. J. Clin. Virol. 13, 131–139.

Garcia, F. Jr, Garcia, F., Bernal, M.C., Leyva, A., Piedrola, G., Maroto, M.C., 1995. Evaluation of enzyme immunoassay for hepatitis B virus DNA based on anti-double-stranded DNA. J. Clin. Microbiol. 33 (2), 413–415.

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 (2), 242–256.

Jouvenne, P., Richarson, C.D., Schreiber, S.S., Lai, M., Talbot, P.J., 1990. Sequence analysis of the membrane protein gene of human coronavirus 229E. Virology. 169, 142–151.

Levy, R., Najioullah, F., Thouvenot, D., Bosshard, S., Aymard, M., Lina, B., 1996. Evaluation and comparison of PCR and hybridization methods for rapid detection of cytomegalovirus in clinical samples. J. Virol. Methods 62 (2), 103–111.

Myint, S.H., 1994. Human coronavirus: a brief review. Medical Virol. 4, 35–46.

Myint, S.H., 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.

Nokso-Koivisto, J., Pitkäranta, A., Blomvist, S., Kilpi, T., Hovi, T., 2000. Respiratory coronavirus infections in children younger than two years of age. Pediatr. Infect. Dis. 19 (2), 164–166.

Rottier, P.J.M., 1995. The coronavirus membrane glycoprotein. In: Siddell, Ed Stuart G. (Ed.), The Coronaviridae. Plenum Press, New York, pp. 115–139.

Sizun, J., Arbour, N., Talbot, P.J., 1998. Comparison of immunofluorescence with monoclonal antibodies and RT-PCR for the detection of human coronaviruses 229E and OC43 in cell culture. J. Virol. Methods 72 (2), 145–152.

Stephensen, C.B., Casebolt, D.B., Gangopadhyay, N.N., 1999. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. Virus Res. 60, 181–189.

Stewart, J.N., Mounir, S., Talbot, P.J., 1995. Detection of coronaviruses by the polymerase chain reaction. In: Becker, Y., Daraï, G. (Eds.), Diagnosis of Human Viruses by Polymerase Chain Reaction Technology. Springer-Verlag, New York, pp. 316–327.

Trigg, C.L., Nicholson, K.G., Wang, J.H., 1996. Bronchial inflammation and the common cold: a comparison of atopic and non-atopic individuals. Clin. Exp. Allergy 26, 665–676.

Vabret, A., Brouard, J., Petitjean, J., Eugène-Ruellan, G., Freymuth, F., 1998. Infections à coronavirus humains, Importance et diagnostic. La Presse Médicale 27 (35), 1813–1818.

Van Der Most, R.G., Spaan, W.J.M., 1995. Coronavirus. Replication. Transcription, and RNA Recombinaison. In: Siddell, Ed Stuart G. (Ed.), The Coronaviridae. Plenum Press, New York, pp. 11–33.