ELSEVIER

Molecular and Cellular Probes 18 (2004) 75–80

MOLECULAR AND CELLULAR PROBES

www.elsevier.com/locate/ymcpr

Detection of human Coronavirus 229E in nasal specimens in large scale studies using an RT-PCR hybridization assay

Sophie Vallet[a,*], Arnaud Gagneur[b], Pierre J. Talbot[c], Marie-Christine Legrand[a], Jacques Sizun[b], Bertrand Picard[a]

[a] *Laboratoire de Virologie, Department of Microbiology, Centre hospitalier Universitaire Morvan, 5 avenue Foch, 29200 Brest Cedex, France*
[b] *Pediatric Intensive Care Unit, Department of Pediatrics, University Hospital, Brest, France*
[c] *Laboratory of neuroimmunovirology, INRS-Institut Armand-Frappier, University of Quebec, Laval, Quebec, Canada*

Received 2 July 2003; accepted for publication 18 September 2003

Abstract

A novel human Coronavirus (HCoV) was this year recognized as the etiological agent of the Severe Acute Respiratory Syndrome. Two other HCoV (HCoV-229E and HCoV-OC43) have been known for 30 years. HCoV-229E has been recently involved in nosocomial respiratory viral infections in high-risk children. However, their diagnosis is not routinely performed. Currently, reliable immunofluorescence and cell culture methodologies are not available.

As part of a four-year epidemiological study in a Pediatric and Neonatal Intensive care unit, we have performed and demonstrated the reliability of a reverse transcription-PCR-hybridization assay to detect HCoV of the 229E antigenic group in 2028 clinical respiratory specimens.

In hospitalized children (children and newborns) and staff members we found a high incidence of HcoV-229E infection.

This reverse transcription-PCR-hybridization assay gave a high specificity and a sensitivity of 0.5 50% Tissue Culture Infective Dose per ml. This technique is reliable and its application for screening large number of clinical samples would improve the diagnosis of HCoVs respiratory infection and our knowledge of these viruses epidemiology.

Keywords: Human Coronavirus; Neonates; Reverse transcription-PCR; Nosocomial respiratory viral infections

1. Introduction

Nosocomial Respiratory Viral Infections (NRVI) are increasingly documented in pediatric units. Respiratory Syncytial Virus (RSV), Influenza viruses and Rhinoviruses are the most common etiological viral agents. A novel human Coronavirus (HCoV) was this year identified as causative agent of the Severe Acute Respiratory Syndrome (SARS CoV) [1]. Two others HCoV have been known for 30 years (HCoV-229E and HCoV-OC43) [2] and only recently has HCoV-229E been recognized as an important cause of NRVI in high-risk infants [3,4]. We reported previously a high incidence of HCoV among NRVI in hospitalized preterm neonates concomitant with HCoV isolation from staff members of a Pediatric and Neonatal Intensive Care Unit [5].

HCoV are enveloped viruses with a long (more than 30 kb) single-stranded infectious RNA [6]. Since monoclonal antibodies are not available for routine immunofluorescence (IF) detection, and clinical strains of both antigenic groups of HCoV are not well adapted to conventional cell culture systems, their diagnosis is not routinely performed. A reliable diagnostic tool is needed to study their epidemiology in hospital care units. An appropriate tool for HCoV diagnosis has to be sensitive and suitable for large scale testing in respiratory specimens. The feasibility of reverse transcription-PCR (RT-PCR) and its higher sensitivity compared to IF for detection of HCoV were previously demonstrated [7]. Several other amplification strategies have already been reported [8,9]. In this study, we used a previously described HCoV-229E RT-PCR

* Corresponding author. Tel.: +33-298223308; fax: +33-298223987.
E-mail address: sophie.vallet@univ-brest.fr (S. Vallet).

0890-8508/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2003.09.005

methodology [7] followed by hybridization detection to screen directly near 2000 nasal samples from hospitalized children and staff members, without previous cell passage. We evaluated the reliability of this technique in a large prospective study.

2. Materials and methods

2.1. Viruses and cell lines

HCoV-229E was propagated by inoculation of susceptible L132 cell lines. L132 (from human embryonic lung) and the 229E strain of human Coronavirus were obtained from Professor Talbot (Quebec, Canada). They originally came from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2 in Earle's minimal essential medium: Hank's M199 (1/1, v/v) (Eurobio, Les Ulis, France), supplemented with 0.13% (w/v) sodium bicarbonate, 20 μg/ml gentamicin (Panpharma, Fougères, France), 100,000 U/l penicillin (Eurobio), 0.1 g/l streptomycin (Eurobio) and fetal bovine serum (5% (v/v)) (Gibco, Cergy Pontoise, France). The fourth passage of HCoV-229E in the susceptible L132 cell line, yielded a viral suspension with a titer of 3.16×10^5 50% Tissue Culture Infective Dose per ml ($TCID_{50}$/ml). It was kept at −80 °C and used for RT-PCR experiments. The infectious virus titer of the HCoV-229E reference strain used for evaluation of RT-PCR methods was measured by an indirect IF test with a monoclonal antibody (mouse IgG1 mAb, 5-11H.6, obtained from Professor Talbot, Quebec, Canada), as described previously [7].

To check for the specificity of the RT-PCR-Enzyme Immunoassay (EIA), we used clinical strains of RSV, Influenza types A and B, Parainfluenza and Adenovirus.

2.2. Clinical specimens

The nasal specimens used in this study were clinical specimens from a prospective survey of NRVI in a Pediatric and Neonatal Intensive Care Unit from November 1997 to October 1999 and March 2001 to December 2001. Nasal specimens were collected on admission and thereafter on a weekly basis from all hospitalized children, using a cytological brush [10]. The entire experimental protocol of collecting samples was previously described [5]. Briefly, nasal specimens were collected once a month from all staff members (nurses and physicians involved in direct patient care). A total of 1981 nasal specimens were analyzed by the RT-PCR hybridization method. Community-acquired infection was defined as a positive viral detection on admission and nosocomial infection as a negative specimen on admission, with a positive viral detection more than 48 h later.

One aliquot of each suspension of nasal cells collected was stored at −70 °C before RNA extraction. The other part was washed in Phosphate Buffered Saline (PBS pH 7.2) and centrifuged at $430 \times g$ for 10 min. Cells were resuspended in PBS and then distributed on a 10-well slide, dried and fixed for 10 min in cold acetone. Slides were frozen at −20 °C before IF analysis for Adenovirus, RSV, Influenza A and B and Parainfluenza 1, 2 and 3, as described below.

Positive and negative controls were included and treated in the same way as patients' samples.

2.3. Molecular detection of HCoV-229E

2.3.1. RNA extraction

Total RNA was extracted from 140 μl of frozen nasal cells diluted in PBS with the QIAamp viral RNA kit (Qiagen, Courtaboeuf, France), according to the manufacturer's protocol. RNA was eluted in 40 μl of the kit's elution buffer. To preserve the quality of RNA extracts and to limit the loss of viral titer, they were stored at −70 °C until RT-PCR was performed.

2.3.2. One step RT-PCR

RT-PCR was carried out in 96-well microplates (Perkin Elmer Applied Biosystem, Courtaboeuf, France) in a final volume of 30 μl. Primer pair E7(+) 5′-TCTGCCAAGAGTCTTGCTCG-3′, E9(−) 5′-AGCATAGCAGCTGTTGACGG-3′ were used, they target the nucleocapsid gene, respectively, in position 819–838 and 1035–1054 and 969–988 [11]. Reverse transcription and amplification of cDNA were performed in the same tube, as recommended by manufacturers (Access RT-PCR System, Promega, Charbonnières, France). Briefly, 30 μl of reaction mix contained 1X AMV/Tfl reaction buffer, 0.250 mM dNTP mix, 0.6 μM each of E7 and E9 primers, 0.2 mM of $MgSO_4$, 3 U of Avian Myeloblastosis Virus reverse transcriptase and 3 U of Tfl DNA polymerase and 15 μl of RNA extract RT-PCR was performed as follow in a 9600 Perkin Elmer thermocycler (PE Applied Biosystem, Courtaboeuf, France): 45 min at 48 °C and 2 min at 94 °C, 40 three-step cycles with 30 s denaturation at 94 °C, 1 min annealing at 55 °C, and 2 min extension at 68 °C and a final 7 min extension step at 68 °C. Products were stored at −20 °C.

Each RT-PCR carried out included as many as 85 patient RNA extracts, 1 negative and 1 positive control (RNA extract from 10^{-4} dilution of HCoV-229E with a known titer of $3.16 \times 10^5 TCID_{50}$/ml).

2.3.3. Hybridization

PCR amplification products were detected by a DNA Enzyme Immunoassay (GEN-ETI-K, DEIA, DiaSorin, Antony, France). First, RT-PCR products were denatured at 94 °C for 2 min and immediately placed on ice. HCoV single-stranded DNA hybridized with the 5′ biotinylated E8 probe GGAAGTGCAGGTGTTGTGGC which targets

the nucleocapsid gene in 969–988 [11]. E8 was previously coated on streptavidin-coated wells of a microtiter plate. Then, an anti-double stranded DNA mouse monoclonal antibody was used to detect the hybrid, if present. This specific hybridization was revealed with an enzyme tracer (protein A conjugated to horseradish peroxidase). We determined the optimal probe concentration for coating: 0.5 ng/μl. The test was carried out according to the manufacturer's recommendations. Briefly, 20 μl of the RT-PCR product was added to the streptavidin-coated microplate wells in 100 μl of binding buffer. After one hour at 50 °C followed by three washing steps with the kit's washing buffer, 100 μl of anti-double stranded DNA diluted 1/50 was added to each well. The samples were incubated at room temperature for 30 min and washed, then 100 μl of 1/50 diluted enzyme tracer was added and the microplate incubated for a further 30 min at room temperature, then washed. The reaction was developed by addition of 100 μl of substrate. Absorbance was read at 450/630 nm in a photometer. A cut-off value of positivity, expressed in optical density (OD), was defined as the mean of the OD for negative controls plus the constant value of 0.150 absorbance units, as given in the kit recommendations (a positive sample showed an OD value more than 0.150 above the mean of the negative controls).

False-positive results linked to contamination were prevented by performing each step (extraction, amplification, hybridization) in different areas of the laboratory, and by including negative controls at each step of the RT-PCR-EIA protocol.

2.4. Detection of other human respiratory pathogenic viruses

Each clinical sample was tested by indirect IF to detect RSV, Influenza types A and B, Parainfluenza types 1, 2 and 3, and Adenovirus. First, 25 μl of a monoclonal antibody specific for each virus (Argene, Varhiles, France) was added to the wells, left for 30 min at 37 °C, washed in PBS and air-dried. This was followed by addition of fluorescein-labeled anti-mouse immunoglobulin and incubation for 30 min at 37 °C, PBS washes, rinsing in water and mounting with glycerol. Slides were observed immediately with a fluorescence microscope, and stored at −20 °C.

3. Results

3.1. RT-PCR hybridization assay

To determine its sensitivity, the RT-PCR-EIA was performed on 100 μl of successive logarithmic dilutions (10^{-1}–10^{-8}) of HCoV-229E reference strain (titer of 3.16×10^5TCID$_{50}$/ml). The highest dilution yielding a positive signal was considered as the detection limit of the assay.

Dilutions of up to 10^{-5} were scored as positives after the RT-PCR hybridization assay. This dose was equivalent to approximately 0.5TCID$_{50}$/ml, as determined by titration of the virus stock in microwell L132 cell cultures.

To evaluate the specificities of the HCoV primers and probe, clinical strains from other human respiratory viruses were tested by RT-PCR hybridization assay (RSV, Influenza types A and B, Parainfluenza and Adenovirus). No amplification occurred with any of these virus strains (data not shown).

3.2. Detection of HCoV-229E in human nasal secretions

A cohort of a total of 639 children was eligible in the two time periods (respectively, $n = 460$ for 1997–1999 and $n = 179$ for 2001). A total of 1598 and 383 specimens were analyzed from children and staff, respectively. Viral infection was diagnosed for 181 children (incidence 28.3%) with 256 positive samples. HCoV-229E was the main pathogen observed, with 166 infected children. Eighty community-acquired infections were detected in 71 children (incidence 11.1%). Sixty HCoV, nine RSV, seven Influenza A, three Adenovirus and one Parainfluenza type 2 were involved (Table 1). Nine samples were positive for two viruses (four HCoV–RSV, three HCoV–Influenza A, two Influenza A–Adenovirus,). General incidence of NRVI was 17.2% (110 infants) with 106 HCoV and five parainfluenza type 3 (one neonate presented two nosocomial infections

Table 1
Community-acquired, nosocomial infections in infants and staff infections during each period of the study

Periods	Community-acquired infections								Nosocomial infections								Staff infections							
	HCoV	RSV	ADV	IA	IB	P1	P2	P3	HCoV	RSV	ADV	IA	IB	P1	P2	P3	HCoV	RSV	ADV	IA	IB	P1	P2	P3
1997–1998	29	7	3	6	–	–	–	–	53	–	–	–	–	–	–	–	67	–	–	1	–	–	–	–
1999	15	1	–	1	–	–	1	–	31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2001	16	1	–	–	–	–	–	–	22	–	–	–	–	–	–	5	2	–	–	–	–	–	–	–
Total	60	9	3	7	–	–	1	–	106	–	–	–	–	–	–	5	69	–	–	1	–	–	–	–

HCoV, Human Coronavirus; RSV, Respiratory Syncytial virus; ADV, Adenovirus; IA, Influenza A; P1, Parainfluenza type 1; P2, Parainfluenza type 2; P3, Parainfluenza type 3. 460 investigated children and newborns in 1997–99 and 179 in 2001. 201 staff samples in 1997–99 and 182 in 2001.

with HCoV and P3). Seventy samples were positive in staff involving 69 HCoV and one Influenza A.

During the first period (from November 1997 to October 1999), there was a high incidence of HCoV positive samples in children (128 of 1101 specimens) and in staff members (67 of 201 specimens). Sixty-three community-acquired infections were found of which 44 were HCoV and there were 84 HCoV-related nosocomial infections (incidence 18.3%) mostly in 1998 (Table 1).

During the second epidemic period (March 2001 to December 2001) there was a lower incidence of HCoV positive samples than during the first period in both children (52 of 497 specimens) and staff members (2 of 182). Moreover, the incidence of HCoV-related nosocomial infections was low with only 22 infected children (incidence 12.3%) (Table 1).

4. Discussion

Diagnostic laboratories do not routinely screen for Coronavirus infections. Most epidemiological studies conducted mainly in the 1970s used serological assays to detect a Coronavirus infection [12]. However, these techniques are not suitable for the diagnostic of respiratory infections because of poor sensitivity and the requirement for two successive blood samples. Cell cultures have proved to be unreliable for the primary isolation of all human respiratory Coronaviruses. Indeed, only some HCoV strains grow in cell culture after adaptation [6]. Diagnosis of respiratory viral infections is mainly based on IF. For diagnosis of HCoV infection, one anti-229E specific monoclonal antibody (Argene, Varilhes, France) was available but is no longer commercialized. One of us (P.J. Talbot, INRS-Institut Armand-Frappier research center) developed two other monoclonal antibodies (5-11H.6 for HCoV-229E and 1-10C.1 for HCoV-OC43) directed against the S protein [7]. For diagnosis procedure, the IF assay using these antibodies appears to be less sensitive than the RT-PCR-DEIA we describe in the current study [7].

To test for HCoV-229E in the course of large scale epidemiological studies, we used a RT-PCR hybridization method for rapid, specific, and more sensitive detection of HCoV-229E DNA products. Hybridization is more specific and sensitive than agarose gel visualization [13]. The microplate enzyme immunoassay format allows the handling of large number of samples needed for epidemiological studies. Another advantage of RT-PCR methodology is that it can detect the virus genome when it is present at a low titer or when the virus is not replication competent. The value of RT-PCR techniques for certain important respiratory viruses, namely Human Rhino-, and Coronaviruses, is clear because no other practical and rapid techniques are available [14,15]. Several recent studies have used molecular techniques such as RT-PCR followed by a hybridization step for viral diagnosis [16,17]. Here, we have adapted and validated such a methodology for a large scale epidemiological study. This requires processing numerous samples while minimizing the time and labor associated with the original molecular HCoV-specific detection protocols. The time needed for diagnosis of HCoV-229E by the RT-PCR-DEIA methodology, which can be carried out in microplates that enable rapid multichannel pipetting, is shorter than that required for cell culture associated with IF detection (4–5 days) but longer than for IF performed directly on nasal aspirates. However, these techniques are not as sensitive as RT-PCR [7]. Eighty-five clinical samples can be tested in parallel within 10 h for the RT-PCR-DEIA described in the current study, which makes it a reliable screening technique suitable for epidemiological studies. HCoV-229E was detected by this RT-PCR-DEIA with a sensitivity of $0.5TCID_{50}$/ml. This limit of detection compares well with the values previously obtained by others [9,16,17] (20, 0.05 and $0.3TCID_{50}$/ml, respectively). Moreover, such a threshold represents an acceptable titer for the detection of viruses implicated in clinical events.

Vabret et al. [16] used a system targeting the gene for the membrane protein M, the most abundant HCoV protein. However, the RNA encoding N is more abundant than that encoding the M protein in infected cells [18] and thus was chosen as a reliable target for RT-PCR.

We performed a survey of NRVI during a 4-year period to modulate interannual variation of the epidemiology of viral infection. Coronavirus-related NRVI mirrors community infections, of which there are epidemics every 3 or 4 years [12]. A study with a shorter time frame may underestimate HCoV infections.

HCoV-229E and OC43 are widespread and responsible for one-third of common colds in children and adults [6]. They have been detected in nasal swab samples from patients with acute flu-like illness [19] and also in human brain autopsy samples [18]. It has been suggested they may also be involved in the etiology of more severe diseases in all age groups (pneumonia, perimyocarditis, etc.) [20]. The ability of Coronaviruses to cause severe disease in animals raises the possibility that Coronavirus could also cause more severe disease in humans. SARS-related Coronavirus appears to be an example of a Coronavirus associated with severe disease in humans.

In pediatric units, the pathogenic role of HCoV remains undefined because there have been few techniques and reagents available for diagnosis. Their role in lower respiratory infection is unclear. However, they are suspected to be involved in exacerbation of asthma in children [21–23]. HCoV has also been isolated in the digestive tract, but their role in gastrointestinal disease is unproven [24].

A neonatal screening program for HCoV has been in place in neonatal and pediatric units in Brest for 10 years. The first study was a retrospective one, with 19 respiratory specimens found HCoV-229E positive in newborns by IF [25]. In 1995, these results were confirmed by a prospective

study, which showed an HCoV incidence of 25% in symptomatic neonates of gestational age under 32 weeks [26]. A prospective observational study was carried out to determine the role of HCoV in nosocomial infections in a pediatric ICU from 1997 to 2001. Preliminary results showed an 11% incidence during the first winter [3,5]. HCoV nosocomial transmission via medical staff is suspected because transmission of these viruses is possible via air or hand contamination [27–29]. Moreover, during the year 1998, a HCoV-related outbreak was identified correlated with a concomitant high prevalence of HCoV in staff members' [29]. During this 3-year prospective study, HCoV was identified as the main pathogen causing NRVI in infants and newborns.

In conclusion, little is known about HCoV epidemiology because no appropriate diagnostic method has been available. In this study, we demonstrate the reliability of RT-PCR associated with detection by hybridization for analysis of large number of clinical specimens.

This technique would facilitate cost-effective diagnosis and improve clinical management of respiratory tract symptoms in hospitalized infected children, thereby limiting unnecessary antibiotic use. Such a technique will be invaluable in epidemiological studies that are needed to firmly establish the medical importance of human Coronaviruses.

Acknowledgements

This work was supported in part by research funds from the French Ministry of Health (PHRC 1997), The CCLIN Ouest and the Société Française de Pédiatrie. We thank Anne Gagneur, Marie-Annick L'Her, Michèle Odermatt and Christine Roger for excellent technical assistance, PICNU staff members and Alex Edelman for reviewing the manuscript.

References

[1] Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE DS, Ling AE, Humphery CD, Shieh WJ, Guarner J PC, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ ALSWG. A novel Coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003;348:1947–58.

[2] Hamre D, Procknow JJ. A new virus isolated from the human respiratory tract. Proc Soc Exp Biol 1966;121:190–3.

[3] Gagneur A, Legrand MC, Picard B, Baron R, Talbot PJ, De Parscau L, Sizun J. Nosocomial infections due to human Coronaviruses in the newborn. Arch Pediatr 2002;9:61–9.

[4] Sizun J, Gagneur A, Legrand MC, Baron R. Respiratory Coronavirus infections in children. Pediatr Infect Dis J 2001;20:555–6.

[5] Gagneur A, Sizun J, Vallet S, Legr MC, Picard B, Talbot PJ. Coronavirus-related nosocomial viral respiratory infections in a neonatal and paediatric intensive care unit: a prospective study. J Hosp Infect 2002;51:59–64.

[6] Myint S. Human Coronavirus. In: The Coronaviridae. 1995. Siddell SG, editor. New York: Plenum Press. p. 389–401.

[7] Sizun J, Arbour N, Talbot PJ. Comparison of immunofluorescence with monoclonal antibodies and RT-PCR for the detection of human Coronaviruses 229E and OC43 in cell culture. J Virol Meth 1998;72:145–52.

[8] Stewart JN, Mounir S, Talbot PJ. Detection of coronaviruses by the polymerase chain reaction. In: Diagnosis of human viruses by polymerase chain reaction technology. 1995. Becker Y, Daraï G, editors. New York: Springer-Verlag. p. 316–27

[9] Myint S, Johnston S, Sanderson G, Simpson H. Evaluation of nested polymerase chain methods for the detection of human coronaviruses 229E and OC43. Mol Cell Probes 1994;8:357–64.

[10] Barnes SD, Leclair JM, Forman MS, Townsend TR, Laughlin GM, Charache P. Comparison of nasal brush and nasopharyngeal aspirate techniques in obtaining specimens for detection of respiratory syncytial viral antigen by immunofluorescence. Pediatr Infect Dis J 1989;8:598–601.

[11] Schreiber SS, Kamahora T, Lai MM. Sequence analysis of the nucleocapsid protein gene of human coronavirus 229E. Virology 1989;169:142–51.

[12] Monto AS, Lim SK. The Tecumseh study of respiratory illness. VI. Frequency of and relationship between outbreaks of coronavirus infection. J Infect Dis 1974;129:271–6.

[13] Freymuth F, Eugene G, Vabret A, Petitjean J, Gennetay E, Brouard J, Duhamel JF, Guillois B. Detection of respiratory syncytial virus by reverse transcription-PCR and hybridization with a DNA enzyme immunoassay. J Clin Microbiol 1995;33:3352–5.

[14] Ieven M, Goossens H. Relevance of nucleic acid amplification techniques for diagnosis of respiratory tract infections in the clinical laboratory. Clin Microbiol Rev 1997;10:242–56.

[15] Halonen P, Rocha E, Hierholzer J, Holloway B, Hyypia T, Hurskainen J, Pallansch M. Detection of enteroviruses and rhinoviruses in clinical specimens by PCR and liquid-phase hybridization. J Clin Microbiol 1995;33:648–53.

[16] Vabret A, Mouthon F, Mourez T, Gouarin S, Petitjean J, Freymuth F. Direct diagnosis of human respiratory coronaviruses 229E and OC43 by the polymerase chain reaction. J Virol Meth 2001;97:59–66.

[17] Nokso-Koivisto J, Kinnari TJ, Lindahl P, Hovi T, Pitkaranta A. Human picornavirus and coronavirus RNA in nasopharynx of children without concurrent respiratory symptoms. J Med Virol 2002;66:417–20.

[18] Arbour N, Day R, Newcombe J, Talbot PJ. Neuroinvasion by human respiratory coronaviruses. J Virol 2000;74:8913–21.

[19] Lina B, Valette M, Foray S, Luciani J, Stagnara J, See DM, Aymard M. Surveillance of community-acquired viral infections due to respiratory viruses in Rhone-Alpes (France) during winter 1994 to 1995. J Clin Microbiol 1996;34:3007–11.

[20] Riski H, Hovi T. Coronavirus infections of man associated with diseases other than the common cold. J Med Virol 1980;6:259–65.

[21] Freymuth F, Vabret A, Brouard J, Toutain F, Verdon R, Petitjean J, Gouarin S, Duhamel JF, Guillois B. Detection of viral, Chlamydia pneumoniae and Mycoplasma pneumoniae infections in exacerbations of asthma in children. J Clin Virol 1999;13:131–9.

[22] Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, Tyrrell DA, Holgate ST. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. BMJ 1995;310:1225–9.

[23] Vernotte E, Legrand MC, Gagneur A, Salmon J, Sizun J, de Parscau L. Asthma exacerbation: declenching role of coronaviruses is not confirmed. Arch Pediatr 1999;6:583s.

[24] Resta S, Luby JP, Rosenfeld CR, Siegel JD. Isolation and propagation of a human enteric coronavirus. Science 1985;229:978–81.

[25] Sizun J, Soupre D, Giroux JD, Alix D, De Parscau L, Legrand MC, Demazure M, Chastel C. Nasal colonization with coronavirus and apnea of the premature newborn. Acta Paediatr 1993;82:238.

[26] Sizun J, Soupre D, Legrand MC, Giroux JD, Rubio S, Cauvin JM, Chastel C, Alix D, De Parscau L. Neonatal nosocomial respiratory infection with coronavirus: a prospective study in a neonatal intensive care unit. Acta Paediatr 1995;84:617–20.

[27] Sizun J, Yu MW, Talbot PJ. Survival of human coronaviruses 229E and OC43 in suspension and after drying onsurfaces: a possible source of hospital-acquired infections. J Hosp Infect 2000;46:55–60.

[28] Ijaz MK, Brunner AH, Sattar SA, Nair RC, Johnson-Lussenburg CM. Survival characteristics of airborne human coronavirus 229E. J Gen Virol 1985;66:2743–8.

[29] Gagneur AVS, Legrand MC, Picard B, Talbot P, Sizun J. Human Coronavirus-related outbreaks in a neonatal and pediatric intensive care unit. Pediatric Academic Societies' Annual Meeting; 2002.