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Genetic Variability of Human Coronavirus OC43-, 229E-, and NL63-Like Strains and Their Association With Lower Respiratory Tract Infections of Hospitalized Infants and Immunocompromised Patients

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In the winter–spring seasons 2003–2004 and 2004–2005, 47 (5.7%) patients with acute respiratory infection associated with human coronavirus (hCoV) 229E-, NL63-, and OC43-like strains were identified among 823 (597 immunocompetent and 226 immunocompromised) patients admitted to hospital with acute respiratory syndromes. Viral infections were diagnosed by either immunological (monoclonal antibodies) or molecular (RT-PCR) methods. Each of two sets of primer pairs developed for detection of all CoVs (panCoV) failed to detect 15 of the 53 (28.3%) hCoV strains identified. On the other hand, all hCoV strains could be detected by using type-specific primers targeting genes *1ab* and *N*. The HuH-7 cell line was found to be susceptible to isolation and identification of OC43- and 229E-like strains. Overall, hCoV infection was caused by OC43-like, 229E-like, and NL63-like strains in 25 (53.2%), 10 (21.3%), and 9 (19.1%) patients, respectively. In addition, three patients (6.4%) were infected by untypeable hCoV strains. NL63-like strains were not found to circulate in 2003–2004, and 229E-like strains did not circulate in 2004–2005, while OC43-like strains were detected in both seasons. The monthly distribution reached a peak during January through March. Lower predominated over upper respiratory tract infections in each age group. In addition, hCoV infections interested only immunocompetent infants and young children during the first year of life, while all adults were immunocompromised patients. Coinfections of hCoVs and other respiratory viruses (mostly interesting the first year of life) were observed in 14 of the 47 (29.8%) patients and were associated with severe respiratory syndromes more frequently than hCoV single infections ($P = 0.002$). In conclusion, the use of multiple primer sets targeting different genes is recommended for diagnosis of all types of hCoV infection. In addition, the detection of still untypeable hCoV strains suggests that the number of hCoVs involved in human pathology might further increase. Finally, hCoVs should be screened routinely for in both infants and immunocompromised patients with acute respiratory infection. ***J. Med. Virol. 78:938–949, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: human coronavirus infections; RT-PCR; phylogenetics; respiratory tract infections; bronchiolitis; pneumonia

INTRODUCTION

Following the first reports in the 1960s [Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966; McIntosh et al., 1967], human coronaviruses (hCoVs) were shown to be pathogenic in volunteer studies [Bradburne and Somerset, 1972] and widespread in the community in a number of seroepidemiological surveys of the two known strains, hCoV-229E and hCoV-OC43 [McIntosh et al., 1970; Kaye et al., 1971, 1972; Hamre and Beem, 1972;

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Monto and Lim, 1974; Gerna et al., 1978, 1980; MacNaughton, 1982]. Initially, several other hCoVs were described, such as B814 [Tyrrell and Bynoe, 1965], hCoV-OC16, hCoV-OC37, and hCoV-OC48 [McIntosh et al., 1967]. However, these viruses could not be grown in tissue culture or animal models. Attempts to identify hCoVs in clinical specimens or cell cultures by using either polyclonal or monoclonal antibodies were not satisfactory [McIntosh et al., 1978; Sizun et al., 1998].

Animal CoVs were then investigated extensively in a variety of animal species, while the study of the antigenic relationship of human and animal CoVs was addressed [Kaye et al., 1975; Gerna et al., 1981], and the unique strategy of replication of CoVs was described [Lai and Holmes, 2001]. This allowed the rapid identification of the etiologic agent of the severe acute respiratory syndrome (hCoV-SARS) in 2003 [Peiris et al., 2003]. In parallel, the potential pathogenic role of hCoVs, and, in particular, hCoV-OC43 in diseases of the gastrointestinal tract was investigated by the study of the antigenic relationship of hCoV-OC43 and neonatal calf diarrhea coronavirus or bovine CoV (BCoV), and hCoV-OC43 and human enteric CoVs [Chany et al., 1982; Gerna et al., 1985].

In addition, molecular techniques permitted the identification of known group I (229E-like) and group II (OC43-like) hCoVs in respiratory secretions [Sizun et al., 1998; El-Sahly et al., 2000; Vabret et al., 2001, 2003; Falsey et al., 2002; Pene et al., 2003; Vallet et al., 2004] and the discovery of new hCoVs. Recently, two groups of researchers from The Netherlands [Fouchier et al., 2004; van der Hoek et al., 2004] and one group from USA [Esper et al., 2005] reported a new hCoV, referred to as NL63 and New Haven, respectively. This virus was related distantly to hCoV-229E, and described as one viral agent associated with acute respiratory disease in hospitalized children. Furthermore, another hCoV (referred to as HKU1), distantly related to hCoV-OC43, was detected in two Hong-Kong patients with pneumonia [Woo et al., 2005a], and then reported elsewhere [Woo et al., 2005b; Sloots et al., 2006; Vabret et al., 2006]. A similar virus was also reported in Sweden by molecular screening of respiratory tract samples [Allander et al., 2005]. Finally, a few studies have addressed the circulation of hCoVs with respect to other respiratory viruses in different countries, as well as their involvement in respiratory syndromes other than the common cold, and their association with asthma and chronic obstructive bronchopneumopathies, including hCoV pneumonia in transplanted patients [Lina et al., 1996; Folz and Elkordy, 1999; El-Sahly et al., 2000; Falsey et al., 2002].

At present, several issues remain to be investigated or defined: (i) the reliability of currently available immunological and molecular methodologies for diagnosing infections caused by different groups of hCoVs; (ii) the epidemiology of respiratory infections caused by hCoVs; (iii) the role of known hCoVs in causing admission to the hospital of patients with respiratory infections as well as their relationship to the severity of the relevant respiratory syndromes; (iv) the comparative pathogenicity of different hCoVs in different age groups. In the present study, these issues were investigated in a hospitalized patient population affected by 229E-, NL63-, and OC43-like infections during two consecutive winter–spring seasons. To date, HKU1 has not been detected in Italy.

MATERIALS AND METHODS

Patients

From December 2003 through May 2005, nasopharyngeal aspirates were collected prospectively from 823 patients admitted to hospital with an episode of acute respiratory infection. Of these, 333 were less than 1 year old, 168 2–5 years old, 87 6–20 years old, and 233 >21 years old. Study patients were further subdivided based on immunocompetence. In the four age classes, immunocompetents included 333, 143, 44, and 77 patients, while immunosuppressed patients included 2, 25, 43, and 156 subjects, respectively. Of the 226 immunosuppressed patients, 70 included in the first three age groups were hematopoietic stem cell transplant recipients. In the >21-year group 108 were solid organ transplant recipients, 46 stem cell transplant recipients, and two AIDS patients. Specimens were handled and aliquoted as reported previously [Sarasini et al., 2006], and used for immunological (monoclonal antibodies) and molecular (RT-PCR) assays, as well as for short-term and long-term virus isolation in cell culture. Respiratory samples were examined routinely for influenzaviruses A and B, parainfluenzaviruses 1-4, human respiratory syncytial virus, and human adenoviruses by both direct fluorescent staining and culture. In addition, human metapneumoviruses were examined by both monoclonal antibodies and RT-PCR as recently reported [Percivalle et al., 2005; Gerna et al., 2006]. Finally, hCoVs were tested by monoclonal antibodies and RT-PCR, as detailed below, and human rhinoviruses were searched for by RT-PCR [Steininger et al., 2001]. In this study, the term coinfection indicates the simultaneous detection of two or more respiratory viruses in the same sample taken from a patient with respiratory infection, while the term sequential infection refers to the sequential identification of two different respiratory viruses in two nasopharyngeal aspirates taken from the same patient within 30 days from each other.

Direct Fluorescent Antibody Staining of NPA Cells and Cell Cultures

Direct fluorescent antibody staining was applied to both slides containing smears of respiratory epithelial columnar cells from nasopharyngeal aspirates and cell cultures 48 hr after inoculation of respiratory secretions, as reported [Rovida et al., 2005]. In either case, as a first step, cells were stained with a pool (SimulFluor Respiratory Screen reagent, Chemicon International, Inc., Temecula, CA) of fluorescein-labeled monoclonal antibodies to conventional respiratory viruses

(influenzavirus types A and B, human parainfluenzavirus types 1-4, human respiratory syncytial virus, and human adenovirus). Then, as a second step, positive samples were stained with individual monoclonal antibodies obtained from the same source (Chemicon). Group- and type-specific monoclonals to human metapneumovirus were developed in the laboratory as reported [Percivalle et al., 2005; Gerna et al., 2006]. As for hCoVs, commercially available monoclonal antibody to OC43 (Chemicon) was found to perform satisfactorily, while monoclonal antibodies to 229E were developed in the laboratory and found to be highly specific. Finally, monoclonal antibodies to NL63 were not available.

Inoculated cell cultures included mixtures (MIX) of A549 and Mv1Lu (ratio 1:1) cells [Huang and Turchek, 2000], as well as LLC-MK2 and MDCK cell lines. Cell cultures were processed for virus isolation and identification as reported previously [Rovida et al., 2005]. In addition, in the last part of the study, HuH-7 [[Nakabayashi et al., 1982] provided by Maura Pizzuti, Institute for Biomedical Research, Rome, Italy], Vero and LLC-MK2 cell lines, as well as secondary African green monkey kidney cell cultures, were inoculated with six NPAs RT-PCR-positive for hCoVs (one positive for 229E, one positive for OC43, and four positive for NL63) to investigate the susceptibility of these cells to isolation and propagation of hCoVs.

RT-PCR Protocol for Respiratory Virus Detection

RT-PCR assays for respiratory viruses were optimized to detect at least 10 input plasmid copies, as described previously [Sarasini et al., 2006]. In addition, as reported in Table I, NPAs were tested for hCoVs by RT-PCR by using: (i) a primer set (referred to as PanCoV-03) developed for detection of all coronaviruses and relevant to gene *1ab* [Poutanen et al., 2003]; (ii) a second set of primers (referred to as PanCoV-05) also developed for detection of both human and animal coronaviruses and relevant to gene *1ab* [Moës et al., 2005]; (iii) three sets of primers specific for OC43, 229E, and NL63 hCoVs, respectively, and relevant to gene *N*; and, finally, three sets of primers specific for OC43, 229E, and NL63, respectively, derived from PanCoV-05, thus, relevant to gene *1ab*.

Phylogenetic Analysis of hCoV Strains

The following fragments of the *1ab* gene were amplified (Table I): nt 14321–14536 of 229E (amplicon size, 215 nt) with Pan CoV-03, nt 14098–14348 of 229E (size, 250 nt) with Pan CoV-05, nt 14922–15172 (size, 250 nt) with OC43-specific primers, nt 14098–14348 (size, 250 nt) with 229E-specific primers, and nt 14017–14267 (size, 250 nt) with NL-63-specific primers. In addition, the following fragments of the *N* gene were amplified: nt 715–1073 (size, 378 nt) with OC43-specific primers, nt 578–921 (size, 343 nt) with 229E-specific primers, and nt 563–876 (size, 313 nt) with NL63-specific primers. The indicated fragments of genes *1ab* and *N* of hCoVs OC43, 229E, and NL63, following amplification with specific primers, were sequenced with the ABI PRISM 3100 automatic sequencer (Applied Biosystems, Foster City, CA). Viral sequences of the amplified fragments of genes *1ab* and *N* of different hCoV strains as well as reference strains were aligned with the Clustal W program version 1.7. Distances between pairs of nucleotide sequences were calculated by using the DNAdist modules (with Kimura's two-parameter method) in the Phylip package, version 3.572 (Felsenstein, Department of Genetics, University of Washington, Seattle, WA). The Phylip (njplot) program was used to construct phylogenetic trees with nucleotide sequences by means of the neighbor-joining method from the same distance matrices. Bootstrap support was determined by 100 resamplings of the sequences.

Statistical Analysis

Comparison of the distribution frequencies was performed with the Pearson's Chi square test.

TABLE I. Primer Pairs Used for Detection of All hCoVs (PanCoV) or Specific for OC43, 229E, and NL63 *N* and *1ab* Genes

Primer pair	Primer sequences	Target gene, nt → nt	Reference
PanCoV-03	5′-tgatgggatgggactatcctaagtgtga-3′ 5′-ttgcatcaccactagttgtgccaccaggtt-3′	*1ab*, 14321 → 14536 (229E)	Poutanen et al. [2003]
PanCoV-05	5′-acwcarhtvaayytnaartaygc-3′ 5′-tcrcayttdggrtartccca-3′	*1ab*, 14098 → 14348 (229E)	Moës et al. [2005]
OC43-specific	5′-cttgttctggcaaaacttggcaaggatg-3′ 5′-ctaattgcgccgttatagcgcaattca-3′	*N*, 715 → 1073	This study
229E-specific	5′-taggttttgacaagcctcaggaaaaaga-3′ 5′-gtgactatcaaacagcatagcagctgt-3′	*N*, 578 → 921	This study
NL63-specific	5′-ctgttactttggctttaaagaacttagg-3′ 5′-ctcactatcaaagaataacgcagcctg-3′	*N*, 563 → 876	This study
OC43-specific	5′-actcaaatgaatttgaaatatgc-3′ 5′-tcacacttaggataatccca-3′	*1a*[a], 14922 → 15172	Moës et al. [2005]
229E-specific	5′-actcagttaaatcttaaatacgc-3′ 5′-tcacacttaggatagtccca-3′	*1ab*[a], 14098 → 14348	Moës et al. [2005]
NL63-specific	5′-acacagctgaatcttaagtatgc-3′ 5′-tcacatttgggataatccca-3′	*1ab*[a], 14017 → 14267	Moës et al. [2005]

[a]Specific primers derived from PanCoV-05.

RESULTS

Methodological Approach to Molecular Detection of hCoVs in Respiratory Secretions

On the whole, 53 hCoV strains were identified from 47 patients admitted to hospital as follows: 27 (50.9%) OC43-like strains, 14 (26.4%) 229E-like strains, 9 (17%) NL63-like strains, and 3 (5.7%) untypeable hCoV strains (Fig. 1A). Six strains (four 229E-like and two OC43-like strains) were recovered twice from the same patients within 2 weeks after initial virus recovery. All hCoV-positive samples were tested by two sets of primers aimed at detecting all hCoV strains (Table I): (i) panCoV-03 [Poutanen et al., 2003]; and (ii) panCoV-05 [Moës et al., 2005]. Of the 53 hCoV-positive samples, 23 (43.4%) were found positive by both assays, while 15 (28.3%) were detected by panCoV-03 only, and 15 (28.3%) by panCoV-05 only (Fig. 1B,C). All of the nine NL63-like strains were detected by the panCoV-05 primer pair only. In conclusion, 38/53 (71.7%) samples tested by panCoV-03 were positive for hCoV: 21 (39.6%) for OC43-, 14 (26.4%) for 229E-like strains, and three (5.7%) for untypeable strains, while 15 strains (28.3%) were negative. Similarly, of the 53 samples tested by panCoV-05, 38 (71.7%) were positive: 22 (41.5%) for OC43-like, 7 (13.2%) for 229E-like, and 9 (17%) for NL63-like strains, while 15 strains (28.3%) were negative (no untypeable strains with this primer set).

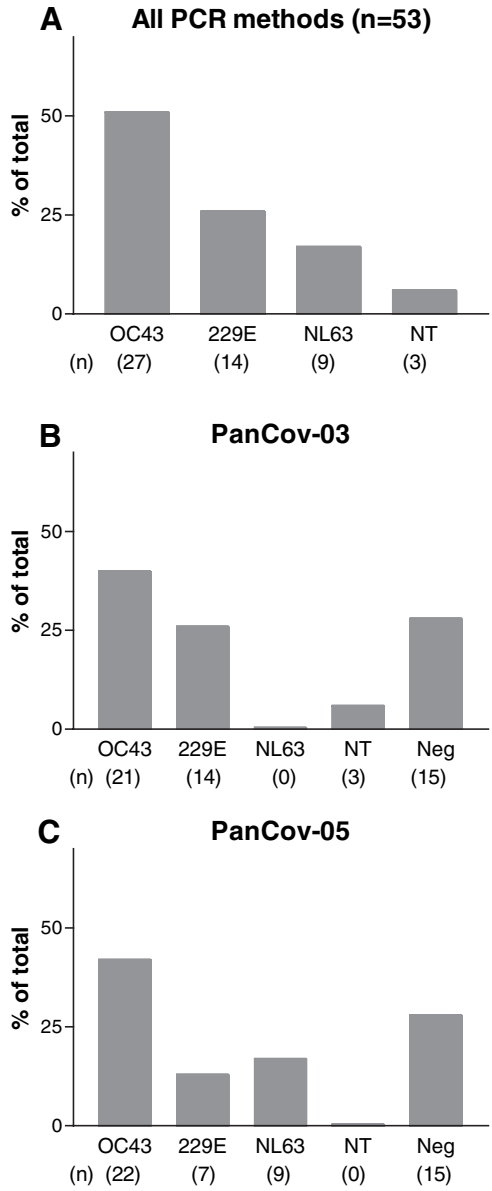

Fig. 1. Total number and relative percentage of human coronavirus strains detected in hospitalized patients during the winter–spring seasons 2003–04 and 2004–05 by (**A**) all PCR methods, (**B**) PanCoV-03, and (**C**) PanCoV-05 primer pair. (n) = absolute number of strains. NT, not typed.

All 53 hCoV strains were typed by sequencing and phylogenetic analysis using the amplification products provided by both or either panCoV. Subsequently, to confirm results, each of the 53 hCoV strains were amplified by using two primer pairs specific for genes *1ab* and *N* of OC43, 229E, and NL63 prototypes, respectively (Table I). PanCoV typing results were consistently confirmed by type-specific primer sets, with no cross-reactivity among primers.

Phylogenetic Analysis

Primer-specific amplification products of gene *1ab* (Fig. 2) and *N* fragments were used for phylogenetic analysis. OC43-like strains clustered in a group distinct from reference strain OC43 and much more distant from the new hCoV HKU1, except for a single strain (I-PV 02/04-2645) distinct from both OC43 and HKU1. Similarly, 229E-strains clustered into a single group distinct from the reference strain 229E and distant from NL63. Finally, NL63-like strains were grouped together substantially, and distinct from the reference strain NL63 and very distant from 229E. A summary of the genetic variability (nt and aa changes) of different hCoV strains (within the limits of the amplified fragment of genes *1ab* and *N*) with respect to reference strains reported in Gene Bank is given in Table II. Weakness of PanCoV-03 amplification of the three untypeable strains did not allow its sequencing. However, negative results following amplification of *N* gene with 229E-, OC43-, and NL63-specific primers did exclude their similarity with the reference strains.

Recovery Attempts of hCoVs in Cell Cultures

Following identification of hCoV-positive nasopharyngeal aspirates by RT-PCR, retrospective attempts were made to grow short-term OC43-, 229E-, and NL63-like strains in cell cultures from a small number of specimens. One OC43- and one 229E-like strain were inoculated and found to grow in the HuH-7 cell line. Viruses were identified (Fig. 3A–D) by monoclonal antibodies to OC43 (available commercially) and 229E (developed in the laboratory). Further propagation of recovered strains gave high virus yields for both OC43-like

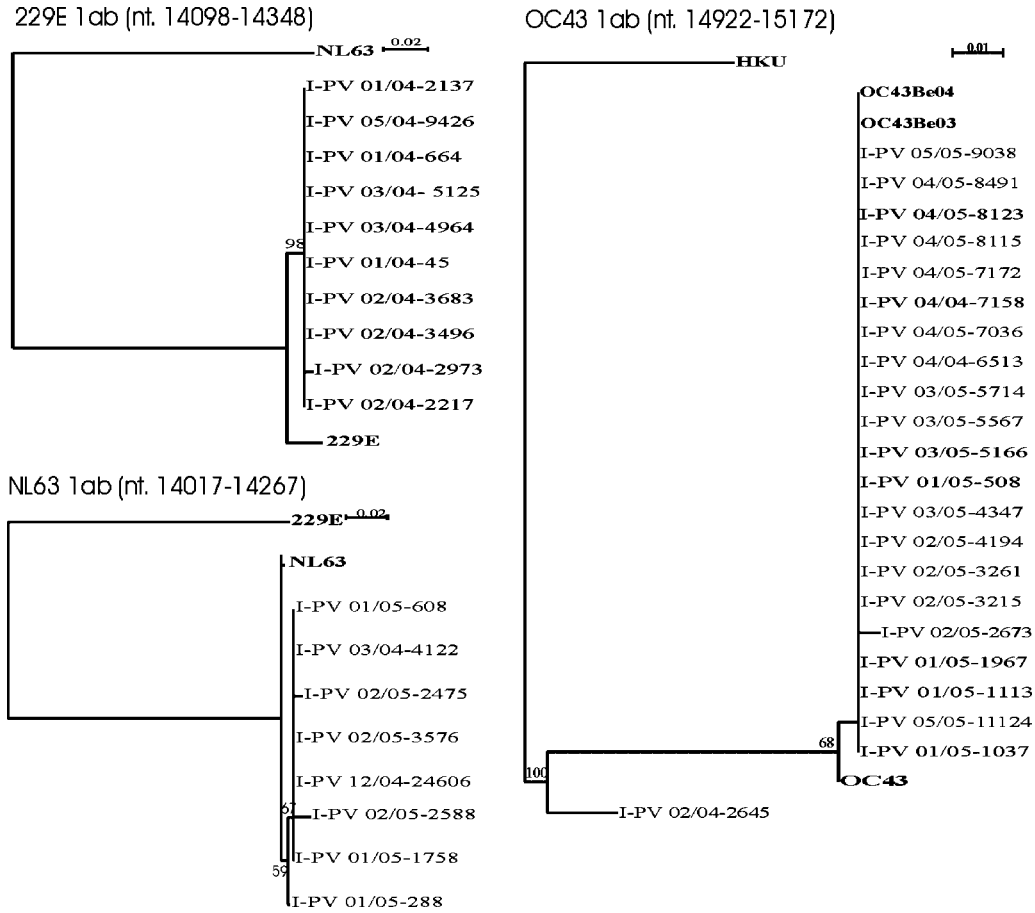

Fig. 2. Phylogenetic analysis of 229E-, NL63-, and OC43-like strains with respect to the relevant reference strains, based on the relevant sequence of gene *1ab* fragment. Accession numbers for the complete genome are as follows: AF304460 for 229E; AY567487 for NL63; AY391777 for OC43, AY903460 for OC43Be04, AY903459 for OC43Be03.

TABLE II. Genetic Variability of 229E-, NL63-, and OC43-Like Strains Detected in Hospitalized Patients

HCoV, gene sequenced (number of strains examined)	Changes[a] with respect to reference strain [aa change] (% homology)	
	nt	aa
229E (AN[b]: AF304460)		
1ab (n = 10)	6/10 (97.6)	1/1 [K4737I] (98.8)
N (n = 9)	2/9 (99.4)	1/9 [S228I] (99.1)
	1del/1 (95.6)[c]	1/1 [A225D] (99.1)
NL63 (AN[b]: AY567487)		
1ab (n = 8)	2/6 (99.2)	None
	1/1 (99.6)	
	4/1 (98.4)	
N (n = 5)	2/1 (99.4)	2/5 [A188S, V293I] (98.1)
	3/1 (99.0)	
	1del/1 (99.0)[d]	
OC43 (AN[b]: AY391777)		
1ab (n = 22)	1/1 (99.6)	1/1 [R5038H] (98.8)
	15/1 (94.0)[e]	
N (n = 19)	3/5 (99.2)	1/3 [S306N] (99.2)
	4/8 (98.9)	
	5/6 (98.6)	

[a]No. changes/no. strains with identical changes.
[b]AN, accession number of prototype strains.
[c]Strain I-PV 05/04-9426 with a 15 nt deletion.
[d]Strain I-PV 01/05-288 with a 3 nt deletion.
[e]Strain I-PV 02/04-2645.

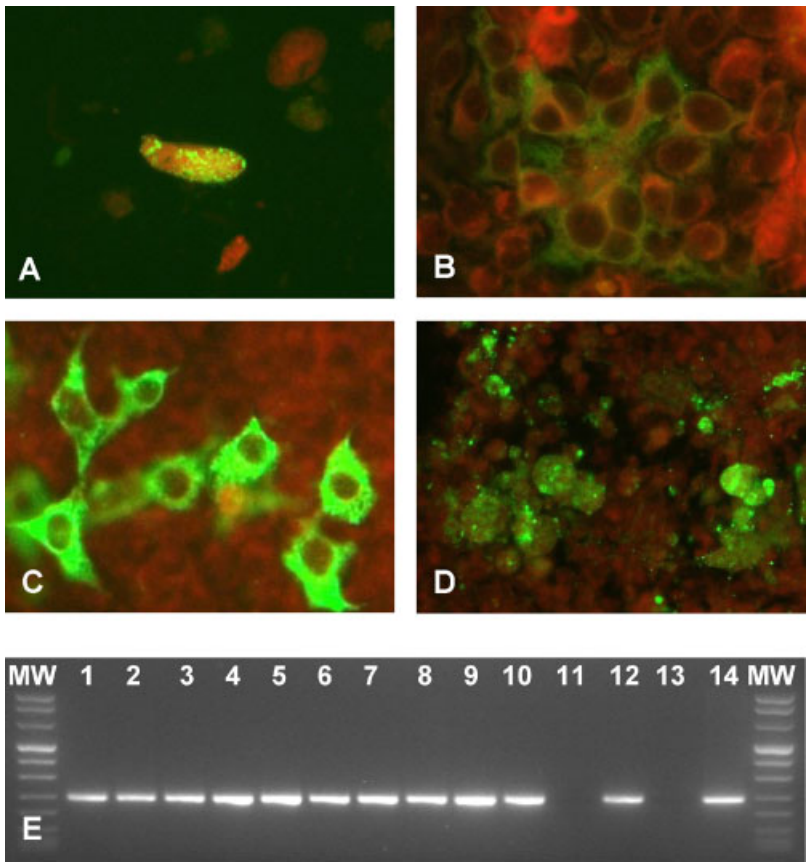

Fig. 3. Isolation and identification of hCoVs. **A**: Direct fluorescent antibody staining of an OC43-like strain in a nasopharyngeal aspirate from a patient with acute respiratory tract infection. **B**: OC43-like strain on primary isolation in HuH7 cells, following identification by monoclonal antibody. **C**, **D**: Early (2 days) and late (6 days) identification of a 229E-like strain in HuH7 cells by a specific monoclonal antibody. **E**: RT-PCR identification of 4 NL63-like strains on primary isolation in LLC-MK2 (**lanes 1**, **4**, **7**, **10**), Vero (**lanes 2**, **5**, **8**, **11**), and primary AGMK (**lanes 3**, **6**, **9**, and **12**) cell cultures, following inoculation of nasopharyngeal aspirates from four patients with acute respiratory tract infection. Lane 11 shows negative results (no viral growth) in Vero cells. **Lane 13**: "0" (buffer) control. **Lane 14**: NL63 plasmid (100 copies).

and 229E-like strains (10^6 to 10^7 $TCID_{50}$/ml). No cross-reactivity of these strains with other human respiratory viruses was detected either by using RT-PCR or monoclonal antibodies. On the other hand, four NL63-like-positive samples showed initial growth in secondary African green monkey kidney cell cultures as well as Vero and LLC-MK2 cell lines, as indicated by RT-PCR using type-specific primers (Fig. 3E). However, further propagation was unsuccessful. These preliminary results suggest the possible recovery and long-term propagation of 229E-like and OC43-like hCoV strains in cell culture, whereas a cell culture system suitable for long-term propagation of NL63-like strains remains to be identified.

Epidemiology of hCoV Infections

Among 823 patients admitted to hospital with an episode of acute respiratory tract infection in the winter–spring seasons 2003–04 and 2004–05, 47 (5.7%) patients were found to be affected by hCoV infections. The list of respiratory viruses associated with acute respiratory infections in 447/823 (54.3%) patients is reported in Table III. The most frequently detected virus was hRSV (26.7%), followed by rhinoviruses (24.2%) and influenza viruses (13.9%), while hMPV and hCoVs were circulating at a comparable rate (8.1 and 7.4%, respectively).

A great variation in the circulation of different hCoVs was observed between the two seasons examined. In fact, 229E-like strains circulated only in 2003–04 (10 infected patients), and NL63-like strains only in 2004–05 (9 infected patients), while OC43-like strains were detected in both seasons, thereby infecting 25 patients (Fig. 4A,B). The peak circulation of hCoVs was reached in January through March, while a few strains were observed from November to May.

Stratification by age showed that 20/333 (6.0%) patients were affected by hCoV infection in the first year of life, while 8 (4.8%) were affected in the 2–5 years old group, 13 (14.9%) in the 6–20 years old group, and only 6 (2.6%) were > 20 years old (Fig. 5A). In 33/47 patients (70.2%), respiratory infections were attributed to a single hCoV strain, while in 14/47 patients (29.8%) hCoVs were associated with one or more other respiratory viruses. Analysis of the distribution of single infections and coinfections by age showed that they affected an equal number (n = 10) of patients in the first

TABLE III. Prevalence of Different Respiratory Virus Infections During Two Subsequent Winter–Spring Seasons

Patients	Season		Total
	2003–04	2004–05	
No. examined	378	445	823
No. positive for respiratory viruses (%)	178 (47.1)	269 (60.4)	447 (54.3)
No. (%)[a] positive for:			
hRSV	53 (29.8)	67 (24.8)	120 (26.7)
Rhinoviruses	32 (18.0)	76 (28.2)	108 (24.2)
Influenza viruses	23 (12.9)	39 (14.5)	62 (13.9)
hMPV	31 (17.4)	5 (1.9)	36 (8.1)
hCoVs	10 (5.6)	23 (8.6)	33 (7.4)
AdVs	7 (3.9)	2 (0.7)	9 (2.0)
hPIVs	6 (3.4)	1 (0.4)	7 (1.6)
Coinfections	10 (5.6)	33 (12.3)	43 (9.6)
Sequential infections	6 (3.4)	23 (8.6)	29 (6.5)
hCoV coinfections	5 (2.8)	9 (3.3)	14 (3.1)
Total hCoV infections	15 (8.4)	32 (19.0)	47 (10.5)

[a]The percentage of each virus refers to the total number of positives.

year of life, while the incidence of coinfections (Table IV) greatly decreased in the other age groups (only four patients in the 2-20 years old groups).

Lower Versus Upper Respiratory Tract Infections Associated With hCoVs

Among the 47 patients affected by respiratory viral infections associated with hCoVs, 35 (74.5%) showed lower (bronchitis, bronchiolitis, pneumonia) and 12 (25.5%) upper respiratory tract (rhinitis, pharyngitis, laryngitis) infections. The predominance of lower versus upper infections was observed in patients of each age group with no significant difference in the incidence among different age groups (Fig. 5B).

Incidence of hCoV Infections in the Immunocompetent and the Immunocompromised Host

As shown in Figure 5C,D, the mean percentage of immunocompetent patients with hCoV infections was 4.5% (27/597), whereas the mean percentage of immunocompromised patients infected by hCoVs was 8.8% (20/226) with a statistically significant difference (χ^2 test, $P = 0.02$). The relative risk of contracting hCoV infection in immunocompromised patients was twice that of immunocompetent patients. The total number of hCoV infections observed during the first year of life included 20/333 (6.0%) immunocompetent infants and young children, while the proportion of immunocompetent children with hCoV infections in the older age groups included 5/143 (3.5%) and 2/44 (4.5%) patients in the 2–5 and the 6–20 years old groups, respectively. No hCoV infection (0/77) was observed in immunocompetent patients older than 20 years (Fig. 5C). Conversely, no immunocompromised patients with hCoV infections, were observed in the first year of life (0/2 patients). In older age groups, hCoV infections of immunocompromised patients increased progressively up to 12.5% (3/25 patients) in the 2–5 years old group, and to 25.6% (11/43 patients) in the 6–20 years old group, including all infected patients older than 20 years (3.8%, 6/156 patients) (Fig. 5D).

Pathogenicity of hCoVs

In the same group of 47 patients examined with hCoV respiratory infections, 25 (53.2%) were infected by OC43-like strains (six in association with other viruses), 10 (21.3%) by 229E-like strains (three coinfections), and 9 (19.2%) by NL63-strains (two coinfections). In addition, three patients (6.3%) were infected by untypeable hCoVs (three coinfections). No significant difference in the circulation of different hCoVs among different age groups was observed (Fig. 4C). The most common symptoms were rhinorrhea (52.2%), fever (47.8%), and cough (47.8%) in OC43-infected patients. Similarly, predominant symptoms were rhinorrhea (70%), fever (50.0%), and cough (50.0%) in 229E-infected patients, while rhinorrhea (57.1%) and cough (42.8%) were most commonly observed in NL63-infected individuals, in association with infrequent episodes of fever (14.2%).

Severe lower respiratory tract syndromes (bronchiolitis, pneumonia) were observed at presentation in 10/15 lower respiratory tract infections associated with hCoV in the first year of life: six (three single infections and three coinfections) were associated with OC43-like strains, two with hCoV untypeable strains (both coinfections), one to 229E-like strain (coinfection), and one to an NL63-like strain (coinfection). Thus, severe hCoV infections in the first year of life appeared to be mostly associated with OC43-like strains. Conversely, none of the five adult immunocompromised adult patients with lower respiratory tract infections showed association with bronchiolitis or pneumonia.

Severe respiratory syndromes were observed in 9/14 (64.3%) coinfected patients (Table IV), and only in

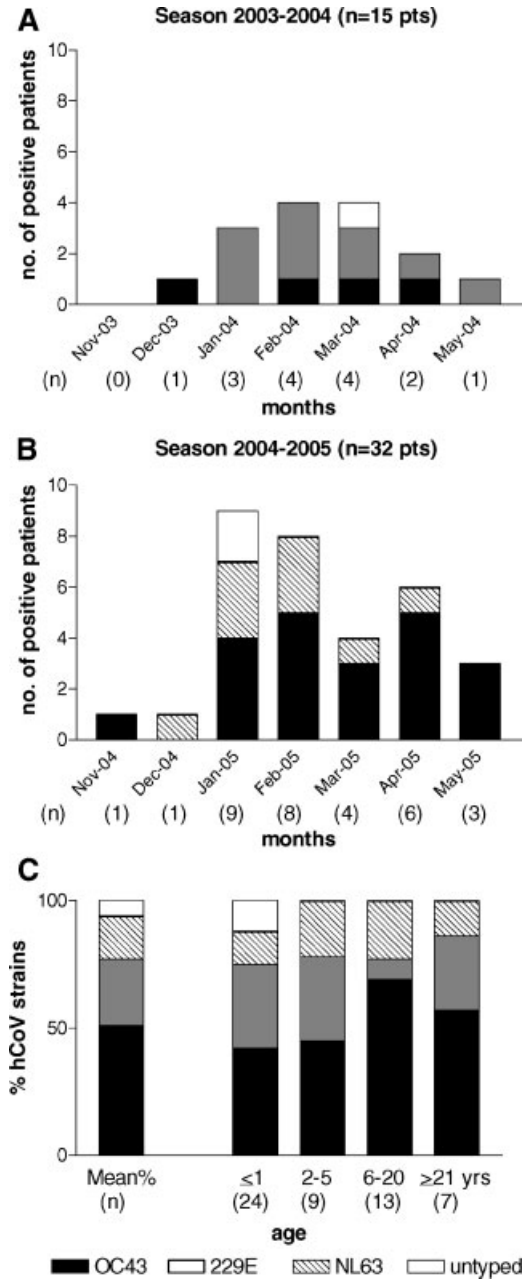

Fig. 4. **A**, **B**: Distribution of 229E-like, NL63-like, OC43-like and untyped hCoV strains detected during two consecutive seasons. **C**: Age distribution of the 53 hCoV strains detected during the present study. (n) = number.

3/33 patients (9.1%) infected by hCoV alone ($P = 0.002$). Thus, hCoV coinfections appeared to be associated with a significantly higher number of severe syndromes involving the lower respiratory tract.

DISCUSSION

After the 1970s, clinical studies on hCoVs were mostly abandoned due to either the presumed low clinical impact of these viruses, or to the great difficulties encountered in their isolation and identification. Renewed interest was triggered recently by the identification of the coronavirus responsible for SARS in 2003. In addition, the development of viral genome amplification procedures has provided a major contribution to the identification of hCoV infections. A major variant of group I hCoVs, with the 229E virus as a prototype, was identified in the NL63 strain detected in the Netherlands [Fouchier et al., 2004; Van der Hoek et al., 2004], and in the USA [Esper et al., 2005]. More recently, a major variant of group II hCoVs, with the OC43 virus as a prototype, was first reported in Hong-Kong as HKU1 [Woo et al., 2005a] and, then, in France, Australia, and other regions of China [Woo et al., 2005b; Sloots et al., 2006; Vabret et al., 2006]. A wide range of antigenic variability has been reported by researchers recovering a series of viruses in organ cultures of human embryonic trachea [McIntosh et al., 1967; McIntosh, 2005]. However, a great deal of controversy has been raised about the genetic stability [St-Jean et al., 2004] or variability [Vijgen et al., 2005] of hCoV OC43-like strains.

The genetic variability of hCoVs is supported by the present study, showing that both sets of PanCoV primers actually did not detect about 30% of circulating strains. This failure of both PanCoV primer sets was confirmed by the use of group-specific primer pairs relevant to two different genes. A similar conclusion was recently reported by another study [Chiu et al., 2005]. As a result, in order to detect the maximal number of circulating hCoVs, specific primers should be used, and results confirmed by a second set of primers, preferably targeting a different gene. In the present study, no HKU1 strain was detected by the two PanCoV primer sets used, while, in the absence of a positive control, HKU1-specific primers were not used.

A complement to the molecular techniques available for detection of hCoVs is represented by the use of the HuH-7 cell line, which has been recently employed for respiratory virus isolation [Vabret et al., 2001; Pene et al., 2003; Freymuth et al., 2005]. This cell line, which was shown to be susceptible to infection by a murine coronavirus, the mouse hepatitis virus [Koetters et al., 1999], has been found to be helpful in increasing the rate of respiratory virus recovery from DFA-negative samples [Freymuth et al., 2005]. In addition, HuH-7 was reported to be permissive for growth of hCoVs, rhinoviruses, and enteroviruses. However, in that study, hCoVs were cultured in HuH7 for 4 days only, and then identified by RT-PCR.

In the present study, it was possible to isolate and extensively propagate two strains of 229E-like and OC43-like strains from respiratory secretions, thus obtaining high virus yields after a few passages. A major morphological characteristic of both 229E-like and OC43-like strains was the presence of syncytial formations in inoculated cell cultures, appearing early (24–48 hr) after inoculation and progressively enlarging until detaching from the cell surface. In this respect, it seems important to recall that SARS-hCoV was reported to produce syncytia in vivo [Ksiazek et al., 2003]. As for

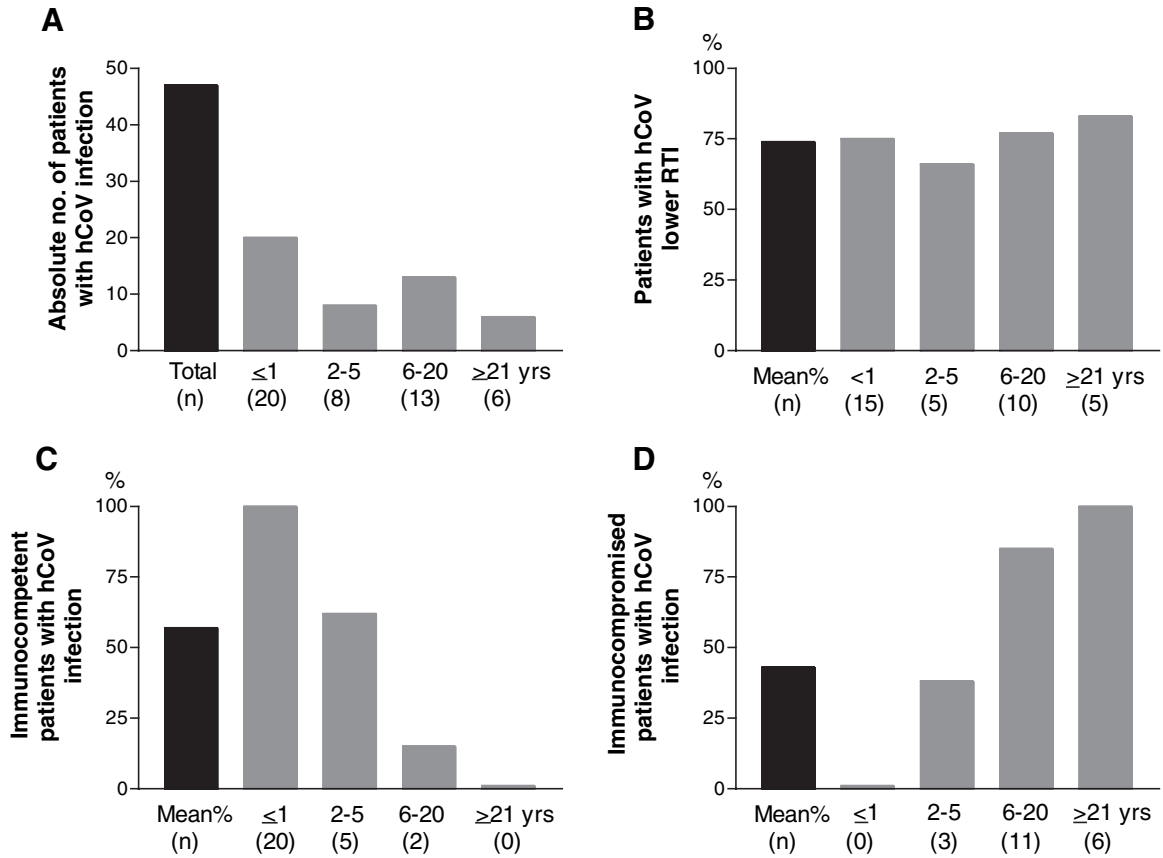

Fig. 5. Distribution by age class of (**A**) patients with hCoV infections (absolute patient number); (**B**) % patients with hCoV lower respiratory tract infections (RTI); (**C**) % immunocompetent patients with hCoV infection; (**D**) % immunocompromised patients with hCoV infection. (n) = number.

NL63-like strains, initial growth was confirmed by the positive RT-PCR results in the supernatant of all three types of cell cultures inoculated with four NL63-positive respiratory samples, that is, two cell lines (LLC-MK2 and Vero) and one secondary culture of African green monkey kidney. However, at subsequent passages, the presence of the viral RNA signal in the medium weakened progressively and finally disappeared. Since inoculated NL63-like-positive samples were freeze-thawed more than once prior to inoculation, new

TABLE IV. List of hCoV Coinfections

Patient #, age	Type of coinfection	Respiratory syndrome	Outcome
1, 2 mos	hMPV-A + hCoV 229E	Rhinitis	Recovered
2, 12 mos	hMPV-A + hCoV 229E	Rhinitis	Recovered
3, 7 mos	hMPV-B + hCoV OC43	Bronchiolitis	Recovered
4, 7 mos	hRSV + hCoV 229E	Pneumonia	Recovered
5, 7 mos	hAdV + hCoV-NT	Rhinopharyngitis	Recovered
6, 8 mos	Rhinovirus + hCoV OC43	Bronchiolitis	Recovered
7, 2 mos	hRSV + hCoV-NT	Pneumonia	Recovered
8, 12 mos	hRSV + hCoV-NT	Pneumonia	Recovered
9, 17 yrs	hRSV + hCoV OC43 (+Aspergillus)	Pneumonia in HSCTR	Deceased
10, 4 mos	hRSV + hCoV NL63	Bronchiolitis	Recovered
11, 3 mos	hRSV + hCoV OC43	Bronchiolitis	Recovered
12, 7 yrs	InfluenzaA + hCoV NL63	Rhinopharyngitis in HSCTR	Recovered
13, 3 yrs	hRSV + Rhinov. + hCoV OC43	Rhinopharyngitis in HSCTR	Recovered
14, 11 yrs	InfluenzaB + hAdV + hCoV OC43	Pneumonia	Deceased

LRTI, lower respiratory tract infection; HSCTR, hematopoietic stem cell transplant recipient; NT, not typed.

isolation attempts will be made as soon as fresh positive specimens become available.

The phylogenetic analysis based on sequencing of small fragments from genes *1ab* and *N* showed that each of the three groups of hCoVs detected in our study belong to a single cluster, which is clearly distinct from the relevant reference strain. The only exception regarded an OC43-like strain (I-PV 02/04-2645), which was distinct from both Pavia strains and the OC43 reference strain and was recovered from a patient who had recently arrived from Albany. These data confirm the genetic similarity of the strains circulating in the same geographical area without excluding the simultaneous presence of other unrelated or poorly related strains.

The pathologic impact of hCoV infections was investigated by analyzing the role of these infections in the context of the patient population admitted to hospital due to an acute respiratory infection during two consecutive winter–spring seasons. On the whole, the overall rate of hCoV infections (including both single infections and coinfections) followed that of human respiratory syncytial virus, rhinovirus, and influenza virus infections, and preceded that of human metapneumoviruses, adenoviruses, and human parainfluenza viruses, with comparable rates of coinfections and sequential infections. Although different studies have reported variations, these data correlate grossly with previous surveys [Lina et al., 1996; Freymuth et al., 2005]. In addition, coinfections by different respiratory viruses have been repeatedly reported [El-Sahly et al., 2000; Rovida et al., 2005].

From an epidemiological standpoint, in the great majority of studies, 229E, OC43, and NL63 [Vabret et al., 2003 van der Hoek et al., 2004] outbreaks have been found to occur in the winter–spring season with recurrences every 2–4 years [McIntosh et al., 1970; Kaye et al., 1971; Monto and Lim, 1974]. In our study, while the peak of hCoV circulation was reached in January through March, the three hCoVs were found to circulate differently during the two consecutive seasons studied. While OC43-like strains were found to circulate in both seasons, 229E- and NL63-like strains were detected in either one of the two seasons, as observed previously by others. On the other hand, all three hCoVs were represented in the four age groups in which the hospitalized patient population was divided, as reported previously [El-Sahly et al., 2000; Vabret et al., 2003].

The study of the age distribution showed a large predominance of hCoV infections in the first year of life, when 15/20 (75.0%) patients were affected by lower respiratory tract infections and 10 (66.6%) suffered from severe lower respiratory tract syndromes, such as bronchiolitis or pneumonia. It is important to emphasize that all infected infants and young children were immunocompetent subjects. These results strengthen previous observations on the great pathologic potential of hCoVs in very young patients [McIntosh et al., 1974; Sizun et al., 1995; El-Sahly et al., 2000; Vabret et al., 2003; Chiu et al., 2005]. In addition to 229E- and OC43-like strains, also the newly identified NL63-like strains were shown to be associated with lower respiratory tract infections, including bronchiolitis and pneumonia [Arden et al., 2005; Bastien et al., 2005; Ebihara et al., 2005; Esper et al., 2005; Vabret et al., 2005]. Therefore, hCoVs, and, as a result of this study, particularly OC43-like strains, appear to behave like other respiratory viruses, such as human respiratory syncytial virus, human metapneumovirus, and human parainfluenza viruses, displaying a greater virulence in infancy and early childhood.

On the other hand, adult patients included in this study were all immunocompromised transplanted patients, who had never suffered from severe respiratory syndromes. However, case reports of hCoV-related pneumonia have been described [Pene et al., 2003], and the increasing number of transplanted patients undergoing immunosuppressive therapy render the investigation of these viruses mandatory in lower respiratory tract secretions from transplant recipients with bronchiolitis and pneumonia.

Finally, the significantly greater association of hCoV coinfections with severe clinical syndromes, that emerged in the present study, shows a rate of severe lower respiratory tract infections greater than 60% in patients with coinfections compared to less than 10% in patients with a single infection. The major pathologic effect of hCoV coinfections was observed again in infants and young children. However, whether the coinfection by hCoV was a factor increasing the severity of the associated viral infection remains hypothetical.

In conclusion, the genetic variability of hCoVs might make the detection of all circulating strains difficult. Cultures might be important for new hCoV detection. The recently renewed interest in hCoVs has allowed the identification of these viruses as major pathogens of infancy and early childhood, while their pathologic role in immunocompromised patients remains to be defined. Coinfection by hCoVs and other respiratory viruses is often detected in severe lower respiratory tract infections of infants and young children.

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