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Human respiratory coronavirus HKU1 versus other coronavirus infections in Italian hospitalised patients

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Abstract

Background: Human respiratory coronavirus (hCoV) HKU1 infections were reported for the first time in 2005 in Hong Kong.
Objective: To investigate epidemiological, clinical, and diagnostic features of HKU1 infections.
Study design: Longitudinal, prospective study from November 2005 through May 2006 in a hospitalised patient population.
Results: Overall, 48/426 (11.3%) patients were found to be infected by hCoV acute respiratory tract infections (ARTI). Of these, 10 (19.2%) were caused by HKU1 (6 single infections and 4 coinfections) during the period January–May 2006. Diagnosis was made by using RT-PCR for all four hCoVs, and in parallel, in-house developed group-specific monoclonal antibodies (MAbs) for HKU1 and 229E. HKU1-specific MAb was able to retrospectively identify 8 of 10 HKU1 strains detected by RT-PCR. Phylogenetic analysis showed that four HKU1 strains were genotype A and six genotype B. In HKU1-infected patients, the predominant clinical symptom was rhinorrhea (nine patients). Within group II hCoV, HKU1-infected patients had a significantly lower rate of lower ARTI compared to OC43-infected patients.
Conclusion: HKU1 hCoV strains circulated in northern Italy during the winter–spring season 2005–2006. Both HKU1 genotypes were detected. HKU1-specific MAb may contribute to the rapid diagnosis of HKU1 infections currently performed by RT-PCR.

Keywords: Human coronavirus HKU1; Acute respiratory tract infections; Monoclonal antibody; Reverse transcription-PCR; HKU1 genotypes

1. Introduction

Following the first reports in the 1960s, human coronaviruses (hCoVs) 229E and OC43 were found to circulate at a high rate in several seroepidemiological studies (McIntosh et al., 1970). However, difficulties encountered in virus isolation and recovery from cell cultures hampered medical investigations of these viruses until the recent identification of the etiologic agent of the severe acute respiratory syndrome (hCoV-SARS) in 2003 (Peiris et al., 2003).

In recent years, the development of molecular techniques prompted the resumption of clinical studies aimed at identifying the role of group I (229E-like) and group II (OC43-like) hCoVs in respiratory syndromes. The new technological tools allowed the recent discovery of two new hCoVs: one, referred to as NL63, belongs to group I and is distantly related to 229E (Fouchier et al., 2004; Van der Hoek et al., 2004), and the second, referred to as HKU1, is more closely related to the group II prototype hCoV-OC43 (Woo et al., 2005a). HCoV-HKU1 was first detected in two patients with pneumonia in Hong-Kong (Woo et al., 2005a), and then reported elsewhere (Esper et al., 2006; Garbino et al., 2006; Sloots et al., 2006; Vabret et al., 2006; Woo et al., 2005b). In addition, a number of studies from different countries have shown that hCoV-NL63 can be recovered from respiratory secretions of both immunocompetent children and immunocompromised adults (Arden et

Abbreviations: hCoVs, human coronavirus; DFA, direct fluorescent antibody staining; MAbs, monoclonal antibodies; RT-PCR, reverse transcription-PCR; NPA, nasopharyngeal aspirate; SVC, shell-vial cultures; ARTI, acute respiratory tract infections; BAL, bronchoalveolar lavage

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al., 2005; Bastien et al., 2005; Boivin et al., 2005; Chiu et al., 2005; Ebihara et al., 2005; Gerna et al., 2006a; Moës et al., 2005).

In Italy, hCoVs 229E, OC43, and NL63 have been shown to circulate both in the past and present time (Gerna et al., 1978, 1980, 2006a). However, the HKU1 epidemiological and clinical aspects have never been investigated either alone or with respect to the other hCoV groups.

2. Patients and methods

2.1. Study population

From 1 November 2005 through 31 May 2006, 555 nasopharyngeal aspirates (NPAs) and 130 bronchoalveolar lavage (BAL) samples were collected prospectively from 426 (283 pediatric and 143 adult) patients admitted to hospital with an episode of acute respiratory tract infection (ARTI). Respiratory secretions were processed and aliquoted as previously reported (Sarasini et al., 2006). NPAs were examined for influenzavirus types A and B, parainfluenzavirus types 1–4, human respiratory syncytial virus, and human adenoviruses by both direct fluorescent antibody (DFA) staining and culture. In addition, human metapneumoviruses were detected by both MAbs (DFA and culture) and RT-PCR as previously reported (Gerna et al., 2006b; Percivalle et al., 2005). Furthermore, all four hCoVs were tested for by RT-PCR, while 229E and HKU1 strains were tested for in parallel by in-house developed MAbs, as detailed below.

2.2. DFA staining of NPA cells and cell cultures

DFA staining was applied to slides containing cells from either NPAs or cell cultures (48 h, p.i.), as reported (Gerna et al., 2006a; Rovida et al., 2005). In addition, in-house developed MAbs were used for identification of: (i) human metapneumovirus group and type (Gerna et al., 2006b; Percivalle et al., 2005); (ii) hCoV 229E strains (Gerna et al., 2006a); (iii) hCoV HKU1 strains. A HKU1-specific MAb was recently developed in our laboratory, and shown not to be cross-reactive with OC43-like strains or other hCoVs or respiratory viruses. In this study, it was used retrospectively for DFA detection of HKU1 strains in cell smears from NPAs. In addition, a commercially available MAb anti-OC43 (Chemicon), already shown to be poorly reactive with OC43-like strains (Gerna et al., 2006a), was used for DFA staining.

The following cell cultures were inoculated with NPAs: primary monkey kidney, Vero, mixtures of A549 and Mv1Lu (Huang and Turchek, 2000), LLC-MK2, MDCK, HRT-18, and HuH7 (Nakabayashi et al., 1982).

2.3. RT-PCR for hCoV detection

NPAs were tested for HKU1 by using: (i) a primer set proposed by Woo et al. (2005b), and relevant to gene *N* (nt 29163–29678); (ii) a primer set derived from the primer pair PanCoV-05 (Moës et al., 2005) and relevant to gene *1ab*, which was used for both result confirmation and phylogenetic analysis, as follows: HKU1-*1ab* for, 5′-ACTCAAATGAATTTAAAATATGC-3′; HKU1-*1ab* rev, 5′-TCACATTTAGGATAATCCCA-3′ (nt 15142–15392). In addition, hCoVs 229E, NL63, and OC43 were sought by using three sets of virus-specific primers relevant to gene *1ab*, used for both virus detection and phylogenetic analysis, as reported (Gerna et al., 2006a). Finally, RT-PCR assays for conventional respiratory viruses were developed to detect at least 10 input plasmid copies, as previously reported (Sarasini et al., 2006).

2.4. Phylogenetic analysis of HKU1 and hCoV strains

The 250 nt-long fragments of genes *1ab* of hCoVs 229E, NL63, OC43, and HKU1, following amplification with specific primers, were sequenced using the ABI PRISM 3100 automatic sequencer (Applied Biosystems, Foster City, CA). Viral sequences of different hCoV strains as well as reference strains were aligned with the ClustalW program version 1.6. The Phylips Megaversion 3.1 (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 sequences.

2.5. Statistical analysis

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

3. Results

3.1. Incidence of HKU1 and other hCoV infections in the hospitalised patient population

During the winter–spring season 2005–2006, 426 patients were admitted to the hospital with an ARTI episode. Of these, 48 (11.3%) had one or two episodes of hCoV infection for a total of 52 ARTI episodes during the entire season. HKU1 was associated with 10 ARTI episodes involving 10 patients, 6 presenting as a single infection and 4 as a coinfection. Other respiratory infections were associated with 229E in 21 episodes (10 infections and 11 coinfections), NL63 in 13 episodes (6 infections and 7 coinfections), hCoV OC43 in eight episodes (4 infections and 4 coinfections) (Table 1). Overall, numbers of single infection ($n = 26$) and coinfection ($n = 26$) episodes were identical.

3.2. Diagnosis of HKU1 and other hCoV infections

Diagnosis of HKU1 infections was performed prospectively using RT-PCR, and retrospectively (whenever back-up smears were available) by MAb staining. Of 15 NPAs found

Table 1
Diagnosis of hCoV infection in 426 patients admitted to hospital

Parameter	hCoV (%)				Total
	HKU1	OC43	229E	NL63	
No. positive/426 patients	10[a] (2.3)	8 (1.9)	20 (4.7)	11[a] (2.6)	48[a] (11.3)
No. positive/358 episodes	10 (2.8)	8 (2.2)	21 (5.9)	13 (3.6)	52 (14.5)
No. positive/685 samples	15 (2.2)	11 (1.6)	21 (3.1)	15 (2.2)	62 (9.1)
DFA	8/10 (80)	1/11 (9.1)	11/14 (78.6)	ND	20/35 (57.1)
SVC	0	1/11 (9.1)	7/21 (33.3)	0	8/32 (25.0)
RT-PCR	15/15 (100)	11/11 (100)	13/21 (61.9)	15/15 (100)	54/62 (87.1)
No. patients with a single infection	6[a] (60)	4 (50)	10 (47.6)	5 (45.4)	25 (51.0)
No. patients with a coinfection	4 (40)	4 (50)	10 (52.4)	6[a] (54.5)	24 (49.0)
Type of coinfection	HCMV ($n=1$)	FluA + Rhino ($n=1$)	hPIV ($n=2$)	Rhino ($n=1$)	
	Rhino ($n=1$)	Rhino ($n=2$)	hRSV ($n=2$)	hRSV ($n=3$)	
	hMPV-B ($n=1$)	FluA ($n=1$)	Rhino ($n=1$)	hMPV-B ($n=1$)	
	Rhino + hPIV3 ($n=1$)		Rhino + AdV ($n=1$)	hPIV + Rhino ($n=1$)	
			HCMV ($n=4$)		

DFA, direct fluorescent antibody; SVC, shell-vial culture; RT-PCR, reverse transcription-polymerase chain reaction; HCMV, human cytomegalovirus; Rhino, rhinovirus; hMPV-B, human metapneumovirus B; hPIV, human parainfluenzavirus; Flu A, influenzavirus type A; hRSV, human respiratory syncytial virus; AdV, adenovirus; ND, not done.

[a] One patient had an HKU1 infection and NL63 coinfection within 30 days from each other.

to be positive by RT-PCR, 10 were available as back-up cell smears for MAb staining. Eight of these (80%) were detected as positive by MAb. Two staining patterns were observed: one involved large syncytial formations patchy stained on the cell membrane, the other apparently interested the cytoplasm of singly infected cells (Fig. 1). In addition, repeated attempts to isolate HKU1 from HKU1-positive NPAs in different types of cell cultures were unsuccessful.

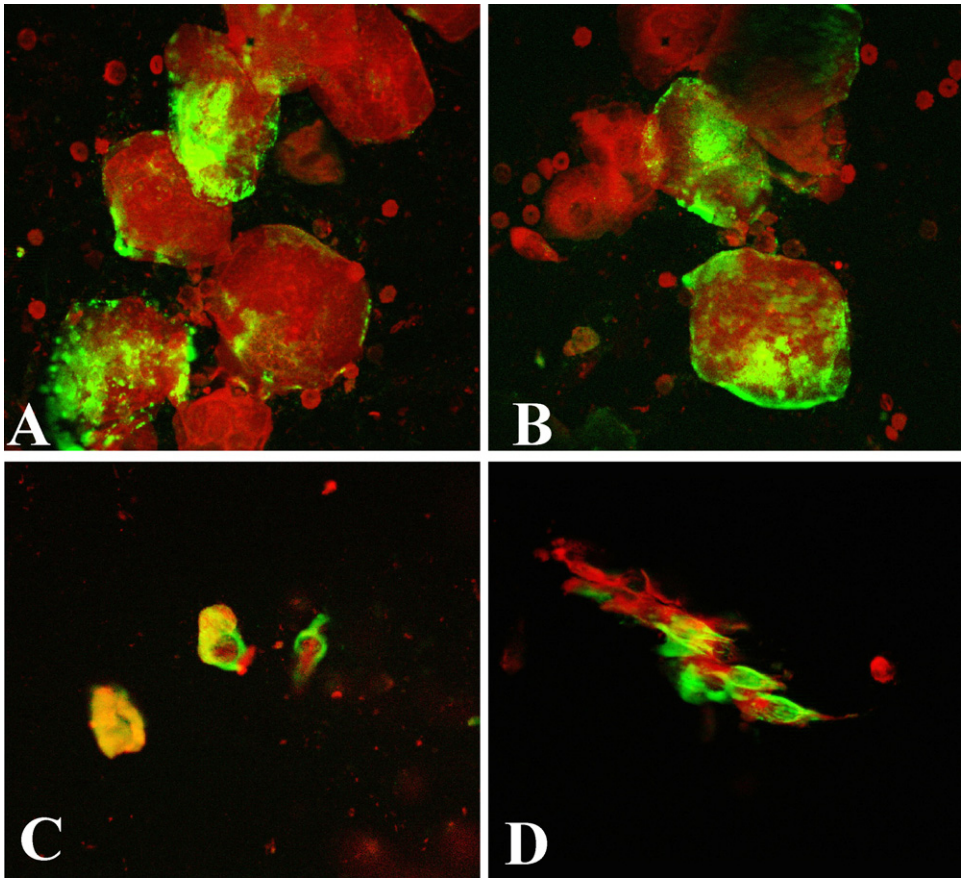

Fig. 1. Detection of HKU1-positve respiratory cells from nasopharyngeal aspirates by direct staining using HKU1-specific monoclonal antibody. (A and B) Membrane staining of large syncytial formations. (C and D) Cytoplasmic staining of respiratory cells.

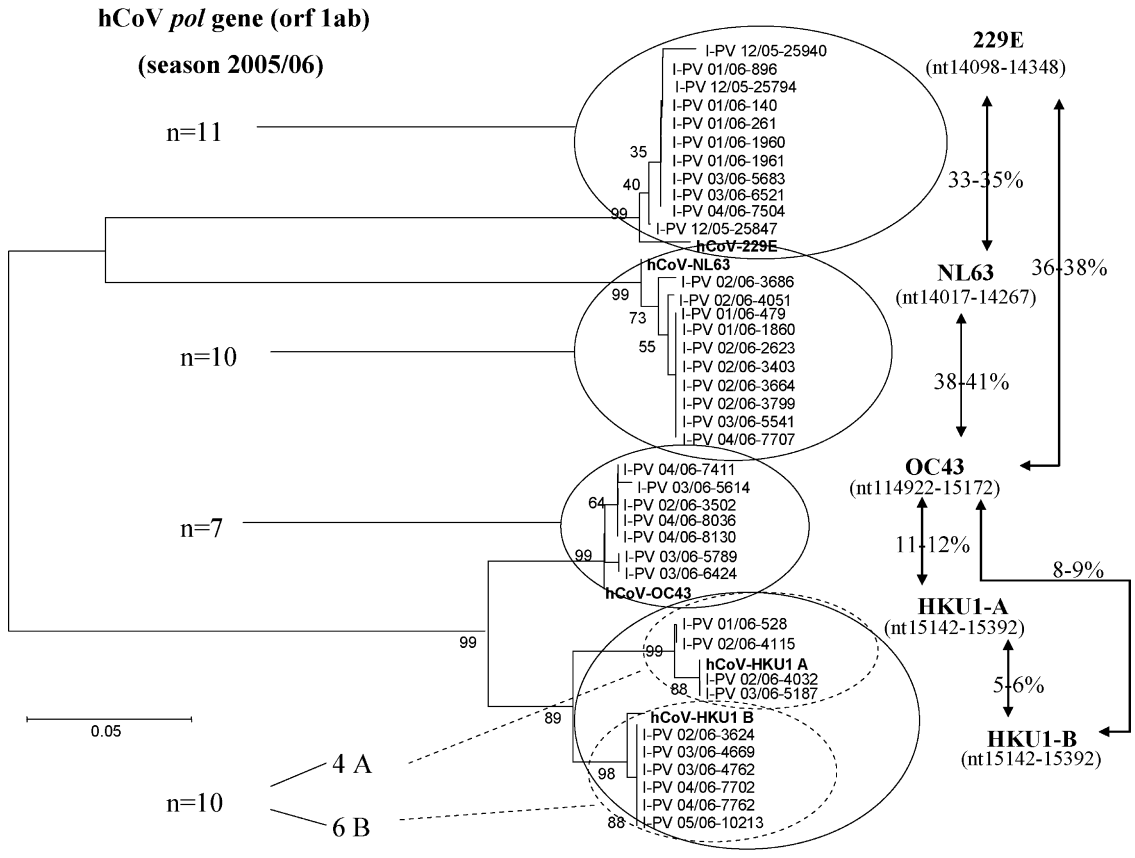

Fig. 2. Phylogenetic tree of HKU1 and other hCoV strains infecting different patients in the winter–spring season 2005–2006 based on sequencing of the indicated homologous fragment of ORF *1ab*. On the left, the number (*n*) of different strains examined is indicated, while on the right the genetic distance (range) between different hCoVs is reported. The two genotypes (A and B) of HKU1 are also indicated.

Interestingly, in an immunocompromised child (a 12-year-old boy with severe combined immune deficiency syndrome) NL63 in association with parainfluenzavirus and rhinovirus infections was followed by a HKU1 infection within one month. The longest duration of virus excretion observed in this study was 38 days in a 3-year-old child undergoing hematopoietic stem cell transplantation, and 25 days in a 1-year-old infant, who resolved HKU1 infection 5 days after admission.

The diagnosis of the other hCoV infections was usually based on RT-PCR (Table 1). However, diagnosis of 229E infections was done by both DFA in 11/14 samples (78.6%) and shell vial cultures (SVC) in 7/21 (33.3%) samples, using in house developed MAbs, while RT-PCR detected 229E in 13/21 (61.9%) samples.

3.3. Phylogenetic analysis of HKU1 and the other hCoV strains

As shown in Fig. 2, based on the comparison of a fragment of 250 nucleotides of the ORF *1ab*, HKU1 strains circulating in the winter–spring season in 2005–2006 in northern Italy belonged to either genotype A or B (range of genetic distance between the two subtypes 5–6%).

3.4. Epidemiology of HKU1 and hCoV infections

HKU1 infections were detected during the period, January through May 2006 (Fig. 3). As shown in Table 2, among the 10 HKU1-infected patients, 5 were children (one immunocompromised), and 5 were adults (all five immunocompromised). Overall, if the other hCoVs were considered (Table 3), 3/30 (10%) hCoV-infected children, and as many as 18/19 (94.7%) hCoV-infected adults were immunocompromised ($p < 0.01$).

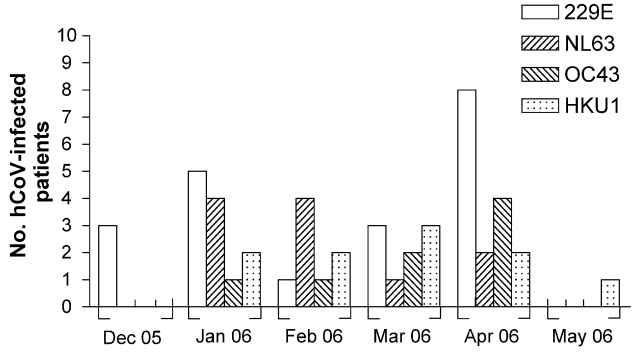

Fig. 3. Monthly distribution of hCoV-infected patients in the winter–spring season 2005–2006 in northern Italy.

Table 2
Characteristics of 10 patients infected by hCoV HKU1 in the winter–spring season 2005–2006

Parameter	Patient number									
	1	2	3	4	5	6	7	8	9	10
Sex	M	F	M	M	M	M	M	F	F	M
Age (year)	66	1	1	2	12	51	1	50	3	20
Underlying/concomitant disease	Multiple myeloma	Gastroenteritis	No	No	SCID	LTR	Pericarditis	LTR	HSCT, ALL	HSCT, AML
URTI	+	+	+	+	–	–	+	+	+	+
LRTI	–	–	–	–	+	+	–	–	–	–
HKU1 subtype	A	B	A	A	B	A	B	B	B	B
Clinical symptoms										
Fever	+	+	–	+	–	–	–	–	–	+
Otalgia	–	–	–	+	–	–	–	–	–	–
Cough	–	–	–	–	+	–	–	–	–	–
Rhinorrhea	+	+	+	+	+	–	+	+	+	+
Sore throat	+	+	–	+	–	–	–	–	–	–
Wheezing	–	–	–	–	+	–	–	–	–	–
Bronchiolitis	–	–	–	–	–	–	–	–	–	–
Pneumonia	–	–	–	–	–	–	–	–	–	–
Diarrhea	–	+	–	–	–	–	–	–	–	–
Coinfection	–	–	Rhino	–	–	HCMV	hMPV-B	–	hPIV-3 + Rhino	–

M, male; F, female; SCID, severe combined immune deficiency; LTR, lung transplant recipient; HSCT, hematopoietic stem cell transplant; ALL, acute lymphocytic leukemia; AML, acute myelocytic leukemia; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; HCMV, human cytomegalovirus; hMPV-B, human metapneumovirus B; hPIV-3, human parainfluenzavirus type 3; Rhino, rhinovirus.

3.5. Clinical syndromes and symptoms associated with HKU1 infections versus other hCoV infections

As reported in Table 2, in HKU1-infected patients the number of upper episodes greatly exceeded that of lower ARTI episodes (80% versus 20%). On the contrary, in OC43-infected patients, lower ARTI episodes were predominant (88% versus 12%) (Fig. 4). This difference was statistically significant ($p = 0.04$). On the other hand, the incidence of upper and lower ARTI episodes in patients infected by 229E and NL63 was comparable. In HKU1-infected patients the most common clinical symptoms were rhinorrhea (90%), followed by sore throat (30%) and fever (30%). No difference in the spectrum of clinical symptoms was observed between patients infected by HKU1 genotype A or B. Similarly, coinfections were associated with either genotype.

For the other hCoV infections, the predominant clinical symptoms are reported in Table 3.

Table 3
Clinical syndromes and symptoms caused by hCoV infections other than HKU1

Parameter	hCoV (%)		
	229E	NL63	OC43
No. patients	20	11	8
Sex (M/F)	11/9	7/4	3/5
Age	12 d to 71 y	1 m to 69 y	2 m to 45 y
Presence/absence of underlying/concomitant disease	11/9	5/6	3/5
URTI	11 (55)	3 (27)	1 (12)
LRTI	9 (45)	8 (73)	7 (88)
Clinical symptoms			
Fever	8 (40)	4 (36)	4 (50)
Otalgia	1 (5)	0	1 (12)
Cough	7 (35)	7 (64)	7 (88)
Rhinorrhea	10 (50)	4 (36)	4 (50)
Sore throat	3 (15)	2 (18)	2 (25)
Wheezing	7 (35)	5 (45)	3 (37)
Bronchiolitis	0	1 (9)	1 (12)
Pneumonia	4 (20)	3 (27)	4 (50)
Diarrhea	0	0	0
Coinfection	10 (50)	6 (55)	4 (50)

M, male; F, female; d, days; m, months; y, years; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection.

4. Discussion

Results of the present study indicate that hCoV HKU1 has been circulating among hospitalised patients in northern Italy during the winter–spring season 2005–2006 along with the other three known hCoVs. Thus, following initial and subsequent reports from Hong Kong (Lau et al., 2006; Woo et al., 2005a,b), HKU1 has been shown to circulate in several countries on different continents, such as Australia (Sloots et al., 2006), Switzerland (Garbino et al., 2006), France (Vabret et al., 2006), United States (Esper et al., 2006) and, now, Italy. The two recently discovered hCoVs, NL63 (Fouchier et al., 2004; Van der Hoek et al., 2004), belonging to group I, and HKU1 (Woo et al., 2005a), belonging to group II, along with the widespread use of molecular techniques allowing detection of conventional respiratory hCoVs 229E and OC43 (McIntosh et al., 1970), have contributed to the consistent

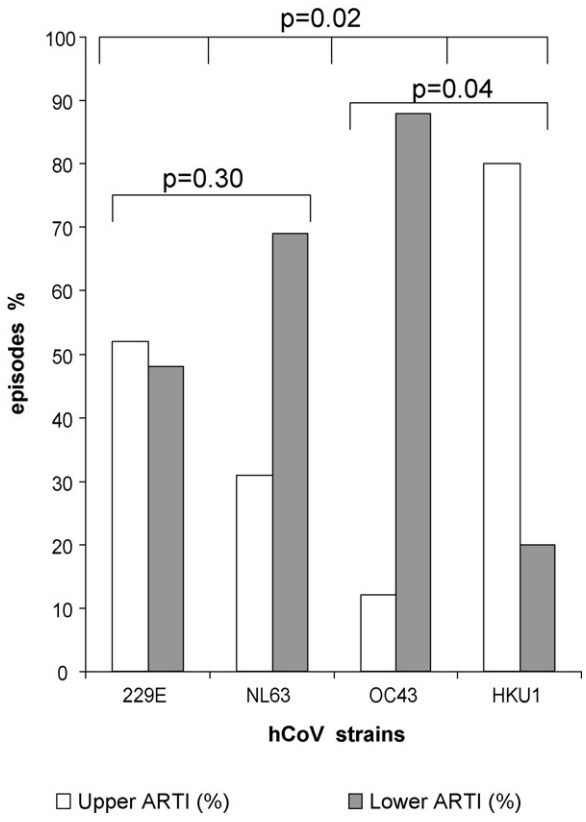

Fig. 4. Comparison of upper and lower acute respiratory tract infections (ARTI) associated with different hCoV infections. The incidence of upper ARTI was significantly higher in HKU1-infected patients compared to patients infected by OC43-like hCoVs.

increase in detection of hCoV-positive respiratory specimens. In this study, more than 10% of positive respiratory samples were due to hCoV infections. HKU1 infection interested mostly immunocompetent infants and young children, as well as immunocompromised adult patients.

The development of RT-PCR techniques has been critical for rapid detection of hCoV as well as other respiratory viruses in clinical samples. However, in parallel, MAbs have been developed for the rapid diagnosis of respiratory viral infections. In this study, we report results obtained with the first HKU1-specific MAb developed. The high specificity of the HKU1-specific MAb was shown by negative DFA results obtained in a large series of NPA smears positive for other respiratory viruses, including OC43, 229E and NL63, while the level of sensitivity appears promising for diagnosis of HKU1 infections. Similarly, a pool of three different 229E-specific MAbs for the prospective diagnosis of 229E infections appeared to be a valuable alternative to RT-PCR.

Two genotypes of HKU1 (A and B) have been reported (Lau et al., 2006; Sloots et al., 2006; Woo et al., 2005b). Correlation between HKU1 genotype and clinical syndrome or clinical symptoms was neither observed in our study, nor in previous studies. However, from a clinical standpoint, a major finding emerging from our study is that, within group II hCoVs, HKU1-infected patients suffered mostly from upper ARTI, while OC43-infected patients were mostly affected by lower ARTI episodes. This difference was statistically significant ($p = 0.04$), and was not observed between the two members of group I hCoVs. Neither bronchiolitis nor pneumonia were detected in our series of HKU1-infected patients. Following the initial report describing the association of HKU1 infection with pneumonia in two adult patients, an epidemiological and clinical study extended to the entire patient population in Hong Kong reported a predominance of upper ARTI, in agreement with the present study (Woo et al., 2005b).

In conclusion, HKU1 was circulating in the winter–spring season in northern Italy among hospitalised immunocompetent children and immunocompromised adult patients. Diagnosis was performed by RT-PCR, but MAb appeared to be a promising complement or alternative to RT-PCR. Phylogenetic analysis showed that genotypes A and B co-circulate. As with other hCoVs, the simultaneous presence in NPAs of other respiratory viruses in association with HKU1 was a frequent event. HKU1 infections were by far predominantly associated with upper respiratory tract syndromes, and the most common symptom was rhinorrhea. No association with bronchiolitis or pneumonia was observed.

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