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Short communication

Phylogenetic analysis of human coronavirus NL63 circulating in Italy

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Abstract

Background: Five known human coronaviruses infect the human respiratory tract: HCoV-OC43, HCoV-229E, SARS-CoV, HCoV-NL63 and HCoV-HKU1.

Objectives: To evaluate the prevalence of HCoV-NL63 in hospitalized adult patients and to perform molecular characterization of Italian strains.

Study Design: HCoV-NL63 was sought by RT-PCR in 510 consecutive lower respiratory tract (LRT) samples, collected from 433 Central-Southern Italy patients over a 1-year period. Phylogenetic analysis was performed by partial sequencing of S and ORF1a. Additional S sequences from Northern Italy were included in the phylogenetic trees.

Results: HCoV-NL63 was detected in 10 patients (2.0%) with symptomatic respiratory diseases, mainly during winter. Phylogenetic analysis indicated a certain degree of heterogeneity in Italian isolates. The ORF1a gene clustering in phylogenetic trees did not match with that of the S gene.

Conclusions: As observed by others, HCoV-NL63 is often associated with another virus. Phylogenetic characterization of HCoV-NL63 circulating in Italy indicates that this virus circulates as a mixture of variant strains, as observed in other countries.

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Keywords: Human coronaviruses; HCoV-NL63; Lower respiratory tract infection; Phylogenetic analysis

1. Introduction

Coronaviruses (CoVs) are large, enveloped RNA viruses of both medical and veterinary importance. Five human CoVs (HCoVs) are known to infect the human respiratory tract: HCoV-OC43, HCoV-229E (Vabret et al., 2001), SARS-CoV

(Peiris et al., 2003), HCoV-NL63 (Fouchier et al., 2004; van der Hoek et al., 2004) and HCoV-HKU1 (Woo et al., 2005).

HCoVs are divided into three distinct groups (van der Hoek et al., 2006; Virus taxonomy, VIIIth report of the ICTV): group 1, including HCoV-229E and HCoV-NL63; group 2, including HCoV-OC43, HCoV-HKU1 and SARS-CoV; group 3 CoVs, including mainly avian viruses.

HCoVs represent the second most frequent causes of common cold and upper respiratory tract infections, after rhinoviruses (Mäkelä et al., 1998). Particularly, HCoV-NL63 can be recovered from the lower respiratory tract (LRT) specimens of patients with pneumonia or LRT complications, mainly involving young infants, and immunocompromised persons (Gagneur et al., 2002; Garbino et al., 2006; Gerna et al., 2006; van der Hoek et al., 2006; Wu et al., 2008). HCoV-NL63 can be considered as a new important cause of

Abbreviations: CoV, coronavirus; SARS, severe acute respiratory syndrome; hCoV, human coronavirus; LRT, lower respiratory tract; S, spike; SP, sputum; BAL, bronchoalveolar lavage; EA, endotracheal aspirates; FLU, influenza virus; hMPV, human metapneumovirus; AdV, adenovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; HRV, Human rhinoviruses; BP, bronchopneumonia; COPD, chronic obstructive pulmonary disease; CAP, community-acquired pneumonia; ARF, acute respiratory failure; RF, respiratory failure.

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respiratory illness, although the available data mainly refer to pediatric populations (Arden et al., 2005; Bastien et al., 2005b; Boivin et al., 2005; Chiu et al., 2005; Ebihara et al., 2005; Kuypers et al., 2007; Moës et al., 2005).

Based on phylogenetic analysis of different genome regions, several subtypes, currently co-circulating in the human population, have been identified in France, Canada, Australia, Belgium, Netherlands, Sweden, China and Korean (Arden et al., 2005; Bastien et al., 2005a; Chiu et al., 2005; Han et al., 2007; Koetz et al., 2006; Moës et al., 2005; Vabret et al., 2005; van der Hoek et al., 2004).

We investigated the prevalence of HCoV-NL63 in samples from the LRT of hospitalized adult patients. Molecular characterization was performed by sequencing a fragment of the spike (S) and of ORF1a genes.

2. Methods

A total of 510 consecutive LRT samples, including 329 sputums (SP), 165 endotracheal aspirates (EA) and 16 bronchoalveolar lavages (BAL), were collected from 433 adult patients, admitted to three Italian hospitals, during April 2004–May 2005.

All specimens were screened with a molecular panel for detecting 12 respiratory viruses. Of the 510 samples tested, 220 (43.1%) were positive for one respiratory virus and 33 (6.5%) were positive for more than one virus. The detailed prevalence for each of these pathogens is published elsewhere (Minosse et al., 2008).

HCoV-NL63 was detected by nested RT-PCR, targeting a 237 bp fragment of the ORF1b (van der Hoek et al., 2004). As a positive control, a clinical sample positive for HCoV-NL63 by RT-PCR and confirmed by sequence analysis, was used. As negative controls, water and a nasopharyngeal swab

from an individual without respiratory symptoms were used in each analytical session. The sensitivity of our RT-PCR protocol, established by probit analysis of serial replicate dilutions of a plasmid containing the RT-PCR amplicon as the insert, was 1 copy/reaction, corresponding to 42 copies/ml of the starting sample. The RT-PCR assay was validated through participation in an external quality assessment (QCMD 2006 Rhinovirus & Coronavirus RNA EQA Pilot Study, Glasgow, UK), where HCoV-NL63 was detected in all positive samples included in the panel.

For phylogenetic analysis, two different genomic regions were amplified and sequenced: a 523 bp fragment of S gene and a 525 bp fragment of ORF1a, by using primers previously described (Bastien et al., 2005a; Vabret et al., 2005).

3. Results

The age of the 433 patients included in the study ranged from 15 to 93 years, mean (\pm S.D.): 56.3 (18.2) years, median (IQR): 57.0 (41.0–72.0) years. Temporal distribution of samples collected over the 1-year study period is shown in Fig. 1. HCoV-NL63 was detected in 10 (2.0%) of 510 samples. Of the 10 positive samples, 3 were co-infected with human rhinovirus (HRV) and 1 with adenovirus (see Table 1). Demographic and clinical characteristics of positive cases are reported in Table 1. The overall frequency of HCoV-NL63 infected patients was equally distributed between genders. The age of HCoV-NL63 positive patients ranged from 29 to 85 years (mean \pm S.D. was 60.1 [18.3] years; median IQR was 64.5 [41.5–76.0] years). All patients were symptomatic, and had clinically relevant respiratory diseases, including bronchopneumonia, chronic obstructive pulmonary disease, community-acquired pneumonia, and acute respiratory failure. Three patients were in the intensive care unit and

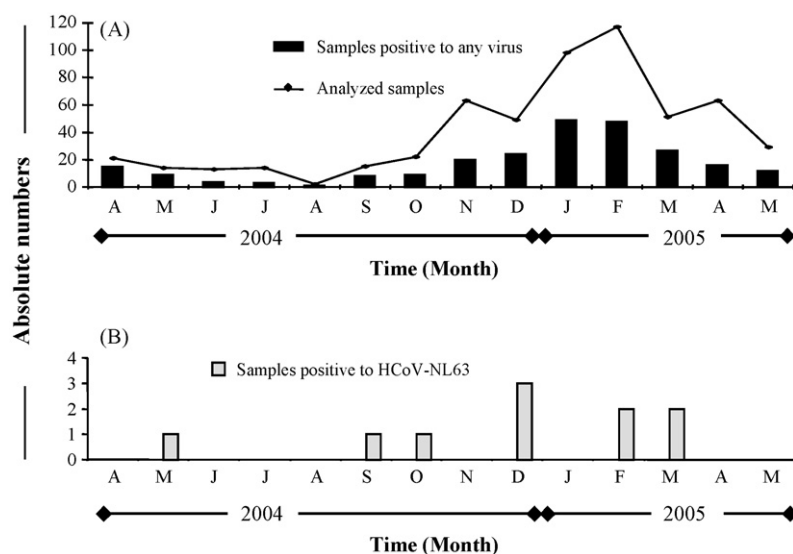


Fig. 1. Monthly distribution of positivity to respiratory viruses and to HCoV-NL63 during the period April 2004–May 2005. (A) Solid line: number of analyzed samples; bars: samples positive to any of 12 tested viruses. (B) Bars: samples positive to HCoV-NL63.

Table 1
Demographic and clinical characteristics of patients with HCoV-NL63 infection

Patient	Age	Sex	Sample date	Specimen*	Symptoms	Respiratory diagnosis**	Immunosuppression	Mechanical ventilation
R1	40	–	22 02 05	SP	Fever, dyspnea, productive cough	BP	Yes (HIV, stage B2)	No
R2	80	M	14 02 05	SP	Dyspnea, productive cough	COPD		No
R3 ^a	53	F	5 03 05	SP	Fever	BP		No
R4	62	F	19 05 04	SP	Dyspnea, chill, productive cough	COPD		No
R5	67	M	18 03 05	SP	Dyspnea, productive cough	COPD, pulmonary emphysema		No
R6 ^b	70	M	27 07 04	EA	Dyspnea, headache	CAP, ARF, COPD	Yes (solid tumour)	Yes
R7	29	M	18 10 04	EA	Dyspnea	ARF		Yes
R8	43	F	14 12 04	SP	Fever	BP		No
R9 ^b	72	F	17 12 04	SP	Fever, dyspnea, productive cough	RF, COPD		No
R10 ^b	85	M	27 12 04	EA	Fever, dyspnea, productive cough	ARF, acute pulmonary oedema,		Yes

* SP, sputum; EA, endotracheal aspirate.

** BP, Bronchopneumonia; COPD, Chronic Obstructive Pulmonary Disease; CAP, community-acquired pneumonia; ARF, acute respiratory failure; RF, respiratory failure.

^a Positive for AdV.

^b Positive for HRV.

needed mechanical ventilation; two patients were immunosuppressed, due to solid organ tumour or HIV infection.

Only 5 of 10 positive samples had sufficient material to allow for genetic characterization. Sequence analysis of the S and ORF1a gene fragments showed 97–99% similarity to the prototype HCoV-NL63 1988 and 2003 Netherland isolates (GenBank accession numbers: AY567487, AY518894). As expected, the phylogenetic tree built on ORF1a contained two clusters: 4 out of the 5 of Italian isolates were included in cluster 1, while one isolate (R3) had the characteristics of an outlier (Fig. 2). The phylogenetic analysis of S gene nucleotide sequences is shown in Fig. 3. Besides the 5 patients described in Fig. 2 (indicated as Southern-Central Italy), 7 additional patients identified in Pavia (indicated as Northern Italy) had S gene sequence data available, and were included in this analysis, together with 12 French isolates and 2 prototype isolates whose sequences were available in GenBank. From this tree it was not possible to identify any cluster with bootstrap values >80. However, one of the Italian isolates (PV5) was included in a subcluster with the French isolate AY994247 (bootstrap values = 78; Fig. 3), that was proposed to be an outlier by Vabret et al. (2005).

The phylogenetic analysis of the predicted amino acid sequences for the analyzed S and ORF1a gene fragments was consistent with that based on nucleotide sequences (not shown).

4. Discussion

Generally, HCoV-229E and HCoV-OC43 cause disease much like the common cold, but they have also been associated with more severe LRT conditions, especially in weakened patients (Kuypers et al., 2007; Pene et al., 2003; Vabret et al., 2001). The spectrum of clinical symptoms associated with HCoV-NL63 infection still needs to be determined.

In this study, we detected HCoV-NL63 in 2.0% of hospital-based adult patients with LRT diseases. This frequency is consistent with prevalence data from previous studies, ranging from 1.0% to 9.3% (Arden et al., 2005; Bastien et al., 2005a,b; Chiu et al., 2005; Ebihara et al., 2005; Kaiser et al., 2005; Kuypers et al., 2007; Moës et al., 2005; Suzuki et al., 2005; Vabret et al., 2005; van der Hoek et al., 2005). Due to the lack of matched healthy subjects for comparison, it is not possible to attribute any causal role to HCoV-NL63 in LRT diseases. Nevertheless, the following points can be derived from our results: (a) HCoV-NL63 was detected in LRT secretions of a small number of adult patients with clinically overt LRT disease; however, it is not possible to rule out that the virus detected could actually derive from contamination by upper respiratory tract secretions; (b) HCoV-NL63 is often combined with other respiratory viruses, in agreement with previous data (van der Hoek et al., 2006); in fact, 3 out of 10 HCoV-NL63 positive samples were co-infected with

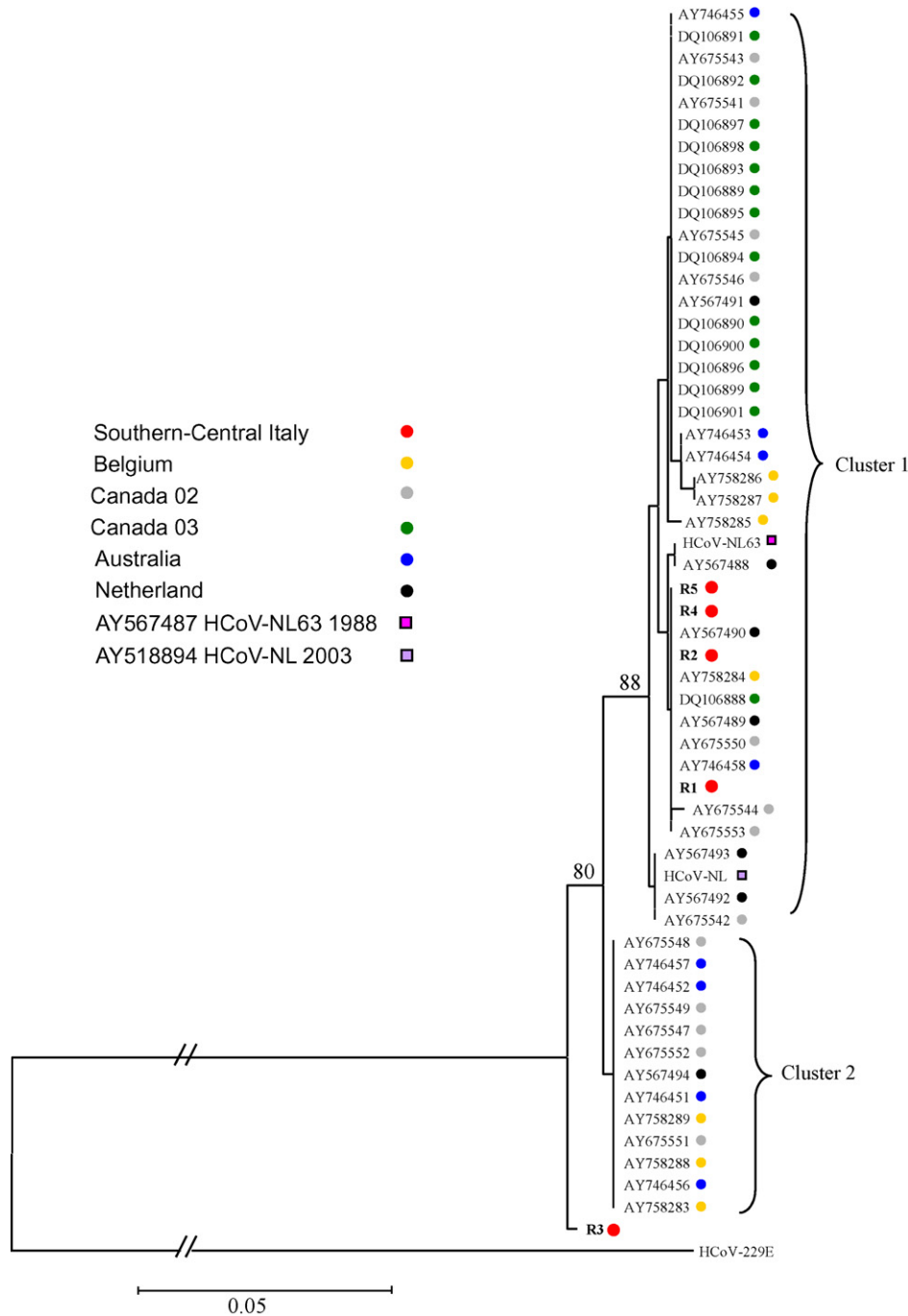


Fig. 2. Phylogenetic analysis of the partial ORF1a nucleotide sequences of HCoV-NL63 isolates from Southern-Central Italy. A region spanning nt position 5844 to 6169 of the reference strain AY518894 was analyzed. Italian isolates are in bold character. The nucleotide sequences of 2 prototype HCoV-NL63 sequences available in GenBank (AY567487 and AY518894, indicated as HCoV-NL63 1988 and HCoV-NL 2003, respectively) were included in the analysis. HCoV-229E was used as an outgroup. Bootstrap analysis with 500 replicates was performed to assess the significance of the nodes; values greater than 80% were considered significant. The ORF1a nucleotide sequences from the 5 strains of HCoV-NL63 from Southern-Central Italy have been lodged in the GenBank sequence database under accession no. EU030684, EU030685, EU125188 to EU1250190.

HRV and 1 with adenovirus; these findings raise the possibility that HCoV-NL63 may be only a bystander virus present in people with LRT disease due to other causative agents, rather than directly being involved in the disease aetiology; in addition, our numbers are too low to draw any conclusion on the role of immunosuppression on prevalence and

clinical presentation of HCoV-NL63 infection; (c) temporal distribution of HCoV-NL63 positive samples (Fig. 1) is consistent with winter seasonality of the infection, as previously reported (Arden et al., 2005; Bastien et al., 2005a; Ebihara et al., 2005; Moës et al., 2005; Vabret et al., 2005; van der Hoek et al., 2005).

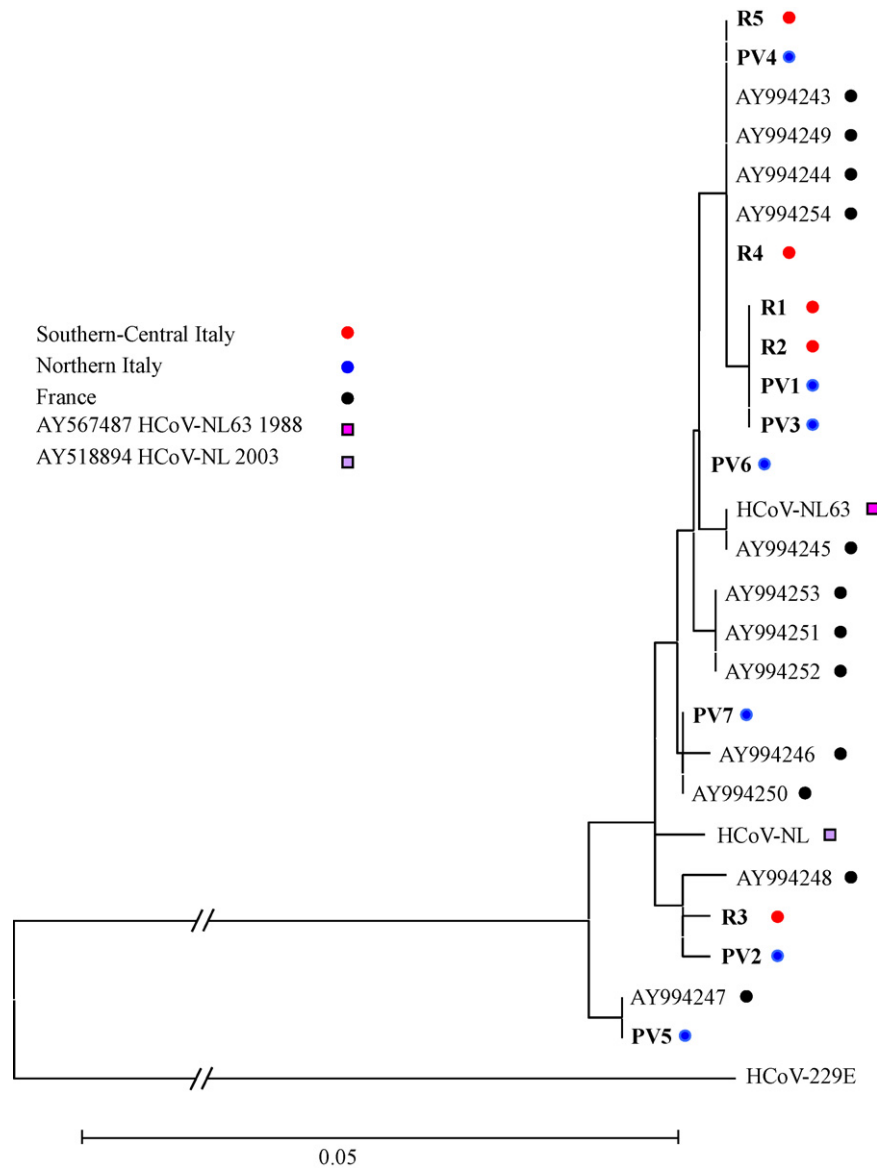


Fig. 3. Phylogenetic analysis of the S gene nucleotide sequences of HCoV-NL63 isolates from Southern-Central and Northern Italy. A region spanning nt position 22567 to 23039 of the reference strain AY518894 was analyzed. Italian isolates are in bold character. The nucleotide sequences of 2 prototype HCoV-NL63 sequences available in GenBank (AY567487 and AY518894, indicated as HCoV-NL63 1988 and HCoV-NL 2003, respectively) were included in the analysis. HCoV-229E was used as an outgroup. Bootstrap analysis with 500 replicates was performed to assess the significance of the nodes; values greater than 80% were considered significant. The S nucleotide sequences from the 5 strains of HCoV-NL63 from Southern-Central Italy have been lodged in the GenBank sequence database under accession no. EU025124 to EU025128.

Recent reports indicate that HCoV-NL63 has a worldwide distribution. Sequence data from Australia, Japan, Canada, France, Belgium, and the Netherlands indicate that this virus circulates as a mixture of variants, without a specific geographical segregation (van der Hoek et al., 2006). In fact, RNA viruses have an enormous potential to generate genetic diversity due to their high mutation rates. As a consequence, HCoV-NL63, like other RNA viruses, may exist as a dynamic distribution of closely related variants.

This report shows the phylogenetic characterization of HCoV-NL63 circulating in Italy. The results, based on two different genomic regions (a portion of the S gene and of

ORF1a), shows a certain degree of heterogeneity in Italian isolates, suggesting that different subtypes may be currently co-circulating, as observed in other countries (France, Netherlands, Australia, Canada, and Belgium), without the emergence of geographically distinct clusters (Moës et al., 2005; Vabret et al., 2005; van der Hoek et al., 2006).

In agreement with literature data, the clustering based on the ORF1a gene does not match with that of the S gene. As suggested in other reports, recombination occurring between different HCoV-NL63 isolates may account for this finding (Pyrç et al., 2007; Moës et al., 2005; van der Hoek et al., 2006).

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