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Co-Circulation of Genetically Distinct Human Metapneumovirus and Human Bocavirus Strains in Young Children With Respiratory Tract Infections in Italy

Alessandra Zappa,[1] Marta Canuti,[1] Elena Frati,[1] Elena Pariani,[1] Silvana Perin,[2] Maria Lorena Ruzza,[3] Claudio Farina,[2] Alberto Podestà,[3] Alessandro Zanetti,[1,4] Antonella Amendola,[1,4] and Elisabetta Tanzi[1,4]*

[1]*Department of Public Health-Microbiology-Virology, University of Milan, Milan, Italy*
[2]*Microbiology Unit, San Carlo Borromeo Hospital, Milan, Italy*
[3]*Pediatric Unit, San Carlo Borromeo Hospital, Milan, Italy*
[4]*Department of Health Sciences, University of Genoa, Genoa, Italy*

The discovery of human Metapneumovirus (hMPV) and human Bocavirus (hBoV) identified the etiological causes of several cases of acute respiratory tract infections in children. This report describes the molecular epidemiology of hMPV and hBoV infections observed following viral surveillance of children hospitalized for acute respiratory tract infections in Milan, Italy. Pharyngeal swabs were collected from 240 children $\leq$3 years of age (130 males, 110 females; median age, 5.0 months; IQR, 2.0–12.5 months) and tested for respiratory viruses, including hMPV and hBoV, by molecular methods. hMPV-RNA and hBoV-DNA positive samples were characterized molecularly and a phylogenetical analysis was performed. PCR analysis identified 131/240 (54.6%) samples positive for at least one virus. The frequency of hMPV and hBoV infections was similar (8.3% and 12.1%, respectively). Both infections were associated with lower respiratory tract infections: hMPV was present as a single infectious agent in 7.2% of children with bronchiolitis, hBoV was associated with 18.5% of pediatric pneumonias and identified frequently as a single etiological agent. Genetically distinct hMPV and hBoV strains were identified in children examined with respiratory tract infections. Phylogenetic analysis showed an increased prevalence of hMPV genotype A (A2b sublineage) compared to genotype B (80% vs. 20%, respectively) and of the hBoV genotype St2 compared to genotype St1 (71.4% vs. 28.6%, respectively). Interestingly, a shift in hMPV infections resulting from A2 strains has been observed in recent years. In addition, the occurrence of recombination events between two hBoV strains with a breakpoint located in the VP1/VP2 region was identified. ***J. Med. Virol. 83:156–164, 2011.***

KEY WORDS: pediatric respiratory infections; phylogenetic analysis; human Metapneumovirus; human Bocavirus; pediatric hospitalization; viral recombination

INTRODUCTION

Acute respiratory tract infections are an important cause of morbidity and mortality worldwide. These infections are ubiquitous and contagious, affecting repeatedly individuals of all ages, particularly children younger than 5 years of age [World Health Organization, 2009] and many infections have unknown etiologies. The recent discovery of the human Metapneumovirus (hMPV) in 2001 [van den Hoogen et al., 2001] and of the human Bocavirus (hBoV) in 2005 [Allander et al., 2005] identified the etiological causes of several respiratory infections. hMPV is a common cause of respiratory disease in children under 5 years old and often accounts for severe clinical manifestations, including bronchiolitis or pneumonia [Foulongne et al., 2006].

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*Correspondence to: Elisabetta Tanzi, PhD, Dipartimento di Sanità Pubblica-Microbiologia-Virologia, Università degli Studi di Milano, Via Pascal 36/38, 20133 Milano, Italy.
E-mail: elisabetta.tanzi@unimi.it

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hMPV is a single-stranded negative RNA virus of the *Paramyxoviridae* family, *Pneumovirinae* subfamily [van den Hoogen et al., 2001; Broor et al., 2008]. Molecular analysis identified two major genotypes (A and B) with genetic lineages A1, A2 and B1, B2, respectively [van den Hoogen et al., 2004]. Lineage A2 has been recently divided into A2a and A2b sublineages [Huck et al., 2006].

hBoV is detected frequently in the respiratory and gastrointestinal tracts, suggesting its pathologic involvement at both sites [Lindner et al., 2008; Schildgen et al., 2008]. hBoV belongs to the *Bocavirus* genus of the *Parvoviridae* family. It is a non-enveloped virus and contains a linear single-stranded DNA molecule. The two major hBoV genotypes correspond to the original St1 (Stockholm 1) and St2 (Stockholm 2) isolates [Allander et al., 2005].

This report describes the molecular epidemiology of hMPV and hBoV identified following surveillance for respiratory viruses in children hospitalized for acute respiratory tract infections in Milan, Italy. A detailed genetic analysis was carried out using the hMPV and hBoV sequences identified.

PATIENTS AND METHODS

Study Population

Pediatric patients (N = 240; 130 males and 110 females; median age, 5.0 months; interquartile range, 2.0–12.5 months) admitted to the San Carlo Borromeo Hospital in Milan between 2004 and 2008 with a diagnosis of acute respiratory infection were enrolled in the study. Lower respiratory tract infections (i.e., bronchitis, bronchiolitis, pneumonia, or asthma) were observed in 66.7% (95% CI: 60.5–72.4) of children. Bronchiolitis and bronchitis were the most frequent outcomes (60.6%, 95% CI: 52.9–68.0 and 37.5%, 95% CI: 30.3–45.2, respectively), pneumonia and asthma being less frequent (16.9%, 95% CI: 11.7–23.3 and 9.4%, 95% CI: 5.5–14.7).

At the time of hospital admission pharyngeal swabs were collected from each patient for the identification and characterization of respiratory viruses using Plain Swabs and ITM-RT (Copan Diagnostics, Murrieta, CA). Informed consent was obtained from parents of eligible children and children were divided into four age groups: ≤6 months (60%, 95% CI: 53.7–66.1), 7–12 months (15%, 95% CI: 10.9–19.9), 13–24 months (18.8%, 95% CI: 14.2–24.1), and 25–36 months (6.2%, 95% CI: 3.7–9.9).

The seasonal distribution of the samples collected is as follows: 35.4% (95% CI: 29.6–41.6) of samples were collected during autumn (October–December), 52.5% (95% CI: 46.2–55.8) during winter (January–March), and 12.1% (95% CI: 8.4–16.7) during spring (April–June).

Nucleic Acid Extraction and Amplification

Nucleic acids were extracted using the RNeasy Mini Kit and QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). For RNA virus detection cDNA was synthesized with $pd(N)_6$ random hexamer primers (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using a M-MLV reverse transcriptase (Invitrogen Tech-Line, Paisley, UK). Viral detection was performed by PCR for the identification and typing of both classic viral respiratory pathogens i.e., influenza virus A and B (FluA/B), parainfluenza viruses 1–4 (hPIV1-4), respiratory syncytial virus A and B (RSVA/B), coronavirus 229E and OC43 (hCoV229E/OC43), rhinovirus (hRV), adenovirus (hAdV), and hMPV and hBoV (Table IA).

Sequencing of Identified hMPV and hBoV Strain Sequences

Molecular characterization was performed by sequence analysis of a 450 bp amplicons of the fusion (F) gene (spanning nucleotides 653–1,102) for hMPV and an 850 bp amplicon of the VP1/VP2 region (spanning nucleotides 1,084–1,933) for hBoV (Table IB).

Following PCR amplification, amplicons were purified with NucleoSpin® Extract II (Macherey-Nagel GmbH & Co. KG, Duren, Germany). Nucleotide sequences were obtained by automated DNA sequencing on the ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The hMPV-F gene and hBoV-VP1/VP2 region nucleotide sequences obtained were deposited into GenBank.

Molecular Characterization and Phylogenetic Analysis

Multiple nucleotide sequences were aligned using ClustalX version 2.0 [Thompson et al., 1997]. Phylogenetic trees were constructed by means of the Neighbor-Joining method and Kimura 2-Parameter model using the MEGA package, version 4.0 [Kimura, 1980; Saitou and Nei, 1987; Tamura et al., 2007]. A bootstrap resampling analysis was performed (1,000 replicates) to test tree robustness [Felsenstein, 1985].

Mean evolutionary distances over all sequence pairs between and within groups were calculated using the Kimura 2-Parameter model in MEGA software [Kimura, 1980; Tamura et al., 2007]. To detect recombination events, a bootscanning analysis was performed using the Simplot software version 3.5.1 (Kimura 2-Parameter model; window size: 200 bp; step size: 20 bp; 1,000 bootstrap replicates, Neighbor-Joining tree analysis), [Lole et al., 1999]. Predicted protein sequences were obtained by means of BioEdit software [Hall, 1999].

Statistical Analysis

Data were expressed as median (interquartile range, IQR) and percentages (95% confidence intervals, 95% CI) as appropriate. Comparisons between groups were performed using the Chi-squared test or Fisher's exact test. A P-value <0.05 was considered statistically significant (two-tailed test). All statistical analyses were

TABLE I. Primers Used in This Study

Primer		Sequence 5′ → 3′	Gene	Amplicon size (bp)	Reference
Primers used for respiratory infection screening[a]					
hAdV nested-PCR					
I step	ADHEX1f	AACACCTAYGASTACATGAAC	Hexon	473	Modified from
	ADHEX1r	ATGGGGTARAGCATGTTRGC			[Avellón et al., 2001]
II step	ADHEX2f	AAYCCMTTYAACCACCACC		170	
	ADHEX2r	ACATCCTTYCKGAAGTTCCA			
hBoV nested-PCR					
I step	162f	CCAGCAAGTCCTCCAAACTCACCTGC	NP-1	399	[Manning et al., 2006]
	561r	GGAGCTTCAGGATTGGAAGCTCTGTG			
II step	188f	GAGCTCTGTAAGTACTATTAC		354	Modified from
	542r	CTCTGTGTTGACTGAATACAG			[Allander et al., 2005]
hCoV multiplex nested-PCR					
I step					
hCoV-229E	CORO9	GCACAGGACCCCATAAAGATGC	N	444	Modified from
	CORO10	GAGAACGAGCAAGACTCTTGGCA			[Dessau et al., 2001]
hCoV-OC43	CORO11	GCAATCCAGTAGTAGAGCGTCC		445	
	CORO12	TTGACATCAGCCTGGTTRCTAGCG			
II step					
hCoV-229E	CORO1	AGGCGCAAGAATTCAGAACCAGAG		308	
	CORO2	AGCAGGACTCTGATTACGAGAAGG			
hCoV-OC43	CORO5	CCCAAGCAAACTGCTACYTCTCAG		228	
	CORO7	GCAGCARTTGACGCTGGTTG			
Flu A/B multiplex real time PCR					
FluA					
	FLUAV_FOR	ACAAGACCAATCCTGTCACCTCT	M	108	[Valle et al., 2006]
	FLUAV_REV	GGCATTTTGGACAAAGCGTCTAC			
	FLUAV_TM	FAM-CAGTCCTCGCTCACTGGGCACGGT(p)-BHQ1			
FluB					
	FLUBV_FOR	CCAGTGGGACAACCAGA	NP	89	
	FLUBV_REV	TGCTCTTTCCGGGGATG			
	FLUBV_TM	JOE-ATCATCAGACCAGCAACCCTTGCC(p)-BHQ1			
hMPV nested-PCR					
I step	HMPV-M1f	GAGTCCTAYCTRGTAGACACC	M	247	This study
	HMPV-M1r	AGTACAGACATDGCWGCACC			
II step	HMPV-M2f	GACCWGCTGTTCAAGTTG		151	
	HMPV-M2r	YTGTGATGYAGCATACAGAG			
hPIV1-4 multiplex nested-PCR					
I step					
hPIV-1	PIP1+	CYTTAAATTCAGATATGTAT	HN	480	Modified from
	PIP1−	GATAAATARTWATTGATACG			[Echevarría et al., 1998]
hPIV-2	PIP2+	AACAATCTGCTGCAGSRTTT	HN	507	
	PIP2−	ATGTCAGAYAATGGRCAAAT			
hPIV-3	PIP3+	CTGTAAACTCAGACTTGGTA	HN	477	
	PIP3−	TTTARGCCYTTGTCAACAAC			
hPIV-4	PI4P+	CTGAACGGTTGCATTCAGGT	P	441	[Aguilar et al., 2000]
	PI4P−	TTGCATCAAGAATGAGTCCT			
II step					
hPIV-1	PIS1+	CCGGHAATYTCTCATACCTATG	HN	316	Modified from
	PIS1−	CYTTGGAGCGGAGTTGTTAWG			[Echevarría et al., 1998]
hPIV-2	PIS2+	CCATTTACCTAAGTGATGGAAT	HN	203	
	PIS2−	GCCCTGTTGTATTTGGAAGAGA			
hPIV-3	PIS3+	ACTCCCAARGTTGATGAAAGAT	HN	102	
	PIS3−	TAAATCTTGTTGTTGAGATTG			
hPIV-4	PI4S+	AAAGAATTAGGTGCAACCAGTC	P	244	[Aguilar et al., 2000]
	PI4S−	GTGTCTGATCCCATAAGCAGC			
hRV eminested-PCR					
I step	PR1f	CGGACACCCAAAGTAG	5'UTR	380	[Ireland et al., 1993]
	PR2r	GCACTTCTGTTTCCCC			
II step	PR1f	CGGACACCCAAAGTAG		202	
	PR3r	GGCAGCCACGCAGGCT			
RSV multiplex nested-PCR					
I step					
RSV-A/B	RSVAB1	ATGGAGYTGCYRATCCWCARRRCAARTGCAAT	F	737	[Coiras et al., 2003]
	RSVAB2	AGGTGTWGTTACACCTGCATTRACACTRAATTC			
II step					
RSV-A	RSVA3	TTATACACTCAACAATRCCAAAAAWACC		363	
	RSVA4	AAATTCCCTGGTAATCTCTAGTAGTCTGT			
RSV-B	RSVB3	ATCTTCCTAACTCTTGCTRTTAATGCATTG		611	
	RSVB4	GATGCGACAGCTCTGTTGATTTACTATG			
B. Primers used for the molecular characterization of hbov and hmpv strains[a]					
hBoV sequencing nested-PCR					
I step	VPs1	GCACTTCTGTATCAGATGCCTT	VP1/2	903	[Smuts and Hardie, 2006]
	VPas1	CGTGGTATGTAGGCGTGTAG			
II step	VPs2	CTTAGAACTGGTGAGAGCACTG CGTGGTATGTAGGCGTGTAG		850	
	VPas1				
hMPV sequencing nested-PCR					
I step	F-f	GTYAGCTTCAGTCAATTCAAC	F	532	Modified from
	MPVF1r	GTCTTCCTGTGCTAACTTTG			[Peret et al., 2002]
II step	MPVF1f	CTTTGGACTTAATGACAGATG		445	[Peret et al., 2002]
	F-r	CCTGTGCTGACTTTGCATG			Modified from [Huck et al., 2006]

[a]Primers used for the molecular surveillance for acute respiratory tract infections (A) and for the molecular characterization of hBoV and hMPV strains (B).

performed using the OpenEPI software, version 2.2.1 [Dean et al., 2009].

RESULTS

Viral Surveillance in Children With Acute Respiratory Tract Infections

One hundred and thirty-one out of 240 (54.6%, 95% CI: 48.3–60.8) samples collected from hospitalized children were PCR-positive for at least one respiratory virus. Forty-six samples (19.2%) were positive for RSVA/B, 11.7% for hAdV, 10% for hRV, 4.6% for Flu A/B, 2.9% for hCoV229E/OC43, and 0.4% for hPIV3. hMPV was detected in 8.3% and hBoV in 12.1% of samples examined (Table IIA). Mixed infections were detected in 22.1% of acute viral respiratory tract infections (Table IIB). Respiratory viruses were detected more frequently in children younger than 2 years of age (52.8%, 61.1%, and 66.7% for children ≤6, 7–12, and 13–24 months of age, respectively) in comparison to children 25–36 months of age (20%, $P < 0.05$, Table IIB).

Viral pathogens were associated with 60.6% (95% CI: 52.9–68.0) of lower respiratory-tract infections, particularly in bronchiolitis cases (60.8%, 95% CI: 50.9–70.2). The most frequently detected viruses were RSV (50.8%, 95% CI: 38.2–63.4), hBoV (20.3%, 95% CI: 11.5–32.0), hRV (16.9%, 95% CI: 8.9–28.1), hAdV (16.9%, 95% CI: 8.9–28.1), and hMPV (11.9%, 95% CI: 5.3–22.1).

Epidemiologic and Molecular Features of hMPV and hBoV Infections

hMPV or hBoV were detected in 37.4% (95% CI: 29.4–45.9) of respiratory virus-positive samples but only in children ≤24 months old. hMPV prevalence was similar in the first three age groups (range: 8.3–9.0%) while hBoV was identified more frequently in the 13–24 months old group compared to the other groups (24.4% vs. 9.7% and 11.1%, $P < 0.05$; Table IIA).

hMPV was identified more often as a single pathogen and only 30% of cases were associated with mixed infections ($P < 0.05$). hBoV was detected as a mixed infection in 58.6% (17/29) of cases, mainly in association with RSV (N = 10), hRV (N = 5) and hAdV (N = 4). Among these, two triple-infection (hBoV-RSV-hRV) cases were detected. The frequency of co-infections was similar in all age groups but different viruses were involved. The median age of hBoV co-infected children was 2.0 months (IQR: 1.5–50.0 months), for hBoV-RSV 8.5 months (IQR: 3.5–13.0 months), and 14 months for both hBoV-hRV and hBoV-hAdV (IQR: 11.5–17.0 months). No hMPV-hBoV co-infections were identified.

hMPV was found as the single source of infection in 7.2% (95% CI: 3.2–13.8) of bronchiolitis cases and part of co-infections in 11.1% of pneumonia (95% CI: 2.9–27.3) and 13.3% of asthma cases (95% CI: 2.3–37.5). hBoV was identified in 12.4% of bronchiolitis cases (95% CI: 6.9–20.1), mainly as a hBoV-RSV co-infection. In addition, a high prevalence of hBoV infections (18.5%, 95% CI: 7.1–36.4) were seen in association with pneumonia, most often as a single viral agent.

Phylogenetic Analysis of hMPV and hBoV Sequences

Ten out of 20 hMPV-positive samples were characterized at the molecular level and a phylogenetic tree of the hMPV F gene was generated (Fig. 1A). The identity range between tree sequences was 81.1–100%. Mean evolutionary distances for all sequence pairs are reported in Figure 1B. Phylogenetic analysis showed a prevalence of genotype A strains (8/10) compared to genotype B strains (2/10). In the context of genotype A, six hMPV-F sequences shared similarity with the A2b sublineage, one with A2a and one with the A1 group that presented with a single amino acid change (N358T). Genotype B sequences belonged exclusively to lineage B1.

Sixteen out of 29 hBoV-positive samples were analyzed and the hBoV VP1/VP2 region described (Fig. 2). All sequences shared a high degree of similarity with reference strains and other defined sequences (identity

TABLE II. Molecular Detection of Respiratory Viruses in Pharyngeal Swabs Collected From Hospitalized Children With Acute Respiratory Tract Infections and Children With Viral Respiratory Infections

	Total 240	0–6 months 144 (60; 53.7–66.1)	7–12 months 36 (15; 10.9–19.9)	13–24 months 45 (18.8; 14.2–24.1)	25–36 months 15 (6.3; 3.7–9.9)
A. Number (95% CI) of infections and age distribution children (N = 240) hospitalized with acute respiratory tract infections					
Viral agents					
RSV	46 (19.2; 14.6–24.5)	35 (24.3; 17.8–31.8)	4 (11.1; 3.6–24.7)	7 (15.5; 7.1–28.4)	0
hAdV	28 (11.7; 8.1–16.2)	13 (9; 5.1–14.6)	7 (19.4; 8.9–34.7)	7 (15.5; 7.1–28.4))	1 (6.7; 0.3–28.7)
hRV	24 (10; 6.7–14.3)	14 (9.7; 5.6–15.4)	5 (13.9; 5.3–28.1)	4 (8.9; 2.9–20.1)	1 (6.7; 0.3–28.7)
FLU	11 (4.6; 2.4–7.8)	5 (3.5; 1.3–7.5)	2 (5.5; 0.9–17.2)	4 (8.9; 2.9–20.1)	0
CoV	7 (2.9; 1.3–5.7)	3 (2.1; 0.5–5.6)	0	3 (6.7; 1.7–17.1)	1 (6.7; 0.3–28.7)
hPIV	1 (0.4; 0.02–2.0)	0	0	1 (2.2; 0.1–10.5)	0
hMPV	20 (8.3; 5.3–12.4)	13 (9; 5.1–14.6)	3 (8.3; 2.2–21)	4 (8.9; 2.9–20.1)	0
hBoV	29 (12; 18.4–16.7)	14 (9.7; 5.6–15.4)	4 (11.1; 3.6–24.7)	11 (24.4;13.6–38.5)[a]	0
B. Number (95% CI) of single and co-infections in patients (N = 131) in the context of age.					
Total	131 (54.6; 48.3–60.8)	76 (52.8; 44.6–60.8)[b]	22 (61.1; 44.6–75.9)[b]	30 (66.7; 52–79.2)[b]	3 (20; 5.4–45.4)
Single infections	102 (77.9; 70.2–84.4)	58 (76.3; 65.8–84.9)	19 (86.4; 67.2–96.4)	22 (73.3; 55.6–86.8)	3
Co-infections	29 (22.1; 15.6–29.9)	18 (23.7; 15.2–34.2)	3 (13.6; 3.6–32.8)	8 (26.7; 13.2–44.4)	0

[a] $P < 0.05$, prevalence of hBoV infections versus that observed in children 0–6 months and 7–12 months of age.
[b] $P < 0.05$, N (%) viral infections versus N (%) of children 25–36 months of age.

A

Within analysis		
Taxa	Mean Similarity	Similarity Range
A1	0.990	0.974-1
A2a	0.989	0.984-0.994
A2b	0.967	0.954-0.984
B1	0.988	0.984-0.989
B2	0.988	0.979-0.994
Between Analysis		
Taxa	Mean Similarity	Similarity Range
A1 vs A2a	0.921	0.913-0.938
A1 vs A2b	0.915	0.897-0.933
A2a vs A2b	0.972	0.954-0.984
B1 vs B2	0.917	0.913-0.938

B

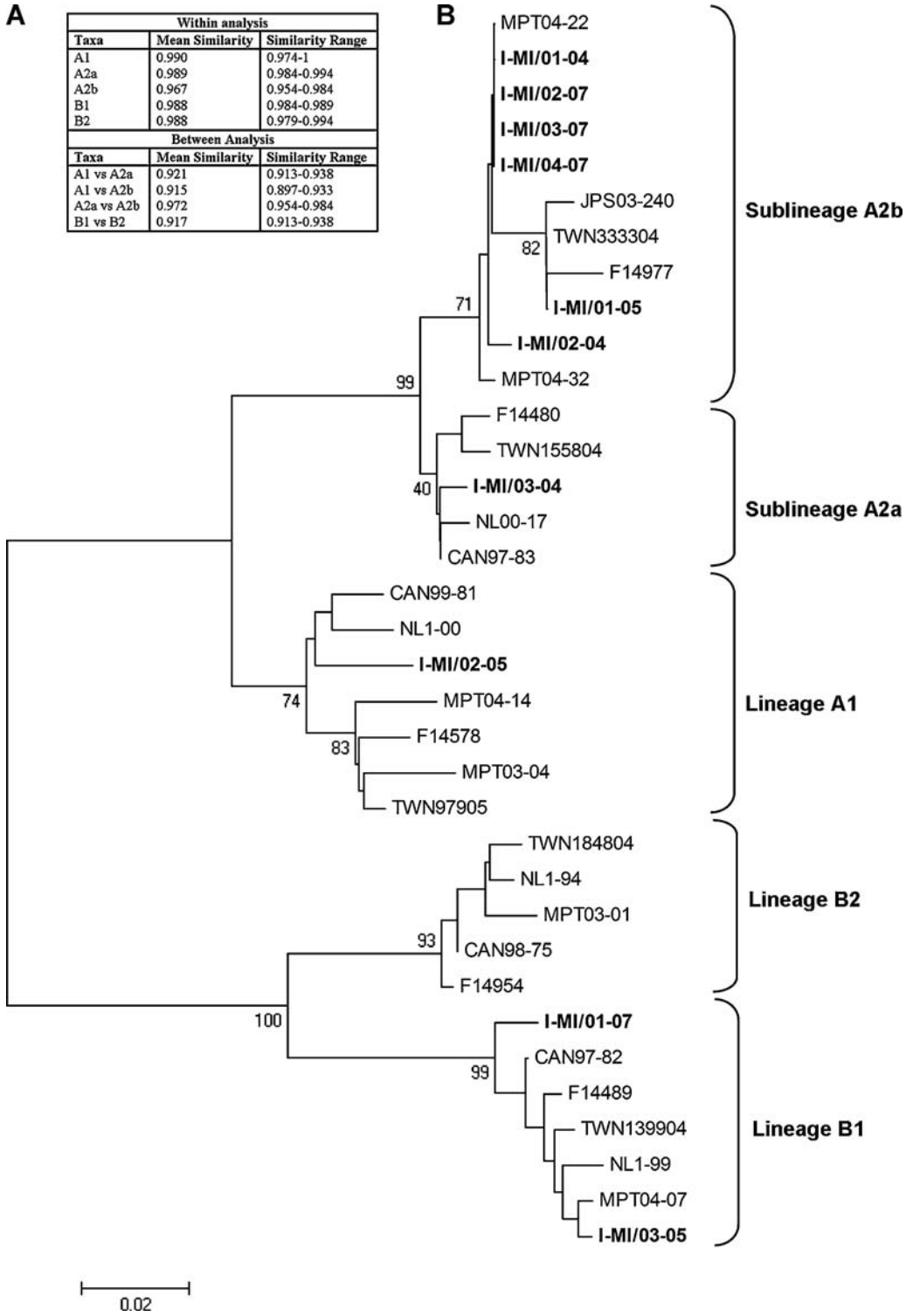

Fig. 1. Genetic analysis of hMPV. **A**: Phylogenetic tree of the Italian I-MI hMPV sequences (**boldface**) demonstrating the phylogenetic relationship of the I-MI sequences based on partial F gene nucleotide sequences. Bootstrap values (in addition to A1, A2a, A2b, B1, and B2 group/sublineages) are indicated. Reference sequences correspond to the following GenBank accession numbers: A1) AF371337, AY145294, DQ453017, AJ867506, AJ867520, EF612444; A2a) AY304360, AY145296, DQ453014, EF612451; A2b) AY530095, DQ453021, AJ867528, AJ867538, EF612457; B1) AY304361, AY145294, DQ453015, AJ867513, EF612440; B2) AY304362, AY145289, DQ452995, AJ867503, EF612441. **B**: Evolutionary mean distances over all sequence pairs within and between groups.

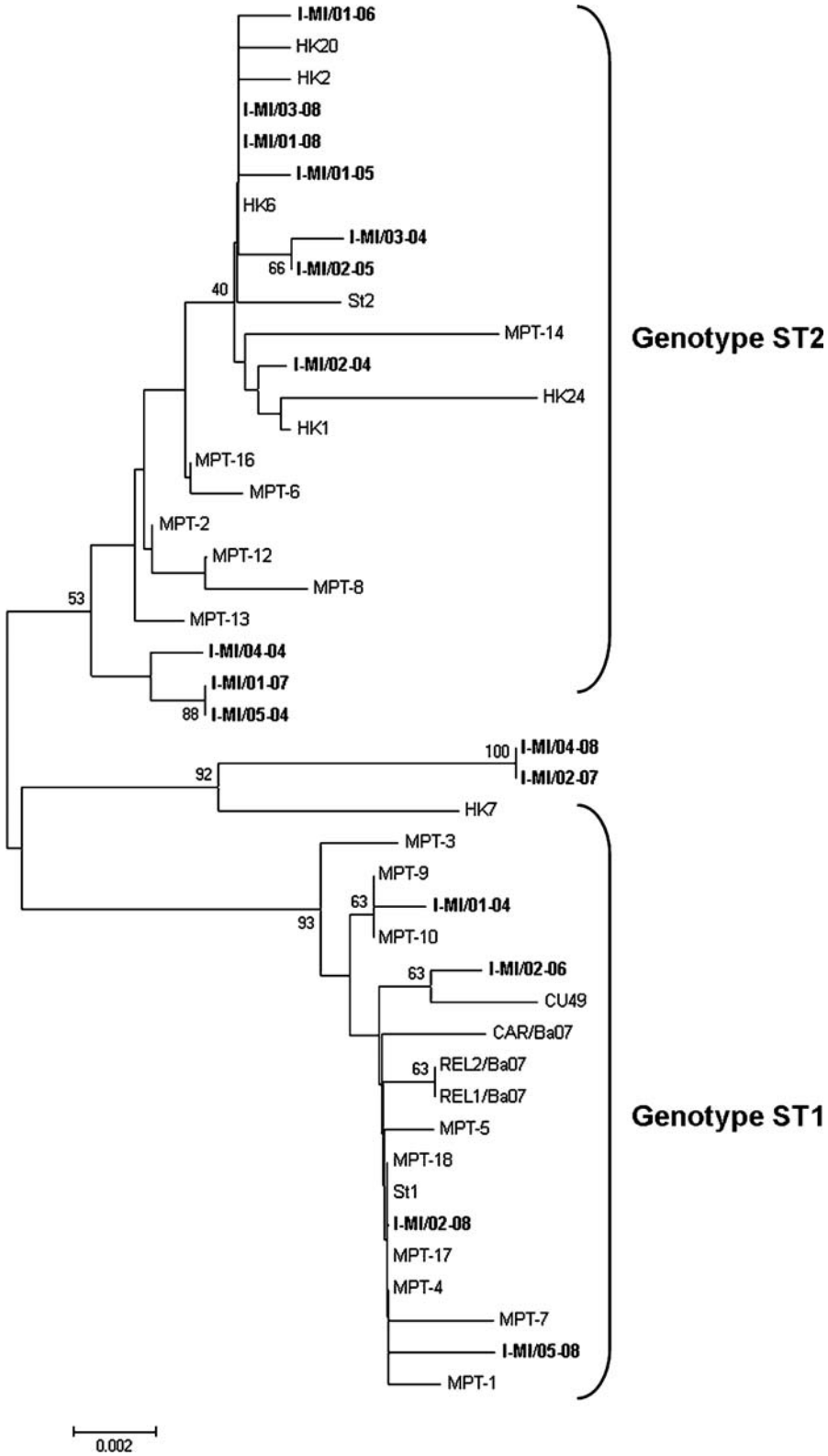

Fig. 2. Genetic analysis of hBoV sequences. Phylogenetic tree of the Italian I-MI human Bocavirus sequences (**boldface**) based on partial VP1/VP2 region nucleotide sequences. Bootstrap values (in addition to St1 and St2 genotype clusters) are indicated. Reference sequences correspond to the following GenBank accession numbers: St-1) DQ000497, AM160609, AM160611–AM160613, AM160615, AM689298, AM689299, AM689306, AM689307, EU069434, EU069436, EU069437, EF203921; St-2) DQ000495, AM160610, AM160614, AM689297, AM689301-AM689303, AM689305, EF450717, EF450718, EF450722, EF450723, EF450736, EF450740.

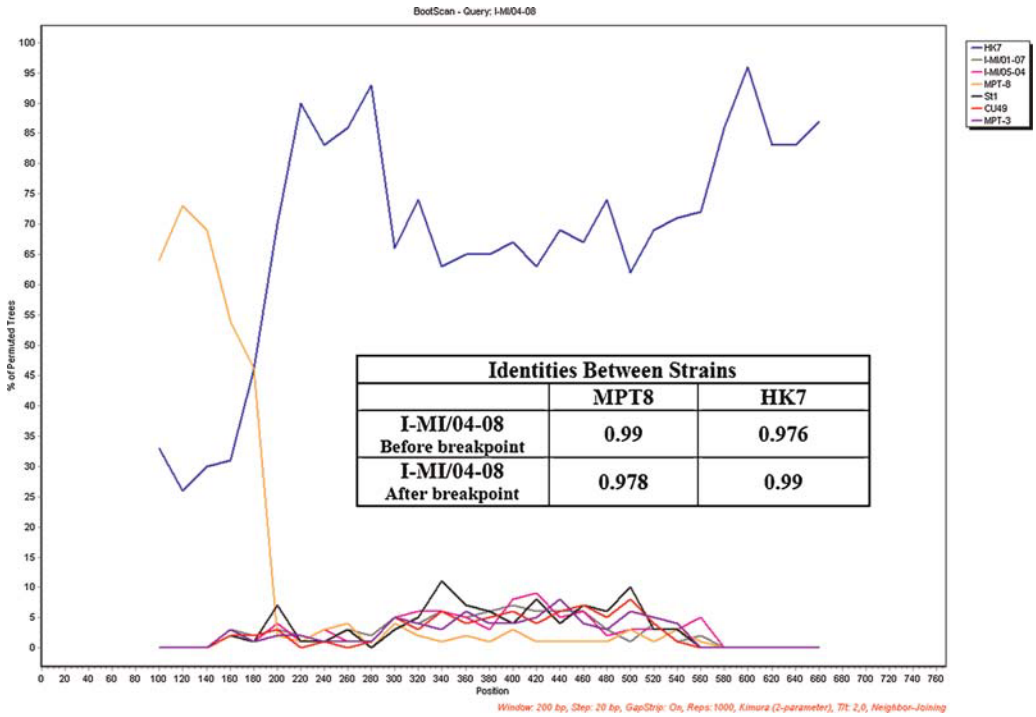

Fig. 3. Recombination recognition of the Italian I-MI/04-08 sequence: bootscan analysis and identities values between the recombinant sequence and the two reference sequence (MPT8 and HK7) before and after the breakpoint (inset).

range: 97.3–100%). Most sequences (14/16) were genetically similar to genotypes St1 (4/14) and St2 (10/14). A third cluster (bootstrap value: 92), including two Italian sequences (I-MI/04-08 and I-MI/02-07, identity 100%) and the Asian HK7 isolate, were identified between the two main branches. The boot scanning analysis revealed a recombination event with a breakpoint located at nucleotide position 1,302 of the VP1/VP2 region (Fig. 3). The 5′ region of these two Italian sequences was more similar to the MPT-8 strain (identity 99% vs. 97.6%) and the 3′ portion more similar to the HK7 strain (identity 97.8% vs. 99%; Fig. 3 inset).

Six amino acid substitutions were found: G415S, N474S, F540Y, N546H, A555T, and G566R. Mutations G415S, F540Y, and N546H were identified in the I-MI/04-08 and I-MI/02-07 sequences.

DISCUSSION

A molecular-epidemiologic survey of acute respiratory infections was conducted in children younger than 3 years old hospitalized between 2004 and 2008 in Milan, Italy. Both hospitalization and positive viral detection were more frequent in children <2 years of age compared to older patients.

The prevalence of both hMPV and hBoV infections (8.3% and 12.1%, respectively) were similar to rates reported previously [Maggi et al., 2003; Bastien et al., 2006; Foulongne et al., 2006; Allander et al., 2007]. Both hMPV and hBoV infections were associated with lower respiratory tract infections. hMPV was present as a single infectious agent in 7.2% of children aged ≤12 months with bronchiolitis. The involvement of hBoV at the onset of bronchiolitis was difficult to asses due to the frequent association with other viruses, particularly RSV. In this study, hBoV was associated with 18.5% of pediatric pneumonias and frequently identified as a single agent.

Twenty percent of hospitalized children had dual or triple co-infections and hBoV was involved in approximately 60% of mixed infections. The extended persistence of hBoV in airways (up to 7 months) increased patient's chances of acquiring a co-infection, explaining the high frequency of co-infections with this virus [Brieu et al., 2008; Lindner et al., 2008; von Linstow et al., 2008]. The seasonal distribution of hBoV, with a peak incidence between December and January, resembled that observed for RSV, hRV, and hAdV. This suggested that the overlapping distribution of these viruses during the seasons could have facilitated hBoV co-infections. The epidemiologic model of hMPV infections was distinct from many other respiratory viruses, with peak incidence rates in late spring, mostly between March and April. This observation may account for the absence of hMPV-hBoV co-infections.

Co-circulation of both hMPV genotypes was observed, with predominance of genotype A (particularly the A2b sublineage), supporting previous reports demonstrating an increase in A2 strain infections in recent years [Boivin et al., 2004; Wang et al., 2008; Herbert et al., 2005]. Guant et al. [Gaunt et al., 2006] suggested a further potential back-shift to genotype B in Scotland

during the winter season of 2007/2008. Unfortunately, no samples from this study were available for sequencing to confirm this hypothesis. As suggested by Herbert et al. [2005], the complex circulation pattern of hMPV may be the reason this strain can escape pre-existing host immune responses, suggesting that additional molecular surveillance studies need to be carried out to confirm genotype B back shift circulation and to clarify the existence of a seasonal distribution of hMPV strains. Phylogenetic analyses demonstrated that both hBoV genotypes co-circulated during the study period and that genotype St2 predominated. Interestingly, evidence of genetic recombination between the two hBoV strains (with a breakpoint site in the VP1/VP2 region) was found. The two mosaic sequences (identity: 100%) were identified in two different years (2007 and 2008), indicating that this variant had been circulating in the population since at least 2007. Genetic recombination has been reported to occur in Parvoviruses [Shackelton et al., 2007; Ohshima and Mochizuki, 2009] and in different hBoV species [Arthur et al., 2009; Kapoor et al., 2009, 2010]. To date, only Lin et al. [2009] have reported an intra-species recombination event involving hBoV1, with a breakpoint located in the NS1 gene. Taken together, these data suggested that intra-species recombination could occur in hBoV1 at different positions of its genome, playing a major role in the evolutionary history of this virus. Further studies will be necessary to identify breakpoint patterns in these viruses and to identify their potential association with viral spread among populations.

In conclusion, this study showed that both hMPV and hBoV circulated in Italy and were involved in the pathogenesis of lower respiratory tract diseases in children. Data presented in this report provided evidence for both a shift in the incidence of hMPV A2 strain infections in recent years and the occurrence of recombination events in hBoV strains. Therefore, continuous molecular surveillance is important for the detection of new viral strains as a means of detecting their spread and assessing their epidemiological impact.

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