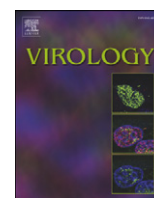Contents lists available at ScienceDirect

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Human metapneumovirus and human bocavirus associated with respiratory infection in Apulian population

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ABSTRACT

We have studied the occurrence of hBoV, hMPV and InfA-B in an Apulian population with respiratory tract infections. During influenza season 2008–2009, 116 oropharingeal swabs were collected from patients affected by Influenza-Like Illness (ILI). The PCR products of hMPV M and HBoV NP-1 genes were sequenced. 78 out of 116 samples were positive for at least one respiratory virus; hBoV was detected in 53, hMPV in 22 and InfA-B in 41 out of 116 swabs. A high rate of hBoV infection in adult (18.9%) and elderly (26.4%) subjects was found. The co-infection rate was higher for hMPV (18/22 cases, 81.8%) compared to hBoV (26/53 cases, 49.1%), and InfA-B (25/41 cases, 61.0%). Co-infections were common in children. hBoV positive samples shared a high level of genetic similarity with the hBoV1 genotype, and hMPV positive samples clustered with A2 subgroup. Our results suggest that hBoV and hMPV play a role in ILI.

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Introduction

Acute respiratory tract infections (ARTI) associate with significant morbidity worldwide, especially in infants and children. Viruses are a leading cause of ARTI, showing a relevant epidemiological variability that depends on climate and region (Armstrong et al., 1999). Etiology is still undefined in a significant proportion of ARTI (Monto, 1994; Henrickson et al., 2004).

The impact of ARTI on health and disease is particularly difficult to address. Similar symptoms can be generated by a variety of agents [influenza virus (Inf), human adenovirus, respiratory syncytial virus (RSV)]. Moreover, respiratory tract samples from healthy subjects or from patients without respiratory symptoms are rarely available.

Viruses, such as Inf, RSV, human adenovirus and coronaviruses NL63 and HKU1 are known to be responsible for the most of respiratory tract infections (Kesson, 2007). In a significant proportion of respiratory tract disease, however, no pathogens are identified.

During the past few years, new respiratory viruses have been discovered. Today, human metapneumovirus (hMPV), family *Paramyxoviridae*, is considered one of the most relevant agents of ARTI and it has been reported worldwide (Van den Hoogen et al., 2001; Debiaggi et al., 2006; Kahn, 2006; Sarasini et al., 2006; Boivin et al., 2007).

The hMPV has been associated with ARTI in all age groups, with more severe disease occurring in young children, elderly individuals and immunocompromised hosts. The virus causes a variety of clinical syndromes in children that are typical of the paramyxoviruses, including upper and lower respiratory tract illness. The clinical characteristics of hMPV infections are not distinctive.

It has been reported that the epidemiological and clinical characteristics of hMPV closely resemble those of RSV (Boivin et al., 2002; Van den Hoogen et al., 2004).

Human bocavirus (hBoV) is a novel parvovirus, family *Parvoviridae*, often associated with respiratory tract disease in adult and pediatric patients worldwide. hBoV has been detected in serum and fecal samples, predominantly in children under the age of 2 years with respiratory infections (Lindner and Modrow, 2008). By means of both genetic organization and sequence homology analyses relations to members of the two genuses, bovine parvovirus and canine minute virus, could be established. Consequently, hBoV has been classified as a bocavirus (Allander et al., 2005).

Recently, three viruses closely related to hBoV have been reported, provisionally named human bocavirus 2 (hBoV2), human bocavirus 3 (hBoV3), and human bocavirus 4 (hBoV4). It remains unclear whether hBoV1, hBoV2, hBoV3, and hBoV4 represent unique viral entities or distant genotypes of a single virus. However, it is a fact that hBoV1 infection has been linked with mild to severe lower respiratory tract infection, but that it has also been detected at low frequency in stool samples, while hBoV2, hBoV3 and hBoV4 have been detected in

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gastrointestinal samples (Kapoor et al., 2009; Arthur et al., 2009; Kapoor et al., 2010).

The impact of hMPV and of hBoV on the global epidemiology of ARTI is still unclear. In fact, most reports are retrospective (Peret et al., 2002; Stockton et al., 2002) or predominantly focus on hospitalized children and on children affected by ARTI (Maggi et al., 2003; Wilkesmann et al., 2006; Wolf et al., 2006).

Epidemiological and clinical features of Influenza-like Illness (ILI) in general population need to be highlighted and ongoing investigations are necessary. We have studied the occurrence of hBoV, hMPV and Inf A and Inf B (InfA-B) in an Apulian population with respiratory tract infections.

Results

Prevalence of respiratory viruses

One hundred and sixteen patients (51.3% males, range 0–93 years) with ILI were enrolled in the study.

The oro-pharyngeal swabs were collected from 52 children (aged 0–14 years), 22 youths and adults (15–64 years) and 42 elderly subjects ($\geq$64 years).

Seventy eight out of 116 samples (67.2%) analyzed by the molecular method were positive for one or more respiratory viruses; particularly, InfA-B was detected in 41 (35.3%), hMPV in 22 (19.0%) and hBoV in 53 (45.7%) out of 116 oropharingeal swabs.

Single infection was found in 40.5% (n = 47) and double infection in 20.7% (n = 24) of the subjects (Table 2).

The percentage of co-infections was higher for hMPV (18/22 cases, 81.8%; P<0.05 vs. hBoV) compared to hBoV (26/53 cases, 49.1%), and to InfA-B (25/41 cases, 61.0%). HBoV was detected mainly in association with InfA-B (72.2%; 13/18). Seven triple infections (6%) were detected.

Age distribution

Viruses were detected only for the period between December and March with a peak on January, with a similar trend for the three viruses.

hMPV exhibited a lower level of viral circulation than the other two viruses (Fig. 1). The distribution of viruses in the age groups showed an increased presence of infections in children (0–14 age group) followed by elderly. In the 0–14 and 15–64 age groups, infections due to hBoV were significantly more represented than those due to hMPV (P<0.05). In the elderly ($\geq$64 years), infections due to hMPV were significantly lower than those due to InfA-B (P<0.05) and to hBoV (P<0.01) (Table 2).

Only for hMPV, the distribution was similar in the 15–64 and $\geq$64 age group. All triple infections were found in children (0–14 years), while 54.2% of double infections were among children, 25.0% in the 15–64 age group and the remaining 20.8% in elderly.

Phylogenetic analysis

The hMPV M gene and hBoV NP1 gene from 13 samples were amplified and sequenced. The phylogenetic analysis of the hBoV nucleotide sequences revealed that all hBoV strains clustered with the hBoV1 (NC_007455) genotype (identity range 75.0–100%) (Fig. 2). The hMPV strains belonged to the A2 subgroup (identity range 74.0–97.0%) (Fig. 3).

Discussion

We have designed a prospective study with the aim of describing the virological impact of three different respiratory viruses (hMPV, hBoV and InfA-B virus) in subjects with ILI in an Apulian population.

At least one out of the three viruses was identified in 67.2% of patients, confirming the widespread distribution in the population (Calvo et al., 2010). hBoV was the most prevalent virus (45.7% of cases), followed by InfA-B virus (35.3%) and hMPV (19.0%). Thus, our results are consistent with data from other groups that attested the presence of at least one of the potential viral pathogens in a 34% to 95% of cases (Allander et al., 2007; Khetsuriani et al., 2007; Kusel et al., 2007; Gendrel et al., 2007; García-García et al., 2010).

The viruses started spreading during late autumn, with a peak on January; the prevalence of hMPV was lower than the other two

Table 1
Flu A/B, hBoV and hMPV primers and thermal profile used in viral genome amplification.

Detection method	Gene	Primer sequences 5'-3'	Location	Thermal profile
hBoV				
Nested-PCR	NP-1	Boca-forward (B1f)		95 °C 5' (×1)
		CCAGCAAGTCCTCCAAACTCACCTGC	162–187	95 °C 30″, 63 °C 30″
		Boca-reverse (B1r)		72 °C 30″ (×35)
		GGAGCTTCAGGATTGGAAGCTCTGTG	536–561	72 °C 7' (×1)
Nested-PCR	NP-1	Boca-forward (B2f)		95 °C 5' (×1)
		GAGCTCTGTAAGTACTATTAC	188–208	95 °C 30″, 54 °C 30″
		Boca-reverse (B2r)		72 °C 30″ (×35)
		CTCTGTGTTGACTGAATACAG	522–542	72 °C 7' (×1)
hMPV				
Nested-PCR	M	Meta-forward (M1f)		95 °C 5' (×1)
		GAGTCCTAYCTRGTAGACACC3	4–24	95 °C 1', 55 °C 1'
		Meta-reverse (M1r)		72 °C 1' (×25)
		AGTACAGACATDGCWGCACC	232–251	72 °C 7' (×1)
Nested-PCR	M	Meta- forward (M2f)		95 °C 5' (×1)
		CAGCWGCTGTTCAAGTTG	44–61	95 °C 1', 53 °C 1'
		Meta-reverse (M2r)		72 °C 1' (×30)
		YTGTGATGYAGCATACAGAG	175–195	72 °C 7' (×1)
Flu A/B multiplex real time PCR				
FluA	M	GAGTCTTCTAACMGAGGTCGAAACGTA	13	FluAv_For
		GGGCACGGTGAGCGTRAA	207	FluAv_Rev
		FAM-TCCTGTCACCTCTGAC-MGB		FluAv_TM
FluB	M	TAGCAGAAGGCCATGAAAGCT	317	FluBv_For
		CGTTCCTAGTTTTACTTGCATTGAAT	411	FluBv_Rev
		JOE-TGTCTCATGGTCATGTACCT-MGBNFQ		FluBv_TM

Table 2
Distribution of hBoV, hMPV, and InfA-B of single and co-infections in subjects with ILI (n = 116) by age.

	InfA/B n (%)	hMPV n (%)	hBoV n (%)	Total n (%)	P value		
					Flu A/B vs hMPV	Flu A/B vs hBoV	hMPV vs hBoV
Age							
0–14 (yrs)	21 (51.2)	16 (72.7)	29 (54.7)	52 (44.8)	0.4126	0.1695	0.0175
15–64 (yrs)	8 (19.5)	3 (13.6)	10 (18.9)	22 (19.0)	0.1623	0.7591	0.0452
>64 (yrs)	12 (29.3)	3 (13.6)	14 (26.4)	42 (36.2)	0.0204	0.8134	0.0055
Total	41 (35.3)	22 (19.0)	53 (45.7)				
Single and co-infections							
Total	41 (35.3)	22 (19.0)	53 (45.7)	–	0.0079	0.1413	0.0001
Single infections	16 (39.0)	4 (18.2)	27 (50.9)	47 (40.5)	0.1547	0.3464	0.0104
Double infections	18 (43.9)	11 (50.0)	19 (35.8)	24 (20.7)	0.0902	0.2500	0.0104
Flu A/B	–	5 (45.5)	13 (68.4)	–	–	–	–
hMPV	5 (27.8)	–	6 (31.6)	–	–	–	–
hBoV	13 (72.2)	6 (54.5)	–	–	–	–	–
Triple infections	7 (17.1)	7 (31.8)	7 (13.2)	–	–	–	–

viruses. However, the samples with suspected respiratory infection were effectively collected by the sentinel physicians only in the period between on October and on March. This short time collection may have limited the evaluation of the real time distribution of the viruses. In order to assess the real viral seasonality, oropharyngeal swabs should have been collected along a 12 month time period for at least three consecutive years. It is natural, then, to assess that further studies need to be performed in order to establish whether there is a real epidemiological trend.

The frequency of respiratory viruses was higher in the 0–14 age group, where the rate of viral detection reached 75.0%. hBoV was detected in 45.7% (53/116 samples) of all the analyzed subjects and in 55.8% (29/52) of the children, a rate higher than previously reported (Maggi et al., 2007). However, these results are not fully comparable because of the different study design. In fact, none of the previous studies was prospective; moreover, one of these studies included only hospitalized children and showed differences in the rate of hBoV detection during a period of years (Maggi et al., 2007).

Recent analyses documented the detection of hBoV also in asymptomatic subjects, which suggests that the virus is not a true pathogen but rather a commensal (Blessing et al., 2009; Salmón-Mulanovich et al., 2011). However, although not yet completely demonstrated, hBoV appears to contribute to the severity of respiratory illness (Martin et al., 2010). In this study, a limitation is the lack of control groups without clinical evidences of illness. To assess the real role of this virus in respiratory infections, it would therefore be useful to compare a symptomatic group with a control group and an asymptomatic period with a symptomatic period.

Phylogenetic analysis of the hBoV positive specimen sequences revealed that all samples clustered in genotype hBoV1 NP1. Consistent with other reports, strains of hBoV1 were more frequently identified in respiratory swabs (Allander et al., 2005; Hishinuma-Igarashi et al., 2009). However, due to the limited number of specimens the presence in the patients of different hBoV genotypes cannot be excluded.

Among the emerging respiratory viruses, hMPV is one of the most frequently reported in recent international studies (Van den Hoogen et al., 2001; Boivin et al., 2007). In Italy, hMPV has been variously associated with Upper Respiratory Tract Infections (URTI) or Lower Respiratory Tract Infections (LRTI), affecting from 3% to 13% of children with ARTI (Bosis et al., 2005; Gerna et al., 2005; Sarasini et al., 2006). Our data do not appear to be in agreement with previous reports, revealing hMPV in 19.0% (22/116) of all samples and in particular in 30.8% (16/52) of children. This virus was detected also in children older than 5 years, which is not in agreement to other serological surveys that showed an early acquisition of infection (Van den Hoogen et al., 2001; Zappa et al., 2011). Nonetheless, hMPV is an emerging cause of ARTI in ILI patients and may have a significant clinical impact, especially in children.

With respect to others, a limitation of this study is the search of only three respiratory viruses. Further studies should include human parainfluenza viruses 1–3 (hPIVs), RSV, adenovirus for a more in depth investigation. Furthermore, in this study the respiratory infections were analyzed for only 1 year and the samples were collected with limited information on the severity of clinical symptoms.

Phylogenetic analysis of hMPV strains in different countries has been based on the comparison of sequences of L, N, F, or P genes (Van den Hoogen et al., 2001, 2004a; Boivin et al., 2002, 2004; Bastien et al., 2003; Mackay et al., 2004). In the present study, the RT-PCR was designed for the M gene to detect hMPV with an equivalent sensitivity for both genotypes. Recent studies have validated the use of the M gene (Bellau-Pujol et al., 2005; Chano et al., 2005) and a genetic discrimination has been established (Van den Hoogen et al., 2001; Bastien et al., 2003; Carr et al., 2005; Legrand et al., 2011). In our study, phylogenetic analysis of the M gene showed that the isolated viruses clustered with A2 strains. Nevertheless, different hMPV genotypes and their sublineages can co-circulate during the year and one of these can be prevalent (Huck et al., 2006; Caracciolo et al., 2008; Xiao et al., 2010; Legrand et al., 2011).

The strains circulating in Italy were described in several studies previously (Sarasini et al., 2006; Gerna et al., 2007; Caracciolo et al., 2008; Larcher et al., 2008; Bosis et al., 2008; Zappa et al., 2011). In all these studies, co-circulation of different hMPV genotypes was observed, but A2 subtype was the most prominent strain. In our study, it has not been possible to assess sub-division of A2 samples in
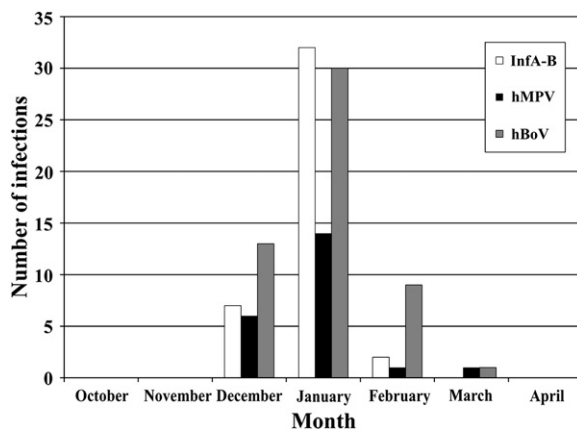

Fig. 1. Number of human bocavirus (hBoV), human metapneumovirus (hMPV) and Influenza A–B virus per month.
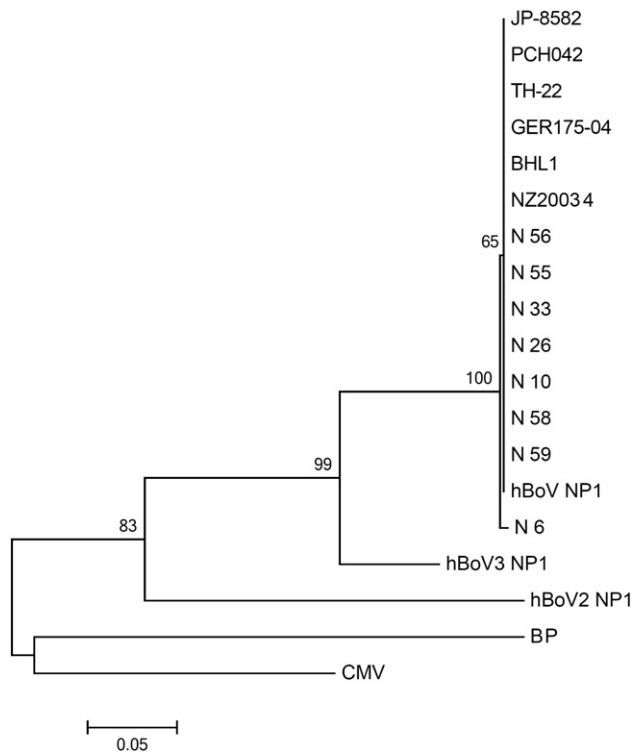
```
          JP-8582
          PCH042
          TH-22
          GER175-04
          BHL1
          NZ2003 4
    65   N 56
          N 55
          N 33
          N 26
   100    N 10
          N 58
          N 59
          hBoV NP1
    99   └ N 6
          hBoV3 NP1
   83     hBoV2 NP1
          BP
          CMV
   ├──┤
   0.05
```

Fig. 2. A dendogram of human and animal BoV based on the NP-1 gene sequences from representative pediatric and adult isolates originating from South Apulia using a 354 pb region of the bocavirus NP-1 region. Additional strains of hBoV [NC_007455; GU563338; FJ767757; GU563333; DQ513330; FJ156727; EF686010], hBoV2 [NC_0012042], hBoV3 [NC_0012564] and the animal parvoviruses: Canine Minute Virus [NC_004442; Region 2389–2949] and Bovine Parvovirus [DQ335247; Region 2659–3300] are included.

A2a and A2b sublineages, most probably due to the short sequence length analyzed in our experimental set up.

Our epidemiological findings support previous observations that demonstrate an increase in A2 strain infections in recent years (Boivin et al., 2004; Wang et al., 2008; Herbert et al., 2005; Zappa et al., 2011). Other authors observed a shift from group A to group B (Agapov et al., 2006; Mackay et al., 2006; Oliveira et al., 2009; Legrand et al., 2011). Taken together, these data suggest a constant genetic evolution of hMPV, which can play a role in the development of severe clinical manifestations.

Simultaneous infections by different viruses have been well documented (Choi et al., 2006; Kaida et al., 2007). In this study, viral co-infections were detected in 26.7% of the cases. This rate is in accordance with the previously published data implicating the rate of dual and multiple infections to vary between 5% and 47.6% of all viral respiratory infections (Aberle et al., 2005; Legg et al., 2005; Calvo et al., 2010; Franz et al., 2010; Wang et al., 2010). It is possible that there were additional co-infections not detected in our study since we did not test our respiratory samples for other probable pathogens such as rhinovirus, human polyomaviruses, RSV and enteroviruses. In this respect, this study should be considered preliminary to further evaluation of other viruses causing respiratory co-infections.

The multiple co-infections and viral genotypes may aggravate the severity of clinical disease in patients suffering from a respiratory infection. However, data in the literature on both hBoV and hMPV provide a mixed message, as some authors show a higher severity of illness (Greensill et al., 2003; Semple et al., 2005), not observed in other studies (Lazar et al., 2004; Williams et al., 2004; Bosis et al., 2005; Caracciolo et al., 2008). In this study, the clinical picture induced by co-infections was not more severe than that observed in patients with only one viral infection. Further studies are needed to clarify the

pathogenesis and interactions of co-infectants. When a single sample contains many viruses it becomes difficult to evaluate the clinical significance of each virus. In these cases, quantitative analysis of each virus could be useful to provide information about the role of each species on the severity of disease. The analysis of oropharingeal swabs is methodologically difficult because the quality of the specimens is dependent on sampling technique and condition of the pharyngeal mucosa. Therefore, an exact quantitation may be difficult to obtain.

Our results suggest that hBoV and hMPV may be partially responsible for the development of ILI in adult and elderly patients. hMPV is one of the leading causes of infection in all of age groups, even in adulthood. hMPV infection induces incomplete immunity, and reinfections later in life are well documented, which justifies the presence of hMPV among adults of all ages (Falsey et al., 2003; Sloots et al., 2006; Walsh et al., 2008), whereas hBoV is rarely detected in adults with respiratory infections (Longtin et al., 2008). In most of the papers, isolated cases or low incidence rates in adults were found. Often, this virus was detected in at high risk or immunocompromised patients (Müller et al., 2009; Ringshausen et al., 2009; Costa et al., 2009). Thus, our results are unexpected for what regards the circulation of the analyzed viruses in all of the age groups and the high hBoV infection rate in adult (18.9%) and elderly (26.4%) subjects. Currently, there are no data that endogenous reactivations or exogenous reinfections do occur for hBoV. So we speculate that the high infection rate in adults could be due to a hBoV secondary infection.

In conclusion, our results provide convincing evidence that in the most of cases of ILI, hBoV and hMPV are involved. Thus, these emergent viral pathogens should be carefully included in routine testing in adult patients with atypical pneumonia but further studies are necessary to prove the clinical relevance of such infections in adults, especially for hBoV. In fact, there is growing consensus on the existence of a relationship between hBoV infections and ARTI, although high rates of co-infections with other respiratory viruses have been reported (Boivin et al., 2007). Additional studies are needed to understand the clinical and epidemiological significance of each virus involved in the co-infections.

Materials and methods

Study population

In a time period between June 2008 and May 2009, 116 oropharingeal swabs were taken from patients with ILI, and demographic and clinical data were collected. Sample collection was carried out by the Italian Network for surveillance of Influenza (InfluNet) organized by the Italian National Institute of Health (ISS) and the Inter-University Centre for Research on Influenza (CIRI) in collaboration with the Regional Health Authorities. Informed consent was obtained from the enrolled patient or from their parents. The main clinical features were acute respiratory problems with rapid and sudden onset with a temperature of >38 °C accompanied by general symptoms (headache, feeling poorly, sweating, asthenia, shivers) and by at least one respiratory symptom (cough, pharyngodinia, nasal congestion) or fever accompanied by rhinitis, rhinopharyngitis, tonsillitis, laryngeal tracheitis, tracheitis and acute bronchitis. The collected samples were stored at −80 °C until processed. Respiratory specimens were tested for hMPV, hBoV and InfA-B by polymerase chain reaction (PCR) or reverse transcriptase-PCR (RT-PCR).

RNA and DNA extraction

Isolation of viral RNA and DNA, from 250 to 400 µl samples, respectively, was performed using Rneasy Mini Kit and QIAamp DNA MiniKit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.
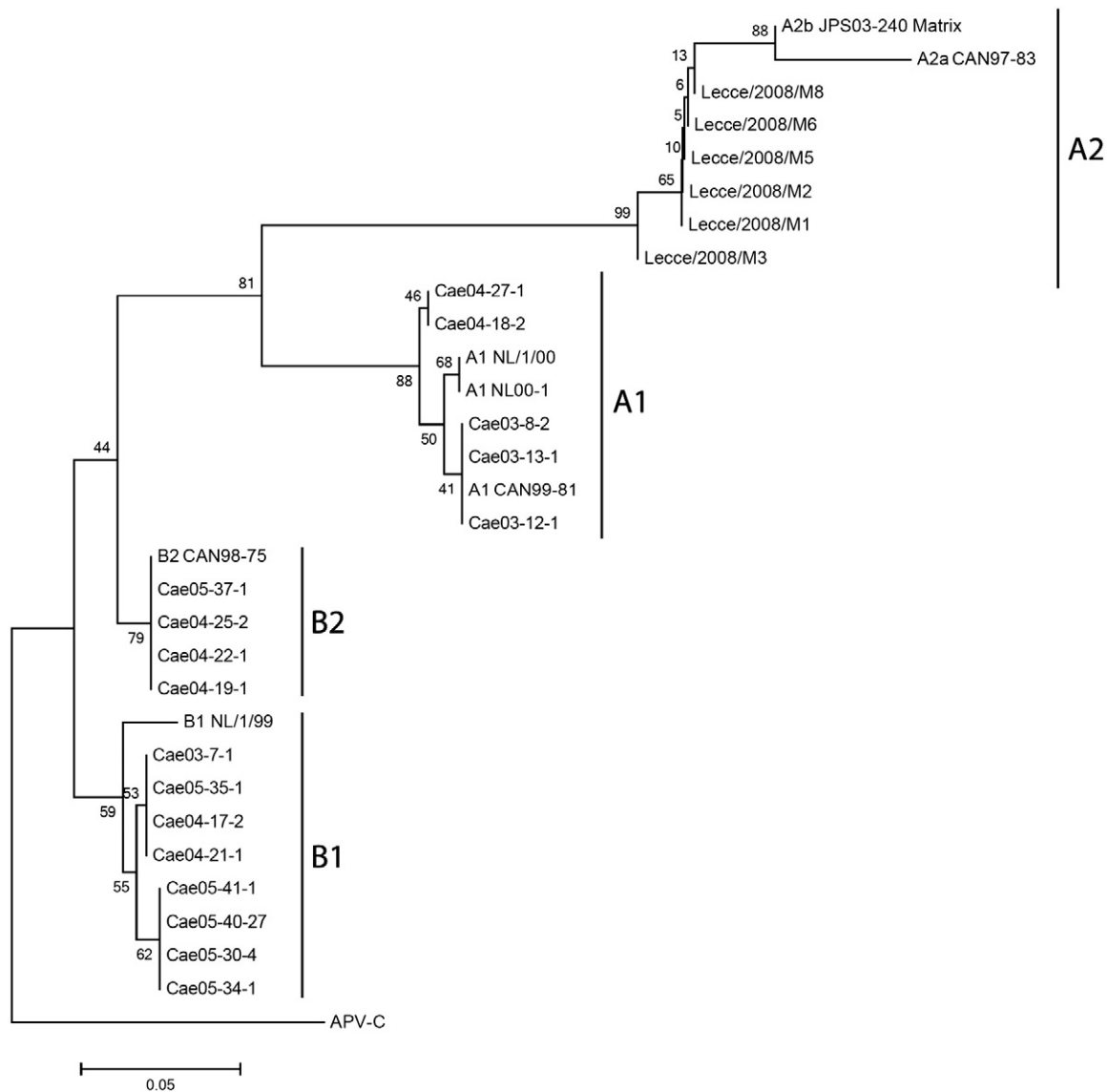
Fig. 3. A dendogram of hMPV based on the M gene sequences from representative pediatric and adult isolates originating from South Apulia using a 151 pb region of the MPV M region. The gene bank access numbers of the referenced strains are: A1 [AF371337, HQ384159, HQ384156, HQ384126, HQ384125, HQ384124, AJ145294], A2 [AJ530095, AJ297749], B1 [HQ384134, HQ384140, HQ384152, HQ384151, HQ384145, HQ384135, HQ384142, HQ384139, AJ525843], and B2 [AJ297748, HQ384157, HQ384146, HQ384150, HQ384154].

PCR assays for respiratory viruses

hMPV and InfA-B detection was performed by RT-nested-PCR and hBoV detection by nested-PCR. For reverse transcription, a commercial kit (RevertAidTM First Strand cDNA Synthesis kit, Fermentas Life Sciences) was used. A subsequent nested PCR was carried out using 5 μl of the first reaction product, which was then added to the reaction mixture to a final volume of 50 μl.

RT-nested-PCR was designed to amplify the conserved region of the hMPV matrix gene corresponding to matrix protein; nested-PCR was performed by using portions of the hBoV NP-1 gene as primers targets. Primer sequences, target genes and thermal profiles are reported in Table 1.

Sequencing and phylogenetic analysis

The PCR products of hMPV M gene (nt 44–61, corresponding to a 151 base-pairs fragment) and hBoV NP-1 gene (nt 108–208, corresponding to 354 base-pairs fragment) were purified with QIAamp nucleic acid purification kits (Qiagen, Germany), according to the manufacturer's protocol. PCR products were sequenced directly or after cloning using the CEQ 8000 (Beckman Coulter) automated sequencer, with the same forward primers (M2f and B2f; see Table 1) used for the PCR. Phylogenetic affiliation of the obtained sequences was determined by using the BLAST search tool at the NCBI web site. Sequence similarity was searched by BLAST (www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment was conducted with ClustalW2 version 2.1 (www.ebi.ac.uk/clustalw) and phylogenetic trees were constructed by the Neighbor-Joining method and Kimura 2-Parameter model using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (www.xmegasoftware.net). A bootstrap resampling analysis was performed (1000 replicates) to test tree robustness. This analysis was performed on hMPV matrix nucleotide sequences and on a portion of the hBoV NP-1 nucleotide sequences.

Statistical analysis

Statistical analysis was performed with StatView 5.1 Software. Categorical variables between groups were compared with the Fisher's Exact-test. $P < 0.05$ was considered to be statistically significant.

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