DOI:10.1111/irv.12061
www.influenzajournal.com

Short Article

Spread of different rhinovirus B genotypes in hospitalized children in Spain

María Teresa Cuevas,[a] Mar Molinero,[a] Francisco Pozo,[a] Cristina Calvo,[b] Maria Luz García-García,[b] Noelia Reyes,[a] Juan Ledesma,[a] Inmaculada Casas[a]

[a]Influenza and Respiratory Virus Laboratory, National Center for Microbiology, ISCIII. [b]Department of Pediatrics, Hospital Severo Ochoa, Leganés, Madrid, Spain.
Correspondence: María T. Cuevas, Laboratorio de Gripe y virus respiratorios, Centro Nacional de Microbiología, Instituto de Salud Carlos III (ISCIII), 28220 Majadahonda, Madrid, Spain.
E-mail: tcuevas@isciii.es

Accepted 30 October 2012. Published Online 06 December 2012.

Human Rhinovirus (HRV) classification is an evolving process. New genotypes have been described within HRV-A and HRV-C species, but only one has been accepted related to HRV-B. From 2003 to 2010, a total of 3987 nasopharyngeal aspirate samples were taken from pediatric patients admitted to the Severo Ochoa Hospital in Madrid (Spain). After viral analysis, 949 (23·8%) tested positive to HRV. A random selection of 397 (42%) positive samples showed that 39 (9·8%) were HRV-B. The sequencing of partial VP4/VP2 coding region revealed the spread of 13 of 25 defined HRV-B serotypes and three putative new genotypes. Such results remark the high diversity of HRV-B.

Keywords Genotype, human rhinovirus, pediatric patients, respiratory infection.

Please cite this paper as: Cuevas *et al.* (2013) Spread of different rhinovirus B genotypes in hospitalized children in Spain. Influenza and Other Respiratory Viruses 7(5), 623–628.

Human rhinoviruses (HRVs) are the most prevalent human respiratory pathogens. The first HRV was discovered in 1956.[1] At the end of the 80s, 100 serotypes were identified using susceptible cell culture and specific antisera.[2] These serotypes were identified as two genetically distinct species: HRV-A and HRV-B with 75 and 25 different genotypes, respectively. A third group HRV-C, described in 2006 and initially called HRV-A2,[3] was accepted as a new species in 2009 by the *Picornaviridae* Study Group of ICTV (http://www.picornastudygroup.com/taxa/species/species.htm).

The description of new genotypes is an evolving process. In this sense, apart from 33 types initially defined within HRV-C,[4] up to 18 new types have been recently accepted, and 12 more are awaiting approval. With regard to HRV-A, serotypes 101, 102, and 103 have been recently accepted (http://www.picornastudygroup.com/taxa/species/species.htm). Up to date, only one new genotype has been defined within HRV-B, the strain CU211, described in Thailand.[5]

The definition of new genetic types is based on the nucleotide sequence divergence defined as the pairwise distance between sequences. According to the analysis of Wisdom *et al.*[6] and Simmonds *et al.*,[4] novel HRVs may be classified following these criteria. In case of HRV-B species, the pairwise distance, based on partial VP4-/VP2-coding region, falls to a minimum value of 10%. In agreement with this classification, other authors suggest that new genetic types may be classified into assigned genetically and assigned until they are confirmed as strict genotypes.[7]

In the present work, we have studied the phylogenetic characterization of HRV-B based on partial VP4-/VP2-coding region, so that we can determine the specific genotypes that have been circulating among children in Madrid from December 2003 to June 2010.

The study population, admitted to the Severo Ochoa Hospital (Madrid, Spain), was composed of children below 14 years of age with acute and severe respiratory infection. Other patients, without respiratory symptoms, were included in the study as healthy controls. After informed consent was obtained from the parents or legal tutors, nasopharyngeal aspirate (NPA) samples were taken from each patient. Samples were sent to the Influenza and Respiratory Virus Laboratory at the National Center for Microbiology (ISCIII, Madrid) for virological studies. An overall of 3987 samples were analyzed throughout the study, of which 949 (23·8%) were positive for HRV. Considering a confidence level of 99% and a confidence interval of 5%, the needing sample size was 391 (http://www.surveysystem.com/sscalc.htm). In our case, we randomly selected 397 samples for sequencing study.

We have studied each species in depth separately, with the aim of this work being to characterize the spread of HRV-B among children in Madrid.

Initially, samples were processed by two multiplex RT-PCR assays, which allow the detection of 14 respiratory viruses.[8] Next, positive results for HRVs were confirmed by a specific HRV RT-PCR assay.[9,10] The amplified product, which comprised the complete sequence of VP4 and partial VP2 proteins, was subsequently sequenced as previously described.[9] Sequences were edited and assembled using SeqMan (dnastar software.http://www.dnastar.com/) and aligned with bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and mafft (http://mafft.cbrc.jp/alignment/server/). After performing a blast algorithm (http://ncbi.nlm.nih.gov/BLAST), similar sequences to those included in our study were retrieved from GenBank.

The relationships between individual viruses were established using neighbor-joining and Kimura-2 parameter methods as nucleotide substitution method. Phylogenetic trees were reconstructed through the neighbor-joining method (MEGA package, version 5·0 http://megasoftware.net/) by 1000 bootstrap resampling to assess the reliability of tree topologies. Bootstrap values of 70% or higher are shown in the tree (Figure 1). The divergence analysis by computing the pairwise distance was also performed with MEGA using the same parameters so as to perform the phylogenetic trees. A chi-square with Yates's correction was used to perform the statistical analysis (http://graphpad.com).

Of the 397 sequenced NPA samples, HRV-A was detected in 204 (51·4%), HRV-B in 39 (9·8%) and HRV-C in 154 (38·8%). The annual distribution of HRV-B was as

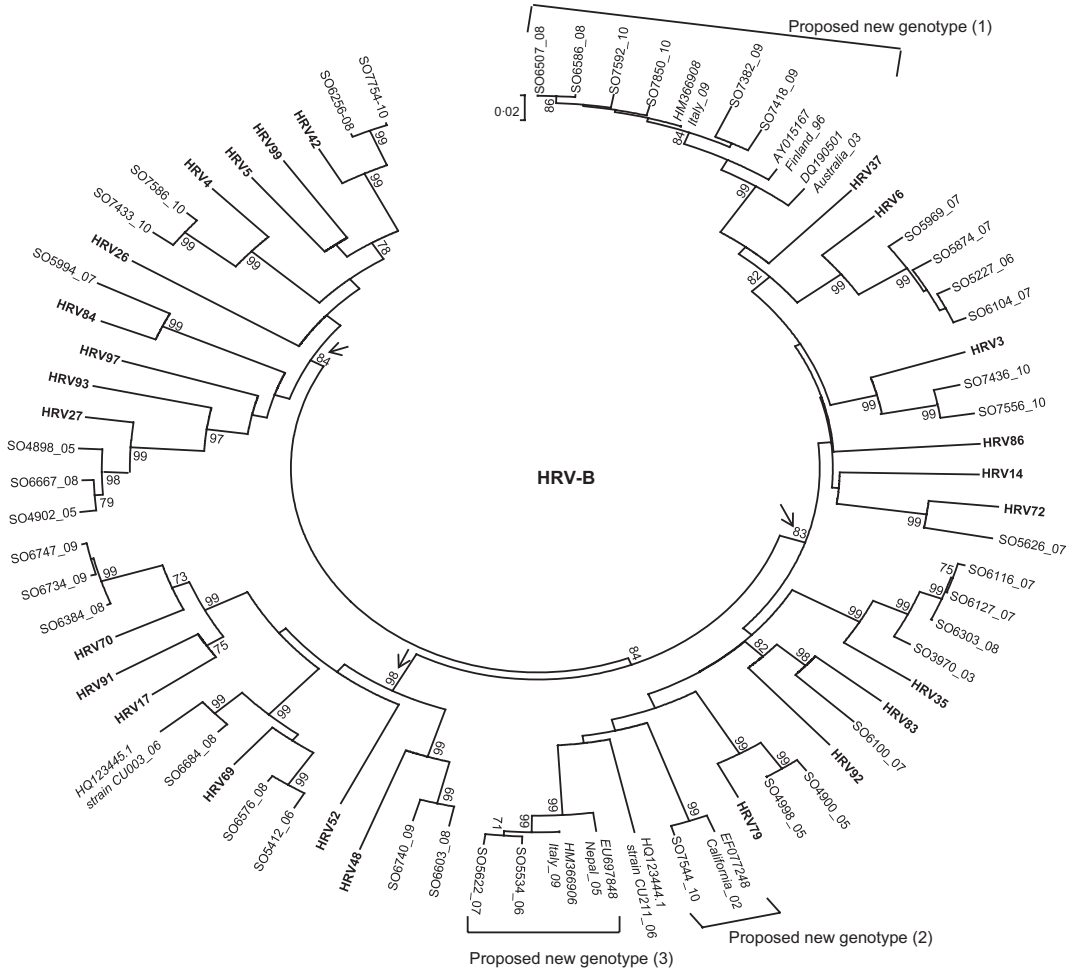

Figure 1. Circular phylogenetic tree of Human Rhinovirus (HRV)-B sequences from NPA samples taken from pediatric patients. Sequences were segregated into three main clades, which are indicated with arrows. The proposed new genotypes are highlighted with the square brackets. HRV prototype strains are pointed out in boldface. Sequences similar to those analyzed in this study and retrieved from GenBank database are marked in italics. The year of sampling is indicated followed by the name of the sample. NPA, nasopharyngeal aspirate.

Table 1. Matrix with the results of the divergence analysis calculated as the pairwise distance. (a) Divergence between the known HRV-B genotypes and the sequences belonging to the three putative new genotypes. Each sequence is preceded by a number which indicates if the sequence belongs to the proposed new genotype 1, 2, or 3. The average of the divergence value of each group with regard to each known genotype is included at the end of the table. (b) Divergence among the sequences from the new genotypes. (c) Intra- and inter-genotype diverge among the three putative new genotypes

(a)

	HRV3	HRV4	HRV5	HRV6	HRV14	HRV17	HRV26	HRV27	HRV35	HRV37	HRV42	HRV48	HRV52	HRV69	HRV70	HRV72	HRV79	HRV83	HRV84	HRV86	HRV91	HRV92	HRV93	HRV97	HRV99
1.SO6507	0·22	0·26	0·27	0·14	0·22	0·26	0·28	0·25	0·23	0·15	0·28	0·29	0·25	0·28	0·26	0·22	0·20	0·23	0·27	0·19	0·29	0·22	0·24	0·27	0·25
1.SO6586	0·23	0·26	0·27	0·14	0·22	0·26	0·28	0·25	0·23	0·15	0·29	0·29	0·25	0·28	0·26	0·22	0·20	0·23	0·27	0·20	0·29	0·22	0·24	0·28	0·26
1.SO7592	0·24	0·26	0·27	0·16	0·23	0·27	0·29	0·25	0·23	0·15	0·27	0·29	0·26	0·29	0·27	0·22	0·19	0·23	0·28	0·20	0·29	0·22	0·24	0·28	0·25
1.SO7850	0·23	0·25	0·27	0·15	0·22	0·26	0·28	0·25	0·23	0·15	0·29	0·29	0·26	0·28	0·26	0·21	0·20	0·24	0·27	0·20	0·29	0·22	0·24	0·26	0·25
1.SO7382	0·26	0·28	0·28	0·16	0·24	0·27	0·28	0·26	0·25	0·18	0·30	0·29	0·26	0·29	0·28	0·25	0·21	0·26	0·27	0·23	0·31	0·23	0·25	0·29	0·27
1.SO7418	0·23	0·27	0·27	0·17	0·22	0·27	0·29	0·26	0·25	0·15	0·29	0·30	0·27	0·29	0·26	0·23	0·20	0·23	0·28	0·21	0·29	0·22	0·25	0·28	0·26
2.SO7544	0·24	0·31	0·29	0·21	0·23	0·28	0·29	0·27	0·21	0·23	0·26	0·30	0·27	0·27	0·29	0·26	0·20	0·22	0·31	0·23	0·29	0·20	0·27	0·23	0·29
3.SO5534	0·26	0·27	0·25	0·21	0·25	0·26	0·27	0·28	0·22	0·24	0·25	0·23	0·21	0·25	0·23	0·24	0·17	0·20	0·31	0·24	0·26	0·22	0·27	0·26	0·23
3.SO5622	0·27	0·29	0·26	0·20	0·25	0·26	0·28	0·28	0·22	0·24	0·24	0·24	0·23	0·26	0·24	0·24	0·18	0·20	0·31	0·24	0·26	0·23	0·26	0·27	0·23
1.Xd 1	0·23	0·26	0·27	0·15	0·22	0·26	0·28	0·25	0·24	0·15	0·29	0·29	0·26	0·28	0·26	0·22	0·20	0·24	0·27	0·20	0·29	0·22	0·24	0·28	0·26
1.Xd 2	0·24	0·31	0·29	0·21	0·23	0·28	0·29	0·27	0·21	0·23	0·26	0·30	0·27	0·27	0·29	0·26	0.20	0·22	0·31	0·23	0·29	0·20	0·27	0·23	0·29
1.Xd 3	0·26	0·28	0·25	0·20	0·25	0·26	0·27	0·28	0·22	0·24	0·24	0·23	0·22	0·25	0·23	0·24	0·17	0·20	0·31	0·24	0·26	0·22	0·26	0.26	0·23

(b)

	SO6507	SO6586	SO7592	SO7850	SO7382	SO7418	SO7544	SO5534
1.SO6507								
1.SO6586	0·00							
1.SO7592	0·02	0·02						
1.SO7850	0·02	0·01	0·02					
1.SO7382	0·05	0·05	0·05	0·04				
1.SO7418	0·04	0·04	0·04	0·04	0·05			
2.SO7544	0·23	0·23	0·22	0·22	0·24	0·24		
3.SO5534	0·20	0·21	0·21	0·21	0·24	0·22	0·18	
3.SO5622	0·20	0·21	0·20	0·21	0·24	0·23	0·17	0·01

(c)

	1	2	3
1	0·03		
2	0·23	–	
3	0·21	0·17	0·01

follows: one sample was detected in 2003, none in 2004, four in 2005, three in 2006, nine in 2007, nine in 2008, five in 2009, and eight in 2010. Although HRVs infections in Spain occurred along the year, the peak of highest activity was observed in winter, December–January (51%).

Human Rhinovirus-B sequences were segregated into three main clades, supported by high bootstrap values of 83%, 98%, and 84%, respectively (Figure 1). Sequences were grouped with high bootstrap values in 13 of the 25 described HRV-B serotypes. Notably, we found three differ-

Table 2. Clinical and virological characteristics of patients included in the study

Sample	Genotype	Diagnosis	Co-infection
SO-3970	HRV35	ALTE	HRV
SO-6576	HRV69	Bronchiolitis	HRV + PIV3
SO-5994	HRV84	Bronchiolitis	HRV + PIV4
SO-6747	HRV70	Bronchiolitis	HRV + RSV-A
SO-5626	HRV72	Bronchiolitis	HRV + RSV-B
SO-5534	PNG3	Bronchiolitis	HRV + RSV-B
SO-5622	PNG3	Bronchiolitis	HRV + RSV-B
SO-6100	HRV83	Bronchiolitis	HRV + RSV-B
SO-7556	HRV3	Bronchiolitis	HRV + RSV-B
SO-4998	HRV79	Bronchiolitis	HRV
SO-6603	HRV48	URTI + febrile convulsion	HRV
SO-6384	HRV70	URTI (no hospitalization)	HRV + Adv + hMPV + PIV4
SO-5412	HRV69	URTI	HRV
SO-5227	HRV6	URTI	HRV + Adv
SO-6507	PNG1	URTI	HRV + Adv
SO-5874	HRV6	Healthy control	HRV
SO-7586	HRV4	Healthy control	HRV
SO-7850	PNG1	Healthy control	HRV + HBoV
SO-6303	HRV35	Asthmatic crisis	HRV + Adv
SO-7433	HRV4	Croup	HRV + RSV-B
SO-5969	HRV6	Pneumonia	HRV
SO-4898	HRV27	Pneumonia	HRV
SO-6740	HRV48	Pneumonia	HRV
SO-7382	PNG1	Pneumonia	HRV
SO-6586	PNG1	Pneumonia	HRV + PIV4
SO-6667	HRV27	Pneumonia + Kawasaki syndrome	HRV
SO-7754	HRV42	Pneumonia + bronchoespasm	HRV
SO-7418	PNG1	Pneumonia + bronchoespasm	HRV + RSV-A
SO-6127	HRV35	Not known	HRV + RSV-A
SO-4900	HRV79	Not known	HRV
SO-7436	HRV3	Recurrent wheezing	HRV + Adv + RSV-B
SO-6684	HRV69	Recurrent wheezing	HRV + HBoV + PIV3
SO-6104	HRV6	Recurrent wheezing	HRV + PIV4
SO-6116	HRV35	Recurrent wheezing	HRV + RSV-A
SO-7592	PNG1	Recurrent wheezing	HRV + RSV-A
SO-6256	HRV42	Recurrent wheezing	HRV + RSV-B
SO-7544	PNG2	Recurrent wheezing	HRV + RSV-B
SO-6734	HRV70	Febrile syndrome	HRV
SO-4902	HRV27	Febrile syndrome	HRV + HBoV

PNG, proposed new genotype; ALTE, apparently life-threatening events; URTI, upper respiratory tract infection; HRV, human rhinovirus; PIV, parainfluenza virus (types 3 and 4); RSV, respiratory syncytial virus (A and B); Adv, adenovirus; HBoV, human bocavirus; hMPV, human metapneumovirus.

ent clades that did not cluster with any serotype previously described. Clade 1 was composed of six viruses collected during 2008 and 2010; clade 2 was defined by one virus detected in 2010; and clade 3 was composed of two viruses circulating in 2005 and 2009, respectively. All of these HRV-B viruses clustered with other ones that were circulating in several countries in different years, as it is shown in the phylogenetic tree.

The pairwise distance was calculated to know whether these sequences fulfilled the criteria to be considered as new genotypes (Table 1). In all the cases, the divergence with the nearest serotype/genotype reference strains was higher than 10%, whereas the intragenotype divergence was lower than 10%. Therefore, the new clades complied with the established criteria.

The two complete sequences recently analyzed in a Thailand study[5] were also included in the phylogenetic tree. The new genotype, CU211, was not detected in our study.

Although HRVs are the most common etiological agents of upper respiratory tract infections referred to as common cold, HRVs have also been associated with more serious illnesses, including pneumonia, otitis media, asthma exacerbation, and chronic obstructive pulmonary disease, as it has been reviewed.[11] In this study, the clinical findings were fever (64·9%), pulmonary infiltrate (41·9%), and hypoxia (29%). Bronchiolitis (23·1%), pneumonia (20·5%), and recurrent wheezing (17·9%) were clinically diagnosed. Genotype, co-infections, and main clinical diagnosis are summarized in Table 2. It was very difficult to establish an association between a specific respiratory syndrome and a specific HRV-B genotype, mainly due to the high percentage of co-infections (64·1%), mostly with respiratory syncytial virus (RSV) detected in 13 patients. Nevertheless, in four of six cases of pneumonia, HRV-B was the unique detected virus, whereas in the other two co-infection with parainfluenza virus 4 and a blood culture positive for *S. pneumoniae* occurred.

The statistical analysis of a specific diagnosis and the presence/absence of other viruses, described in Table 2, revealed that there is a correlation in case of pneumonia and no co-infection ($P = 0{\cdot}0293$), but not in case of bronchiolitis or recurrent wheezing ($P = 0{\cdot}1703$ and $P = 0{\cdot}08$, respectively).

The spread of HRV-B among children in Madrid from 2003 to 2010 has been reported in this article. In our study, the most prevalent species is HRV-A, followed by HRV-C, and HRV-B being the least frequent. Focusing on HRV-B, our sequences clustered initially into three main clades. The presence of different groupings of HRV was also described within HRV-A.[12] A remarkable finding is the high HRV-B diversity found in our study, with 13 of 25 serotypes detected, compared with other surveys. Studies performed in Edinburgh[6] and Switzerland[13] detected six different serotypes each, while four different types were found in China,[14] South Africa,[15] and the USA,[16] respectively. This lower diversity may be due to the number of years spanned in these studies –usually one or two – compared with our study of a 6·5-year period. The most prevalent genotype in our study was the new clade 1, detected in six patients, followed by serotypes 6 and 35, detected in four patients each. On the contrary, in the mentioned surveys, HRV-B52 was the most prevalent serotype which was not detected in our study. Other serotypes detected were HRV-B27, HRV-B83, HRV-B6, and HRV-B4, all of them also detected in our study. These differences may be due to a specific geographical circulating distribution, as it is described for HRV-C,[4] or to a distinct seasonal distribution, where several serotypes could circulate in a continuous way, whereas other serotypes could be detected only in specific seasons.[17]

In addition to this diversity, we have described three putative new clades, which are supported by a high bootstrap value and have fulfilled the molecular cutoff of nucleotide divergence (10%) proposed by Wisdom.[6] Furthermore, these viruses clustered with other viruses that have been circulating in Australia, Nepal, USA, Finland, and Italy, from 1996 to 2009.

In conclusion, our results show the high diversity of the HRV-B that has been circulating among children in Spain.

Nucleotide sequence accession numbers

HRV VP4/VP2 sequences have been submitted to GenBank under accession numbers JF439625–JF439661, EU697836, and EU697832.

Acknowledgements

The Spanish National Health Institute (ISCIII, Fondo de Investigaciones Sanitarias) Grant numbers: PI06/0532 and PI09/0246.

References

1 Price WH. The isolation of a new virus associated with respiratory clinical disease in humans. Proc Natl Acad Sci USA 1956; 42:892–896.

2 Hamparian VV, Colonno RJ, Cooney MK *et al.* A collaborative report: rhinoviruses-extension of the numbering system from 89 to 100. Virology 1987; 159:191–192.

3 Arden KE, McErlean P, Nissen ND, Sloots TP, Mackay IM. Frequent detection of Human Rhinoviruses, Paramyxoviruses, Coronaviruses and Bocavirus during acute respiratory tract infections. J Med Virol 2006; 78:1232–1240.

4 Simmonds P, McIntyre C, Savolainen-Kopra C, Tapparel C, Mackay IM, Hovi T. Proposals for the classification of human rhinovirus species C into genotypically assigned types. J Gen Virol 2010; 91:2409–2419.

5 Linsuwanon P, Payungporn S, Suwannakarn K, Chieochansin T, Theramboonlers A, Poovarawan Y. Complete coding sequence characterization and comparative analysis of the putative novel human rhinovirus (HRV) species C and B. Virol J 2011; 8:5.

6 Wisdom A, Leithc EC, Gaunt E, Harvala H, Simmonds P. Screening respiratory samples for detection of Human Rhinoviruses (HRVs) and Enteroviruses: comprehensive VP4-VP2 typing reveals high incidence and genetic diversity of HRV species C. J Clin Microbiol 2009; 47:3958–3967.

7 Arakawa M, Okamoto-Nakagawa R, Toda S *et al.* Molecular epidemiological study of human rhinovirus species A, B and C from patients with acute respiratory illnesses in Japan. J Med Microbiol 2012; 61:410–419.

8 Coiras MT, Aguilar JC, García ML, Casas I, Pérez-Breña P. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol 2004; 72:484–495.

9 Calvo C, Casas I, García-García ML *et al.* Role of rhinovirus C respiratory infections in sick and healthy children in Spain. Pediatr Infect Dis J 2010; 29:717–720.

10 Calvo C, García ML, Pozo F, Reyes N, Pérez-Breña P, Casas I. Role of rhinovirus C in apparently life-threatening events in infants, Spain. Emerg Infect Dis 2009; 15:1506–1508.

11 Gern JE. The ABCs of Rhinoviruses, Wheezing and Asthma. J Virol 2010; 84:7418–7426.

12 Palmemberg AC, Rathe JA, Ligett SB. Analysis of complete genome sequences of Human Rhinovirus. J Allergy Clin Inmunol 2010; 125:1190–1199.

13 Tapparel C, Cordey S, Junier T *et al.* Rhinovirus genome variation during chronic upper and lower respiratory tract infections. PLoS ONE 2011; 6:e21163.

14 Huang T, Wang W, Bassaud M *et al.* Evidence of recombination and genetic diversity in Human Rhinoviruses in children with acute respiratory infection. PLoS ONE 2009; 4:e6355.

15 Smuts HE, Workman LJ, Zar HJ. Human rhinovirus infection in young African children with acute wheezing. BMC Infect Dis 2011; 11:65.

16 Lee WM, Kiesner C, Pappas T *et al.* A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants. PLoS ONE 2007; 10:e966.

17 Savolainen C, Mulders MN, Hovi T. Phylogenetic analysis of rhinovirus isolates collected during successive epidemic seasons. Virus Res 2002; 85:41–46.