

Correlation of Rhinovirus Load in the Respiratory Tract and Clinical Symptoms in Hospitalized Immunocompetent and Immunocompromised Patients

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While human rhinoviruses (HRVs) are well accepted as a major cause of common cold syndromes (rhinitis), their role in the etiology of lower respiratory tract infections is still controversial, and their detection in asymptomatic patients is relatively common. The HRV pathogenic role in four groups of hospitalized patients (pediatric immunocompetent and immunocompromised patients, and adult immunocompetent and immunocompromised patients) was investigated by quantifying HRV load in nasopharyngeal aspirates or bronchoalveolar lavage samples by real-time reverse transcription PCR (RT-PCR). Real-time RT-PCR was performed in duplicate on all respiratory samples resulting positive by qualitative RT-PCR. In addition, molecular typing allowed detection of all known HRV species (A, B, and C). In immunocompetent pediatric patients HRVs were mostly associated with lower respiratory tract infections (in the absence of other viral agents) and wheezing, when viral load was $\geq 10^6$ RNA copies/ml. In young immunocompromised patients (stem cell transplantation recipients), an inverse correlation between HRV persistence over time and time at which the infection occurred after transplantation was observed, whereas in adult immunocompromised patients (lung transplant recipients) HRVs could be detected at a medium–low level ($< 10^5$ RNA copies/ml) in bronchoalveolar lavage samples taken routinely from asymptomatic patients. In conclusion, when detected at high viral load, HRVs may cause severe upper and lower respiratory tract infections, whereas when detected at a medium–low viral load, an event more frequent in immunocompromised subjects, they may represent only bystander viruses. **J. Med. Virol. 81:1498–1507, 2009.** © 2009 Wiley-Liss, Inc.

KEY WORDS: human rhinovirus load; nasopharyngeal aspirates; bronchoalveolar lavage; respiratory tract infections; immunocompromised patients

INTRODUCTION

Following their identification in 1956, human rhinoviruses (HRVs) were mostly considered as the responsible agent of “common cold” syndromes and, thus, having a minor impact on human pathology [Arruda et al., 1997]. However, more recently, their pathogenic role has been repeatedly claimed in acute sinusitis of adults [Pitkaranta et al., 1997], acute otitis media of children [Pitkaranta et al., 1998], and asthma exacerbations of children, adolescents, and adults [Nicholson et al., 1993; Johnston et al., 1995]. Furthermore, the pathogenic role of HRVs in lower respiratory tract infections of infants, elderly and immunocompromised patients has been reported. In more detail, 12.5% of viral pneumonia cases in neonates were attributed to HRVs [Abzug et al., 1990], as well as 24% of community-acquired pneumonia cases in hospitalized children [Juven et al., 2000], often in association with concurrent bacterial infections. In addition, HRVs are considered the second most common cause of bronchiolitis after RSV, often in association with RSV

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[Ong et al., 2001; Papadopoulos et al., 2002]. Furthermore, HRVs have been reported to be associated with hospitalization of children with a history of wheezing/asthma [Miller et al., 2007]. In a prospective study covering a 3-year follow-up, first year wheezing caused by HRV was the strongest predictor of subsequent 3rd year wheezing [Lemanske et al., 2005].

In adults, the impact of HRV infections on the lower respiratory tract in asthma, chronic bronchitis, and cystic fibrosis exacerbations [Nicholson et al., 1993; Collinson et al., 1996; Seemungal et al., 2001] has been reported along with the frequent association of HRV with lower respiratory tract infections in immunocompromised hosts and lung transplant recipients [Garbino et al., 2006; Kaiser et al., 2006]. In addition, HRVs have been reported to be present in respiratory secretions of asymptomatic individuals or patients [Van Kraaij et al., 2005].

Recently, HRVs, which were classified previously into two genetic groups (HRV-A including 76 serotypes and HRV-B including 25 serotypes), have been extended to include a third group (HRV-C) consisting of many previously unrecognized non-cultivable HRVs [Lau et al., 2007; Lee et al., 2007].

In the present study, the relationship was investigated between HRV load in respiratory secretions and occurrence of clinical symptoms in the respiratory tract in young immunocompetent and immunocompromised subjects as well as in adult immunocompetent and immunocompromised patients admitted to hospital.

MATERIALS AND METHODS

Patients and Specimens

Patients were admitted to the Fondazione IRCCS Policlinico San Matteo, University Hospital, and respiratory specimens were sent to the Viral Diagnostic Service for routine viral diagnosis. Patients examined ($n = 602$) were classified into four groups: (i) pediatric immunocompetent patients admitted to hospital with a diagnosis of acute respiratory tract infection, mostly interesting the lower respiratory tract, and associated with wheezing ($n = 290$); (ii) children and young adults with oncohematologic disorders and an acute respiratory tract infection ($n = 68$), some ($n = 35$) who had received a hematopoietic stem cell transplantation; (iii) adult immunocompetent patients ($n = 95$) admitted to hospital with different pathologies and an acute respiratory tract infection; and (iv) adult immunocompromised patients ($n = 149$) including adult stem cell transplantation recipients ($n = 25$) with an acute respiratory tract infection, and lung transplant recipients examined either because of an acute respiratory tract infection or following a routine bronchoscopy performed to monitor the occurrence of chronic rejection ($n = 67$).

Respiratory specimens, either nasopharyngeal aspirates or bronchoalveolar lavage samples, were collected from November 1, 2006 to May 31, 2007. Samples were collected according to a standardized procedure both at admission and discharge for most patients, and in some

cases also at one or more follow-up medical visits. Respiratory samples positive for respiratory viruses at admission were included even if negative at subsequent visits during follow-up. The period June–October 2007 was not included in this study.

Diagnosis of Respiratory Virus Infection

Nasopharyngeal aspirates and bronchoalveolar lavage samples were aliquoted and handled as previously reported [Rovida et al., 2005; Sarasini et al., 2006]. Respiratory specimens were tested for influenzaviruses A and B (Flu A and B), human parainfluenzaviruses (hPIV) 1–4, human respiratory syncytial virus (RSV), human adenoviruses (hAdV), human coronaviruses (hCoV) 229E and HKU1, and human metapneumoviruses (hMPV) by monoclonal antibodies (MAbs) and direct fluorescent antibody (DFA) staining. MAbs to Flu A and B, hPIV 1–4, RSV and hAdV were obtained from the same source (Chemicon International, Inc., Temecula, CA), while MAbs to hMPV [Percivalle et al., 2005; Gerna et al., 2006b], hCoV 229E [Gerna et al., 2006a] and HKU1 [Gerna et al., 2007] were developed in the laboratory.

Specimens positive by DFA were quantified by real-time reverse-transcription PCR (RT-PCR) for hMPV A and B [Kuypers et al., 2005], and RSV A and B [Perkins et al., 2005]. In addition, specimens were tested by real-time PCR for human bocavirus [hBoV, Lu et al., 2006]. Respiratory samples were also tested on a qualitative basis for HRV by the method of Steininger et al. [2001], and for hCoV by the method of Moës et al. [2005]. Specimens positive for HRV were quantified by real-time RT-PCR [Deffernez et al., 2004]. Finally, the presence of HRV-C strains was verified in the group of young oncohematologic patients by sequencing and phylogenetic analysis using the method of Lee et al. [2007]. This approach was limited to this group of patients, in order to verify whether different types of HRV infected these patients during follow-up.

Real-Time RT-PCR for Rhinoviruses

RNA was extracted from 200 μ l nasopharyngeal aspirates or bronchoalveolar lavage by the automated extraction kit Nuclisens[®] easy MAG[™] (BioMérieux, Lyon, France) and eluted in 50 μ l elution buffer. Reverse transcription (RT) of viral RNA was performed with SuperScript[™] II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a reaction mixture containing 5 μ l extracted RNA (corresponding to 20 μ l nasopharyngeal aspirates or bronchoalveolar lavage), 10 μ l 5 \times buffer (250 mM Tris–HCl [pH 8.3], 375 mM KCl, 15 mM MgCl₂), 5 μ l dithiothreitol (0.1 M), 1 μ l SuperScript II RNase H-Reverse Transcriptase, 5 μ l deoxynucleoside triphosphate (10 mM), 2.5 μ l random primers p(dN)₆, and 21.5 μ l nuclease-free water in a final volume of 50 μ l. The RT thermal profile was: 10 min at 25°C, 50 min at 42°C, and 15 min at 70°C. Real-time PCR was performed on 10 μ l cDNA (corresponding to 4 μ l nasopharyngeal aspirates or bronchoalveolar lavage),

according to Deffernez et al. [2004], in a final volume of 50 μ l reaction mixture containing 25 μ l TaqMan[®] Universal PCR Master Mix, no AmpErase[®] UNG (Applied Biosystems International, Foster City, CA), 900 mM forward, and 600 mM reverse primer, and 200 mM of each of the two probes. Primer and probe sequences were as follows: forward primer, 5'-GCACTTCTGTTTCCCC-3'; reverse primer, 5'-GGCAGCCACGCAGGCT-3'; probe 1, FAM-5'-AGCCTCATCTGCCAGGTCTA-3' TAMRA; probe 2, VIC-5'-AGCCTCATCGACCAAATA-3' TAMRA. Thus, probe 1 (for HRV-A) was labeled at the 5' end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' end with the quencher dye 6-carboxy-tetramethylrodamine (TAMRA), and probe 2 (for HRV-B) was labeled at the 5' end with the reporter dye VIC (Applied Biosystems, PE Europe, Basel, Switzerland), and at the 3' end with the same quencher dye TAMRA. DNA amplification was performed in 96-well microplates using the ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystems) according to the following thermal profile: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 sec at 95°C and 60 sec at 55°C. Each real-time RT-PCR test run included the amplification of two DNA scales (one per each probe) consisting of two linearized plasmids obtained by cloning two reference HRV serotypes (serotype 13 for probe 1, and serotype 3 for probe 2) with the TA cloning kit (Invitrogen). External standards were amplified from 10⁶ to 10¹ DNA copies, allowing the construction of two standard curves, from which amounts of viral load present in clinical samples were extrapolated. Results were then multiplied by a factor of 250 (referring to 1.0 ml nasopharyngeal aspirates or bronchoalveolar lavage). Thus, negative results were indicated as <250 RNA copies/ml. The two curves were adopted in order to detect both HRV-A and HRV-B groups [Deffernez et al., 2004]. RT-PCR methods for HRV detection by Steininger et al. [2001], as well as the RT-PCR for HRV molecular typing (based on phylogenetic analysis of a 260-bp variable sequence in the 5' non-coding region of HRV and performed on the group of young oncohematologic patients only) according to Lee et al. [2007] were performed as reported. In experiment replicas, mean intraassay and interassay coefficients of variability were both less than 10% for the PCR assay (plasmid scales).

Statistical Analysis

Comparison between medians was performed with the Wilcoxon test for paired data, while the Mann-Whitney *U*-test was used for unpaired data. The correlation between onset of HRV infection after transplantation and duration of HRV infection was investigated by means of a nonlinear curve fitting analysis.

RESULTS

Respiratory Virus Infection Rates

During the 2006–2007 winter–spring season, 602 patients admitted to hospital were examined for respiratory viruses in nasopharyngeal aspirates or

bronchoalveolar lavage samples. On the whole, 348 (57.8%) were found to carry one or more respiratory viruses, whereas 254 (42.2%) patients had virus-free respiratory samples collected during the hospital stay or during medical control visits in the out-patient unit. The total number of respiratory samples examined was 967. Of these, 519 (53.7%) were positive for one or more respiratory viruses, whereas 448 (46.3%) were negative. Since a fair number of patients were followed in the out-patient unit for a sustained period of time after hospital discharge, episodes of respiratory virus infection were considered different from one another for the same patient, when the virus responsible for one episode disappeared from respiratory secretions for at least 2 weeks (i.e., upon two subsequent examinations), while a subsequent episode was considered to be caused by a different virus. On this basis, 330 were single infection episodes, and 62 were co-infections by two or three respiratory viruses, resulting in a total number of 392 respiratory infection episodes.

The two viruses causing by far the largest number of episodes of single infection and co-infection were HRVs and RSV (Fig. 1B,C). In detail, HRVs were associated with 107 (27.3%) episodes of single infection and 41 (10.5%) episodes of co-infection (Table I). In parallel, RSV was associated with 120 (30.6%) episodes of single infection and 34 (8.7%) episodes of co-infection (Table I). On the whole, HRVs and RSV were associated with 148/392 (37.8%) and 154/392 (39.3%) episodes of respiratory infection, respectively (Table I). When considering the 18 episodes of HRV + RSV co-infection, the total number of respiratory episodes involving HRV and/or RSV was 284/392 (72.4%).

The absolute numbers of patients and respiratory samples examined and the numbers of patients and samples positive for respiratory viruses are shown in Figure 1A,D, respectively, according to age (pediatric and adult patients) and immune competence.

As for the distribution of the two most represented infections, 188/284 (66.2%) episodes of HRV and RSV infection or co-infection interested immunocompetent infants and young children (15 cases of double infection), while 41 (14.4%) affected young immunocompromised patients (3 cases of double infection), and 55 (19.4%) adult ($n = 13$ immunocompetent, and $n = 42$ immunocompromised) patients (Table I). However, while the absolute number of HRV and RSV infections was significantly higher in young immunocompetent patients compared to young immunocompromised and adult patients, the percentage of HRV infections was comparable in young and adult immunocompetent and immunocompromised patients. On the contrary, the percentage of RSV infections was significantly higher in pediatric immunocompetent patients compared to other groups. In addition, the ratio of HRV/RSV infections was 0.6 (predominant RSV infections) in pediatric immunocompetent patients, whereas it was 1.7 in young immunocompromised patients. In both groups of adults, the ratio was greater than 2.0 in immunocompromised, and greater than 3.0 in immunocompetent patients (Table I).

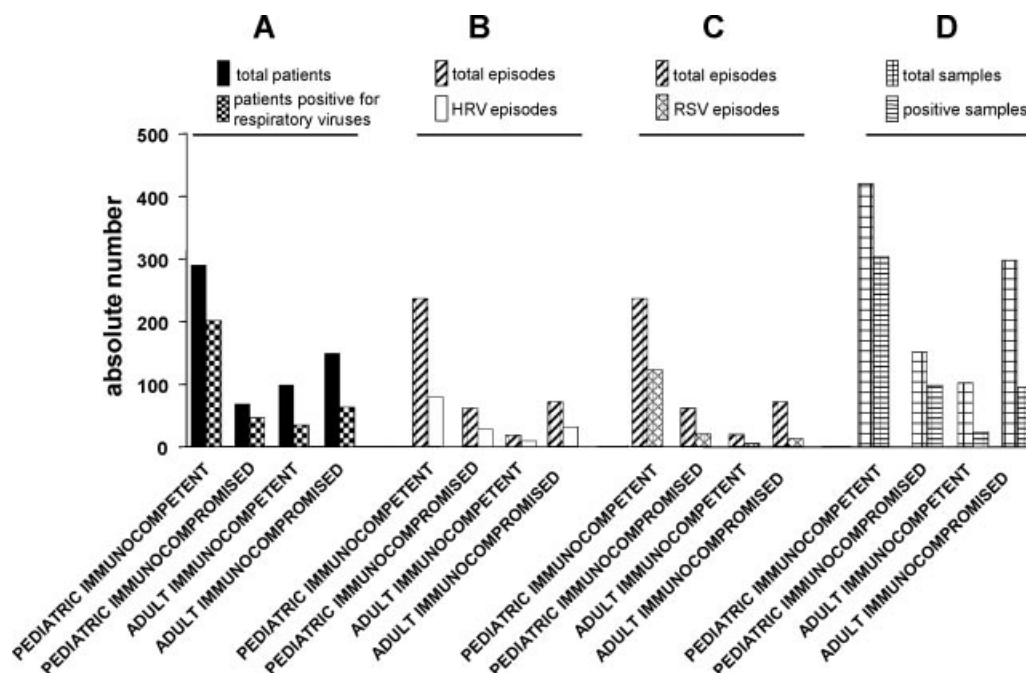


Fig. 1. For the four groups of subjects examined (pediatric immunocompetent and immunocompromised subjects, adult immunocompetent and immunocompromised patients), absolute numbers of the indicated parameters are reported, as follows. (A) Patients positive for respiratory viruses/total number of patients; (B) HRV episodes/total respiratory episode number; (C) RSV episodes/total respiratory episode number; (D) total number of positive respiratory samples/total number of respiratory samples examined.

The most interesting features of the relationship observed in the four groups of patients between HRV load and clinical symptoms are reported as follows.

Pediatric Immunocompetent Patients

On the whole, 40 patients were selected from this group due to the availability of follow-up data and

clinical findings. Out of 40 infants and young immunocompetent children with an episode of acute respiratory tract infection undergoing follow-up, 26 were admitted to hospital within a median time of 3 (range 1–5) days after onset of symptoms and examined within 24–48 hr. Of these, 15 showed the presence of an HRV strain only, and 11 had a co-infection by another respiratory virus associated with HRV. The remaining 14 young patients

TABLE I. Relative Frequencies of Respiratory Episodes Caused by Human Rhinoviruses (HRV) and Respiratory Syncytial Virus (RSV) in Hospitalized Immunocompetent and Immunocompromised Patients During the Winter–Spring Season 2006–2007

Type of virus and infection	No. of episodes (%) of respiratory infection detected in				Total
	Pediatric patients		Adult patients		
	Immunocompetent (n = 290)	Immunocompromised (n = 68)	Immunocompetent (n = 95)	Immunocompromised (n = 149)	
HRV					
Single infection	49 (45.8)	21 (19.6)	10 (9.3)	27 (25.3)	107
Co-infection	31 (75.6)	7 (17.1)	0	3 (7.3)	41
Total	80 ^a (54.1)	28 ^b (18.9)	10 (6.7)	30 (20.3)	148
RSV					
Single infection	94 (78.3)	11 (9.2)	3 (2.5)	12 (10.0)	120
Co-infection	29 (85.3)	5 (14.7)	0	0	34
Total	123 ^a (79.9)	16 ^b (10.4)	3 (1.9)	12 (7.8)	154
Ratio HRV/RSV episodes					
Single infection	0.5	1.9	3.3	2.2	0.9
Co-infection	1.1	1.4	NA	NA	1.2
Total	0.6	1.7	3.3	2.5	0.9

NA, not applicable.

^aFifteen patients had a HRV + RSV co-infection.

^bThree patients had a HRV + RSV co-infection.

TABLE II. Human Rhinovirus (HRV) Load and Clinical Follow-Up of 15 Young Patients Hospitalized for Upper (U) or Lower (L) Acute Respiratory Tract Infection Associated With a Single HRV Infection

Patient #	Age	URTI	LRTI	HRV load	Hospital stay (d)	Interval between paired respiratory samples (d)	Clinical follow-up
2	19m	+	-	1.2×10^6	None		Rhinorrhea
		-	-	$<1.0 \times 10^2$	-	16	Resolution
3	6y	+	+	3.3×10^6			Wheezing
		-	-	4.2×10^4	8	7	Resolution
4	3y	+	+	9.0×10^6			Bilateral bpn
		-	-	1.0×10^5	10	7	Resolution
5	2y	+	+	1.3×10^6	5	NA	Wheezing
9	2m	+	+	6.0×10^6			Hypotransparency area
		-	-	2.4×10^7	7	6	Resolution
18	4y	+	+	2.3×10^5			Wheezing + bpn
		-	-	3.2×10^2	9	14	Resolution
19	3y	+	+	5.1×10^6			Wheezing + pn
		+	-	$<1.0 \times 10^2$	9	6	Resolution
20	3m	+	+	9.3×10^6			bpn
		-	-	8.9×10^6	6	5	Resolution
22	10m	+	+	4.8×10^6			pn-wheezing
		+	-	1.1×10^8	5	4	Partial resolution
23	6m	+	+	4.6×10^6			Wheezing
		-	-	1.2×10^5	6	5	Resolution
25	12m	+	+	7.0×10^5			pn
		+	+	2.0×10^3	16	15	Improvement
26	6m	+	+	6.1×10^7	?	NA	Wheezing
27	2m	-	+	5.7×10^6	?	NA	Wheezing + pn
28	8m	+	+	1.2×10^7			pn
		±	±	5.6×10^6	9	9	Partial resolution
29	6m	+	+	1.1×10^7			Wheezing
		-	-	4.0×10^2	17	17	In resolution

bpn, bronchopneumonia; pn, pneumonia; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; d, days; m, months; y, years; NA, not applicable.

were examined late (10–15 days) with respect to onset of the respiratory episode or during chronic respiratory infection episodes.

Of the 15 patients positive for HRV only 9 showed a fall in viral load from admission through discharge in close association with improvement of clinical symptoms (Table II). In three patients (#5, #26, and #27), a high viral load was detected in the single sample taken at admission concomitantly with acute respiratory symptoms. However, in three patients (#9, #20, and #22), viral load increased or remained stable from admission to discharge in contrast with improvement of clinical symptoms (Table II).

In the group of 11 patients with co-infection (Table IV), HRV was present at a different titer in nasopharyngeal aspirates in association with another respiratory virus (hPIV-3 in 1 patient, RSV-A in 7 patients, and RSV-B in 3 patients). In all patients, the HRV-associated virus was detected at a titer greater than 1.0×10^5 RNA copies/ml nasopharyngeal aspirate at the time of admission to hospital, and a variably lower titer at the time of discharge. As for HRV, the virus was detected at a titer greater than 1.0×10^5 copies/ml nasopharyngeal aspirate in 5/11 patients (#1, #6, #7, #8, and #17), and in four of these patients the HRV titer decreased at discharge with respect to patient admission. However, in the fifth (#6) patient the HRV titer rose from 10^6 to 10^7 , and in two additional patients (#11 and #13) the titer rose from 10^2 to 10^4 during the hospital stay. All 11 patients were

clinically affected by bronchiolitis or pneumonia, often in association with wheezing at the time of admission, while at the time of hospital discharge all patients showed clinical improvement including the three patients showing a rise in HRV titer during the hospital stay (Table III).

As shown in Table IV, in patients with single HRV infection, viral load was significantly higher ($P = 0.03$) at time of admission to hospital (5.1×10^6 RNA copies/ml) with respect to time of dismissal (7.1×10^4 RNA copies/ml). In patients with a co-infection, the HRV load was comparable ($P = ns$) between admission (1.3×10^4 copies/ml) and discharge (1.8×10^4 copies/ml), whereas the co-infecting virus showed a viral load in nasopharyngeal aspirates at admission (1.4×10^7 copies/ml) significantly higher ($P = 0.015$) than that found at discharge (1.0×10^4 copies/ml). On the other hand, at admission, HRV load in patients with a single infection (5.1×10^6 copies/ml) was significantly higher ($P = 0.002$) than that of patients with a co-infection (1.3×10^4 copies/ml), while HRV viral load at discharge was comparable ($P = 0.45$) in patients with single infection (7.1×10^4 copies/ml) and with co-infection (1.8×10^4 copies/ml). Co-infection did not entail a greater duration in the hospital stay (8.8 days) as compared to single HRV infection (8.5 days).

Finally, in 14 additional young patients with delayed admission to hospital with respect to onset of symptoms (not reported), HRV detection at a titer consistently

TABLE III. Human Rhinovirus (HRV) and Co-Infecting Virus Loads, and Clinical Follow-Up in 11 Young Patients Admitted to Hospital for an Acute Respiratory Tract Infection Associated With Double Viral Infection

Patient #	Age	URTI	LRTI	Co-infecting virus	HRV load	Co-infecting virus load	Hospital stay (d)	Interval between respiratory samples (d)	Clinical follow-up
1	12m	+	-	hPIV-3	2.1×10^5	ND	12	11	Bronchitis Improvement
6	3m	+	+	RSV-A	1.8×10^3	ND			
		-	-		2.1×10^6	2.7×10^5	8	5	pn + wheezing Resolution
		-	-		2.6×10^7	2.3×10^2			
7	13m	+	+	RSV-A	3.2×10^5	3.8×10^7	11	25	Resolution pn + wheezing
		-	-		1.8×10^4	1.6×10^3			
		-	-		1.0×10^2	1.0×10^3	19	8	Improvement Bronchiolitis
8	2m	+	+	RSV-B	2.4×10^6	1.8×10^9			
10	3m	+	+	RSV-A	2.2×10^4	3.3×10^6	9	8	pn Improvement
		+	-		2.7×10^3	3.4×10^5			
11	3y	+	+	RSV-A	$<1.0 \times 10^2$	3.3×10^3	7	6	pn Improvement
		-	-		$<1.0 \times 10^2$	2.0×10^5			
12	11m	+	+	RSV-B	1.0×10^2	3.3×10^2	NA	NA	pn
13	5m	+	+	RSV-B	$<1.0 \times 10^2$	2.5×10^8	7	7	Bronchiolitis + pn Improvement
		-	-		7.7×10^4	3.2×10^5			
15	6m	-	+	RSV-A	1.0×10^2	6.6×10^5	9	3	Wheezing Improvement
		-	+		$<1.0 \times 10^2$	8.9×10^4			
		-	-			$<1.0 \times 10^2$	7	7	Recovery Wheezing
17	5m	-	+	RSV-A	1.5×10^7	2.2×10^7			
		-	-		3.9×10^6	3.4×10^5	7	6	Improvement
24	2y	+	+	RSV-A	1.3×10^4	1.8×10^7			

pn, pneumonia; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; d, days; RSV-A, RSV-B, respiratory syncytial virus A, B; hPIV-3, human parainfluenzavirus 3; ND, not done; NA, not applicable; m, months; y, years.

lower than 1.0×10^5 RNA copies/ml nasopharyngeal aspirate at the time of admission to hospital did not provide any useful result in view of the definition of the current respiratory tract infection.

Pediatric Oncohematologic Patients

Of 28 episodes of symptomatic HRV respiratory infection (21 single infections and 7 co-infections) concerning

TABLE IV. Human Rhinovirus (HRV) Load at Admission and Discharge in Patients With Acute Respiratory Tract Infection Associated With HRV Single Infection or HRV + RSV Co-Infection

HRV infection	Median (range) viral load at			Duration of hospital stay (days)
	admission	p	discharge	
Single infection				
HRV load:				
median	5.1×10^6	0.03	7.1×10^4	8.5
range	$(2.3 \times 10^5 - 6.1 \times 10^7)$		$(<1.0 \times 10^2 - 1.1 \times 10^8)$	(5-16)
p	0.002		ns	ns
RSV-coinfection				
HRV load:				
median	1.3×10^4	ns	1.8×10^4	8.0
range	$(<1.0 \times 10^2 - 1.5 \times 10^7)$		$(<1.0 \times 10^2 - 2.6 \times 10^7)$	(2-19)
p	0.001		ns	
RSV load:				
median	1.4×10^7	0.01	1.0×10^4	
range	$(2.2 \times 10^4 - 1.8 \times 10^9)$		$(<1.0 \times 10^2 - 3.4 \times 10^6)$	

ns, not significant.

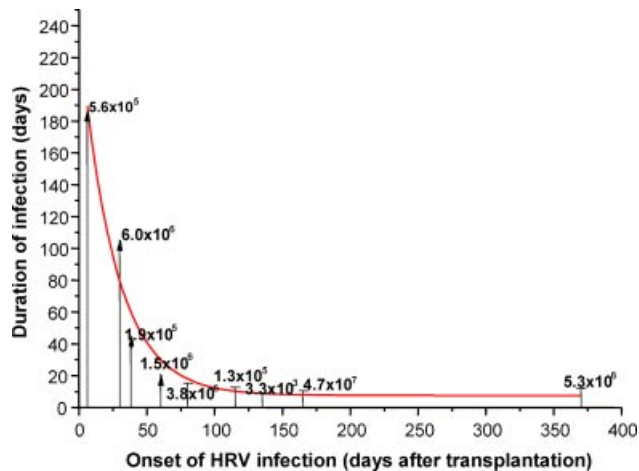


Fig. 2. Inverse correlation between the duration (days) of HRV infection and time (days) elapsed after stem cell transplantation prior to onset of HRV infection in nine young patients. For each patient, vertical arrow (\uparrow) indicates HRV persistence at time of last viral control, whereas HRV disappearance is indicated by (\downarrow). Over each patient, the peak HRV load detected in respiratory secretions for each patient is reported.

as many young oncohematologic patients, nine (with single infection) were followed for at least 6 months. As shown in Figure 2, an inverse correlation (described by an exponential decay model) was observed between time (days) from transplantation to onset of HRV infection and duration of HRV infection (adjusted $R^2 = 0.95$). Patients experiencing HRV infection within 60 days from the allograft showed a high viral load in the last nasopharyngeal aspirate sample tested, thus suggesting sustained viral excretion. In patients experiencing HRV infection >80 days after transplantation, HRV disappeared from nasopharyngeal aspirates within 1–2 weeks after onset of infection. However, in these patients, the peak viral load measured during the infection was not different in magnitude from viral load peaks observed in patients undergoing HRV infection in the early post-transplantation period.

Three patients of this group were infected by HRV-C strains. One of these patients, who had received two T-cell depleted allografts for relapsed leukemia from the HLA-haploidentical mother, died 6 months after transplantation due to respiratory failure (hemorrhagic alveolitis). The patient had been infected by two different HRV-C strains, one detected in nasopharyngeal secretions upon transplant, and the other detected in bronchoalveolar lavage at the time of death.

Adult Immunocompetent Patients

As observed in immunocompetent pediatric patients, immunocompetent patients from the adult population, who exhibited levels of HRV load greater than 1×10^5 RNA copies/ml, as a rule, presented with respiratory symptoms interesting the upper or lower respiratory tract, whereas lower viral levels did not appear to correlate with the kinetics of clinical symptoms. This observation was made during follow-up, when it was noted

that in some patients low HRV load became negative in association with persistent clinical symptoms, and in other patients low viral load remained unchanged in association with improvement of clinical symptoms.

Lung Transplant Recipients

The pathogenic role of HRV infection was also investigated in 12 lung transplant recipients. During follow-up, these patients were examined either in nasopharyngeal aspirates ($n = 8$ positive/24 examined) during acute respiratory tract infection episodes, or in bronchoalveolar lavage (even in the absence of symptoms) following a routine bronchoscopy examination ($n = 14$ positive/84 examined). In detail, nasopharyngeal aspirates from five episodes of acute upper respiratory tract infections interesting five lung transplant recipients (#1, #2, #3, #4, and #6, Table V) were found to be associated with a high HRV load ($>10^6$ RNA copies/ml nasopharyngeal aspirates) in upper respiratory secretions, thus documenting an association between respiratory HRV load and upper respiratory clinical symptoms (no other infectious agents were detected in these patients). On the other hand, of the remaining seven patients, three (#5, #9, and #11) with respiratory tract infection showed a HRV load $>10^5$ RNA copies/ml bronchoalveolar lavage, while the other four patients (#7, #8, #10, and #12) free of acute respiratory symptoms in the lower respiratory tract had a viral load of 10^3 – 10^4 RNA copies/ml bronchoalveolar lavage. This finding documented the HRV presence in the lower respiratory tract in the absence of overt clinical symptoms (Table V). Clinical follow-up excluded the possibility that the low HRV amounts detected in bronchoalveolar lavage in these patients was related to the end of a previous lower respiratory tract infection episode or the onset of a new respiratory infection episode occurring in the lower respiratory tract.

DISCUSSION

Results of the present study focusing on the analysis of the correlation of HRV load in respiratory secretions and presence/absence of clinical symptoms are limited to the winter–spring season and allow the formulation of some general conclusions: (i) viral load greater than 10^5 HRV RNA copies/ml nasopharyngeal aspirate appears to be mostly associated with presence of clinical symptoms both in upper and lower respiratory infections; (ii) in immunocompetent infants and young children, in the case of HRV + RSV co-infection, the quantification of viral load allows to hypothesize the viral agent responsible for the current respiratory episode and, if required, to adopt the adequate therapeutic measures; (iii) in young stem cell transplantation recipients, a 6-month follow-up allowed the detection of an inverse correlation between time from transplantation and duration of HRV infection, while the peak viral load was not influenced by the time elapsed from transplantation to the occurrence of viral infection; (iv) in adult lung transplant recipients examined in bronchoalveolar lavage during post-transplant follow-up, HRV load lower than

TABLE V. Relationship of HRV Load and Clinical Symptoms in 12 Lung Transplant Recipients

Patient # (age)	Days after transplantation	Symptoms		HRV load (RNA copies/ml)		Associated infection
		URTI	LRTI	NPA	BAL	
1 (56y)	1,084	+	+	1.4×10^6	ND	<i>P. aeruginosa</i>
2 (61y)	887	+	-	3.9×10^6	ND	—
3 (59y)	957	+	-	2.5×10^7	ND	—
4 (31y)	1,026	+	-	1.1×10^6	ND	—
5 (36y)	707	+	+	ND	3.7×10^7	—
6 (30y)	551	+	-	7.6×10^6	ND	—
7 (45y)	376	-	-	ND	2.6×10^3	—
8 (56y)	305	-	-	ND	2.0×10^4	EBV (BAL)
9 (57y)	631	+	+	ND	9.2×10^6	<i>P. aeruginosa</i>
10 (36y)	193	-	-	ND	3.4×10^3	—
11 (37y)	510	+	-	ND	4.1×10^5	EBV
12 (50y)	260	-	-	ND	1.0×10^3	—

URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; NPA, nasopharyngeal aspirate; BAL, bronchoalveolar lavage; ND, not done.

10^5 RNA copies/ml did not appear to be associated with the presence of clinical symptoms, which were detected with larger viral loads.

There has been a lot of debate regarding the presence of HRV in the respiratory tract of asymptomatic children and adults [Nicholson et al., 1996; Blomqvist et al., 2002; Nokso-Koivisto et al., 2002; Kusel et al., 2006; Rawlinson et al., 2003; Van Bentem et al., 2003; Winther et al., 2006]. Using molecular assays, given the 1- to 3-week duration of HRV shedding around each infection episode, incidental HRV detection during an independent hospitalization may occur frequently [Turner, 2007]. This event may occur in individuals recently experiencing respiratory symptoms or having contact with household members with an acute respiratory tract infection, or in patients who develop symptoms after sampling. However, even excluding these individuals, it is estimated that overall 5% of apparently healthy children are picornavirus-RNA-positive in the absence of previous or incipient respiratory symptoms [Nokso-Koivisto et al., 2002].

In order to address the clinical significance of HRV present in the respiratory tract, it was decided to quantify HRV load in respiratory secretions and to correlate viral load with clinical symptoms. We found that a viral load greater than 10^5 RNA copies/ml in nasopharyngeal aspirate appeared to be associated with the presence of clinical symptoms either interesting the upper or the lower respiratory tract, while a viral load lower than 10^5 copies/ml was infrequently associated with clinical symptoms. Under experimental conditions, it has been shown that HRVs are able to grow in the lower respiratory tract, as demonstrated by their ability to grow both in vitro in primary human bronchial epithelial cells [Schroth et al., 1999; Papadopoulos et al., 2000] and in vivo in lower airway secretions following experimental infection by intranasal instillation and aerosols [Gern et al., 1997; Papadopoulos et al., 2000]. In addition, it was shown that in bronchoscopy samples, virus titers were similar to those observed in nasal brush samples [Halperin et al., 1983]. These findings support the assumption that virus quantitation in nasopharyngeal

aspirates in lower respiratory tract infections mirror the virus amount present in the lower respiratory tract. Along this line of thought, it appears reasonable to attribute the etiologic role of a lower respiratory episode to HRVs, whenever a high viral load is detected in nasopharyngeal aspirates from patients not shedding other viruses in respiratory secretions.

As reported by Deffernez et al. [2004], all HRV serotypes tested ($n = 29$) and clinical isolates are detectable by their assay. However, it is not possible to exclude a lower sensitivity for detection of other serotypes, including those belonging to the recently identified group C. In this study, some of the amplicons were sequenced, thus showing the ability of the assay to detect strains belonging to A, B, and C species HRV.

As many as 21/62 episodes (33.9%) of dual HRV + RSV (in three episodes a third virus was detected) co-infection were observed. In previous studies on bronchiolitis, no greater disease severity was reported on admission of patients with co-infection compared to patients with single infection [Andreoletti et al., 2000; Papadopoulos et al., 2002]. In our study, HRV + RSV co-infections were predominant among the other combinations of co-infections. Virus quantification allowed to hypothesize that RSV was the etiologic agent of the acute respiratory episode, while HRV, present in lower amounts, was more likely to be considered a bystander virus with respect to the current respiratory infection episode. Although it is relatively infrequent that two viruses are detected at a high concentration in respiratory secretions ($>10^5$ RNA copies/ml NPA), our experience indicates that more than the absolute value of viral load in the admission sample, the kinetics of viral load drop in association with improvement of clinical symptoms may discriminate, among two or three co-infecting viruses, the etiologic agent of a respiratory syndrome [Campanini et al., 2007; Gerna et al., 2008].

In the present study, HRV was the second most common respiratory virus causing hospitalization of immunocompetent children after RSV. In children with single HRV infection, the almost constant association with wheezing and/or pneumonia appears to confirm recently

published data on the frequency of HRV-associated hospitalizations in young children [Miller et al., 2007]. This study reported a rate of HRV-associated hospitalizations of 5/1,000 children <5 years of age. This rate increased from 5 months to 5 years of age and was higher in children with a history of wheezing/asthma. However, whether HRV infection predisposes to asthma or patients with asthma are more susceptible to HRV infection remains to be defined. The current study confirmed that children with a predisposition to wheezing/asthma are at risk of hospitalization when affected by HRV infection.

The median duration of HRV illness is 7 days, some patients, however, experience a 2-week duration [Hayden et al., 2003]. In two stem cell transplant recipients repeat bronchoalveolar lavage samples were HRV-positive at intervals of 31 and 44 days after first virus detection. This finding raised the possibility of prolonged lower respiratory tract replication [Ison et al., 2003]. In the present study, we were able to detect an inverse correlation between duration of HRV infection and time (days) elapsed after transplantation in a group of nine young stem cell transplant recipients who were followed for at least 6 months. This finding supports the recommendation, when feasible, to delay transplantation by at least 1 month, if HRV is detected in nasopharyngeal aspirates in the period preceding the beginning of the preparative regimen to the allograft. On the other hand, peak viral loads were not influenced by the time elapsed from transplantation. In the past, cases of respiratory failure along with HRV respiratory infection were either associated with a co-pathogen or attributed to chemotherapy/radiotherapy [Ghosh et al., 1999; Ison et al., 2003; Van Kraaij et al., 2005]. However, two cases of fatal lower respiratory tract infections in adult stem cell transplant recipients likely caused by HRV have been recently reported [Peck et al., 2007]. In our study, all patients, but one, survived HRV infection during follow-up. The case of a transplanted patient who succumbed to pneumonia following HRV-C infection underscores the potential severe pathogenic role of this newly identified group of HRVs [Lau et al., 2007; Lee et al., 2007].

Finally, in a group of lung transplant recipients examined during a long-term follow-up, it was documented that, even in lower respiratory tract secretions, the presence of low HRV amounts was not associated with overt clinical symptoms, which, by contrast, were consistently observed in the presence of higher viral load. Data previously reported on the prognostic impact of HRV present in bronchoalveolar lavage of lung transplant recipients are contradictory [Holt et al., 1997; Malcolm et al., 2001]. These findings may help to clarify this issue.

In conclusion, this study shows that: (i) HRV quantitation in nasopharyngeal aspirates appears to correlate with clinical symptoms associated with lower respiratory tract infections; (ii) HRV + RSV co-infections do not appear to worsen the prognosis of lower respiratory tract infection episodes; (iii) in young stem cell transplant

recipients, an inverse correlation between duration of HRV infection and time at which the infection occurs after transplantation is observed; (iv) in lung transplant recipients HRV load in bronchoalveolar lavage appears to correlate with clinical symptoms.

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