

Frequent Detection of Respiratory Viruses in Adult Recipients of Stem Cell Transplants with the Use of Real-Time Polymerase Chain Reaction, Compared with Viral Culture

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Background. Respiratory virus infections have been recognized as important causes of severe pneumonia in patients who have undergone stem cell transplantation (SCT). Reported incidences of respiratory virus infection in adult SCT recipients vary in the literature from 3.5% to 36% when determined by viral culture. However, a more sensitive method to assess the presence of respiratory viruses in the lower airways may be important for delineation of the true incidence of respiratory virus-associated pneumonia and may be essential for guidance on implementation of antiviral therapy and prevention or limitation of nosocomial spread of infection with respiratory viruses.

Methods. To determine the incidence and severity of respiratory tract illness (RTI) and to assess the diagnostic value of real-time reverse-transcriptase polymerase chain reaction (RT-PCR) versus viral culture, 72 SCT recipients were monitored during a 6-month period.

Results. A respiratory virus was detected in 21% of episodes of RTI by viral culture and in 63% of RTI episodes by real-time RT-PCR ($P < .0001$). In lower respiratory tract illness, real-time RT-PCR was much more sensitive than viral culture for detection of respiratory virus (73% vs. 9%; $P = .008$). The mortality rate for patients with respiratory virus-associated lower respiratory tract illness (25%) was similar to rates reported elsewhere. Respiratory viruses (predominantly rhinovirus) were detected by real-time RT-PCR in 9% of samples obtained from symptom-free SCT recipients at predetermined times by real-time RT-PCR and by viral culture in 1% ($P < .0001$), indicating that asymptomatic shedding of respiratory viruses also occurs.

Conclusion. We conclude that, although asymptomatic shedding of respiratory virus occurs, respiratory viruses are frequent causes of RTI in SCT recipients.

Pneumonia is one of the most common infectious complications of stem cell transplantation (SCT). During the past decade, infections due to respiratory viruses have increasingly been recognized as important causes of severe pneumonia in patients who have undergone SCT [1–3]. Respiratory syncytial virus (RSV) and parainfluenza virus (PIV) have been especially associated

with severe lower respiratory tract infections after SCT, causing high morbidity and mortality [4–8]. However, the role of other respiratory viruses has not yet been sufficiently elucidated, and it is not clear whether respiratory viruses by themselves cause lower respiratory tract illnesses (LRTIs) or whether respiratory virus infection predisposes patients to additional infections [8–11]. A sensitive method to assess the presence of respiratory viruses in the lower airways may be important to delineate the true incidence of respiratory virus-associated pneumonia. Moreover, a rapid and sensitive method for detection of respiratory viruses may be essential to guide implementation of therapy with current antiviral agents and to prevent or limit nosocomial spread of infection with respiratory viruses [12–14].

Real-time RT-PCR has been proven to be an ex-

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tremely specific, sensitive, and rapid method for detection of respiratory viruses and can be implemented more easily than classic PCR [15, 16]. In a previous retrospective study, we demonstrated that nested PCR was far more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia [17]. Therefore, to determine the incidence and severity of respiratory virus infections after transplantation and to assess the diagnostic value of real-time RT-PCR for the detection of respiratory viruses compared with viral culture, we conducted a prospective study of persons who underwent autologous or allogeneic SCT.

PATIENTS, MATERIALS, AND METHODS

Patients

A single-center prospective study was performed from 1 October 1999 through April 2001 after approval by the local ethics committee. Written informed consent was required from all participating patients who underwent allogeneic or autologous SCT. Included patients were monitored for respiratory viral infections (due to influenza virus A or B, RSV A or B, PIV 1–4, rhinoviruses, enteroviruses, human coronavirus OC43 and 229E, or adenoviruses) during the 6 months after transplantation. If patients reported a respiratory tract illness, a combined nose-throat swab specimen was obtained within 48 h for viral culture and real-time RT-PCR for detection of respiratory viruses. Nose-throat swab specimens were also obtained, if possible, on days 2–3, 4–7, 8–14, and 15–21 after the initial complaint. Samples collected during an episode of respiratory tract illness were defined as “diagnostic samples.” In addition, nose-throat swab specimens were obtained at the time of hospital admission and on weeks 3, 8, 16, and 26 after transplantation to monitor asymptomatic excretion of respiratory viruses and to establish the diagnostic value of detection by real-time RT-PCR; these were referred to as “surveillance samples.” Diagnostic procedures, such as radiography, CT of the thorax, and bronchoscopy with bronchoalveolar lavage (BAL), were performed on the basis of the judgment of the treating physician.

Conditioning Regimens and Transplantation Procedures

Autologous SCT. Patients who underwent autologous SCT for acute leukemia received conditioning regimens either with cyclophosphamide followed by 8 Gy of total body irradiation or with oral busulphan and cyclophosphamide. Recipients of autologous SCT who were treated for lymphoma or multiple myeloma received the BEAM preparative regimen (1,3-bis-(2-chloroethyl)-1-nitrosourea [BCNU], etoposide, cytarabine, and melphalan) or high-dose melphalan, respectively.

Allogeneic SCT. Patients were treated with cyclophosphamide (60 mg/kg iv q.d.) for 2 days followed by two 6-Gy doses of total body irradiation. Patients who underwent vol-

untary allogeneic SCT with an unrelated donor also received antithymocyte globulin (4 mg/kg iv q.d.) during the 5 days before commencement of cyclophosphamide therapy. All transplant recipients received partial T cell–depleted donor marrow ($1\text{--}2 \times 10^5$ T cells/kg).

Infection Prophylaxis and Infection-Prevention Measures

During hospitalization, surveillance oropharynx, feces, and urine samples were obtained at least once per week. Antibacterial prophylaxis consisted of oral ciprofloxacin (500 mg b.i.d.) and oral amphotericin B (200 mg q.i.d.) combined with fluconazole (50 mg q.d.). For prevention of bacteremia due to α -hemolytic streptococci, patients received cephalothin (1 g iv 6 times per day) after transplantation. The antimicrobial regimen was continued until the granulocyte count was $>0.5 \times 10^9$ cells/L. Persons who underwent allogeneic SCT routinely received valaciclovir (500 mg b.i.d.) and cotrimoxazole (480 mg q.d.) during the first 12 months after transplantation. Persons who underwent allogeneic SCT who had positive cytomegalovirus pp65 test results during the first 3 months after SCT received preemptive therapy with ganciclovir [18]. Hospitalized patients convalesced in a single room with free entry for staff and visitors. Careful hand washing and the use of low-count microbial food were the only preventive measures used for these patients.

Definitions of Respiratory Tract Illness

Respiratory tract illness was considered to be hospital acquired if symptoms developed ≥ 4 days after hospital admission. An upper respiratory tract illness (URTI) was defined by clinical symptoms as rhinorrhea, pharyngitis, laryngitis, or cough without clinical or radiological evidence of lower respiratory tract involvement and/or hypoxemia. LRTI was defined by the development of radiographic pulmonary abnormalities in patients with signs and symptoms such as cough, dyspnea, sputum production, and fever. Simultaneous infection with ≥ 2 different viruses was considered to be a single episode of infection.

Diagnostic Methods for Routine Detection of Respiratory Viral Pathogens

BAL fluid samples and nose-throat swab specimens were placed in a tube containing virus transport medium, immediately transported to the laboratory, and processed directly or stored at 4°C for a maximum of 24 h. The nose-throat and BAL fluid samples were vortexed for 10 s and centrifuged at 2000 g for 15 min. Part of the supernatant was used for conventional viral culture and shell vial culture for the detection of respiratory viruses (i.e., adenoviruses, PIV, RSV, influenza viruses, and picornaviruses) [17]. The remaining material was stored at -70°C until further analysis by real-time RT-PCR. BAL fluid samples were also processed for routine bacterial, mycobacterial, and fungal cultures and for examination of herpesviruses.

Viral RNA Extraction, cDNA Synthesis, and Real-Time PCR

Total nucleic acid was extracted from 100 μ L of patient material (BAL fluid or nose-throat swab specimens) according to the method of Boom et al. [19] using the MagnaPure LC Total Nucleic Acid Kit (Roche Diagnostics). The total nucleic acid was directly used for the amplification of the adenovirus (DNA virus), and the remainder of the nucleic acid was used in 1 cDNA reaction, as described elsewhere [16]. The cDNA was subsequently used in 6 different real-time RT-PCRs for the detection of influenza viruses A and B, PIV 1–4, rhinoviruses, enteroviruses, RSV A and B, and human coronaviruses OC43 and 229E.

All of the primers and probes were selected from GenBank and were based on genomic regions of high conservation: the matrix gene was used for influenza A virus, the hemagglutinin gene was used for influenza B virus [16], the 5'-noncoding region was used for the Picornaviruses [20], the N-gene was used for RSV A and B [21] and coronaviruses 229E and OC43 [22], the hemagglutinin-neuraminidase glycoprotein gene was used for PIV 1–4, and the hexon gene was used for adenoviruses. The primer and probe concentrations were optimized, and the real-time Taqman PCR was performed as described elsewhere [16, 20]. To control for correct isolation and amplification, all samples were spiked before extraction with internal control virus (murine encephalomyocarditis virus [RNA virus] and phocine herpes virus [DNA virus]) [23]. The fluorogenic probes recognizing the human respiratory viruses were all labelled with the 5' reporter dye FAM and a 3' quencher dye TAMRA, whereas the fluorogenic probes recognizing the internal control viruses were all labelled with the 5' reporter dye VIC. By using these different fluorogenic labels, amplification of a human respiratory virus can be distinguished from amplification of the internal control virus.

Statistical Analysis

Descriptive statistics were expressed as median values. χ^2 Analysis with use of McNemar's or Fisher's exact test was performed to determine the degree of significance between the various variables.

RESULTS

Eighty-two SCT procedures were performed on 81 patients. Nine patients were excluded from the study (6 patients refused participation, and 3 patients underwent follow-up after transplantation elsewhere). The 72 remaining patients had a complete follow-up after SCT for 6 months (or <6 months in the event of early death). Patient characteristics are shown in table 1.

Data on 52 episodes of respiratory tract illness in 40 patients were evaluable for the detection of a respiratory virus (no nose-throat swab specimens were obtained during 4 episodes). A

Table 1. Characteristics of stem cell transplant (SCT) recipients.

Characteristics	Value
No. of patients	72
No. of patients, male/female	49/23
Age, median years (range)	44 (18–64)
Underlying disease	
Acute myeloid leukemia	15
Acute lymphoblastic leukemia	14
Chronic myeloid leukemia	16
Non-Hodgkin lymphoma	10
Multiple myeloma	10
Myelodysplastic syndrome	3
AL amyloidosis	2
Severe aplastic anemia	1
Hodgkin disease	1
Type of transplantation, no. (%) of patients	
Allogeneic SCT	37 (51)
Allogeneic SCT from a VUD	17 (24)
Autologous SCT	18 (25)
No. (%) of patients with an evaluable respiratory tract illness	40 (56)

NOTE. Data are no. of patients, unless otherwise indicated. VUD, voluntary unrelated donor.

comparison between episodes of respiratory tract illnesses with or without a respiratory virus is shown in table 2.

Detection of respiratory viruses during episodes of respiratory tract illness. A total of 153 nose-throat swab specimens and 11 BAL fluid samples were obtained during the 52 episodes of respiratory tract infection. With use of conventional viral culture, a respiratory virus was isolated in 11 (21%) of 52 episodes (table 3), compared with 33 (63%) of 52 episodes when real-time RT-PCR was used ($P < .0001$; PCR-positive samples included all culture-positive samples). The most frequently detected respiratory viruses were rhinoviruses (19 [58%] of 33 episodes). Adenoviruses and coronaviruses were only detected by real-time RT-PCR. There was no significant difference in the incidence of respiratory tract infection between the 3 different SCT modalities (table 4).

URTI. Forty-one (79%) of 52 episodes of respiratory tract illness were URTIs (table 3). Twenty-five of these episodes were found to have been associated with a respiratory virus by real-time RT-PCR, compared with only 10 episodes for viral culture ($P < .0001$). Rhinovirus was the predominant respiratory virus in URTI (39%). None of the patients had progression to an LRTI after URTI, and all patients recovered completely without the need for antiviral therapy.

LRTI. Eleven (21%) of 52 episodes of respiratory tract illness were LTRIs. All patients with an LRTI were admitted to the hospital. Fever, cough, dyspnea, and malaise were the predominant signs and symptoms in these patients, and the cases

Table 2. Comparison between episodes of respiratory tract illnesses with or without a detectable respiratory virus present.

Characteristic	Patients with episodes of respiratory tract illness		
	With a respiratory virus (n = 33)	Without a respiratory virus (n = 19)	All (n = 52)
Transplant type			
Allogeneic			
Related donor	19 (58)	12 (63)	31 (60)
Unrelated donor	7 (21)	2 (11)	9 (17)
Autologous	7 (21)	5 (26)	12 (23)
URTI	25 (76)	16 (84)	41 (79)
LRTI	8 (24)	3 (16)	11 (21)
Neutropenia	6 (18)	0 ^a	6 (11.5)
Immunosuppressive therapy	16 (48)	7 (37)	23 (44)
Graft-versus-host disease	10 (30)	5 (26)	15 (29)
Source of infection			
Nosocomial	10	2	12
Community acquired	23	17	40
Season when infection was acquired			
Winter ^b	21	9	30
Summer ^c	12	10	22
Time after SCT, median weeks	8	13	8

NOTE. None of the differences between the episodes were significant. LRTI, lower respiratory tract illness; SCT, stem cell transplantation; URTI, upper respiratory tract illness.

^a $P = .075$

^b October through March.

^c April through September.

had not been preceded clinically by a URTI. In 9 episodes, bronchoscopy with BAL was performed, and in 2 episodes, nose-throat swab specimens were obtained. A respiratory virus was detected by viral culture for 1 episode of LRTI and by real-time RT-PCR for 8 (73%) of 11 episodes ($P = .008$; table 3), with use of either BAL fluid samples ($n = 3$), combined nose-throat swab specimens ($n = 2$; no BAL was performed for these 2 patients), or both ($n = 3$). One BAL fluid sample contained 2 respiratory viruses: rhinovirus and parainfluenzavirus.

In the 3 patients who tested negative for a respiratory virus, a bronchoscopic biopsy revealed other causes of LRTI—namely, irradiation pneumonitis, invasive aspergillosis, and bronchiolitis obliterans. Four patients with an LRTI died (1 patient who died of invasive aspergillosis, 1 rhinovirus-positive patient with posttransplantation lymphoproliferative disease, and 2 patients in whom only a respiratory virus was detected [coronavirus for one patient and RSV for the other]). The death of the latter 2 patients was considered have been associated with respiratory virus LRTI (2 [25%] of 8 episodes). These 2 patients were the only ones who had been treated with aerosolized ribavirin.

Duration of viral detection in episodes of respiratory tract illness. Follow-up nose-throat samples were obtained during

35 of 52 episodes of respiratory tract illness (28 [68%] of 41 episodes of URTI and 7 [64%] of 11 episodes of LRTI). Real-time PCR detected respiratory viruses over a longer period of time than did viral culture. The initial respiratory virus could still be detected by means of real-time RT-PCR for 16 (64%) of 25 samples obtained 1 week after onset of symptoms, but it could be detected in none of the samples by viral culture ($P \leq .0005$). Rhinovirus was the predominant virus (11 [69%] of 16 samples), followed by coronaviruses (2 samples), RSV (2 samples), and enterovirus (1 sample).

Detection of respiratory viruses in the absence of respiratory tract illness. A total of 259 surveillance nose-throat swab specimens were obtained from the 72 patients. By viral culture, 3 samples tested positive for respiratory viruses, but by real-time RT-PCR, a respiratory virus could be detected in 24 samples ($P < .0001$) (table 5). Rhinovirus was the predominant pathogen in these surveillance samples (21 of 24 samples), but coronavirus (2 samples) and adenovirus (3 samples) were also detected. Surveillance nose-throat swab specimens obtained before SCT tested positive for a respiratory virus for 9 patients (rhinovirus, 8 patients; coronavirus, 1 patient); none of them had respiratory complaints at the time of collection. Three of

Table 3. Respiratory viruses detected either by viral culture or by real-time RT-PCR in 52 episodes of respiratory tract illness (RTI).

Finding	No. (%) of episodes of RTI					
	URTI (n = 41)		LRTI (n = 11)		Total (n = 52)	
	Viral culture result	Real-time RT-PCR result	Viral culture result	Real-time RT-PCR result	Viral culture result	Real-time RT-PCR result
Any respiratory virus	10 (24)	25 (61) ^a	1 (9)	8 (73) ^b	11 (21)	33 (63) ^c
Double infection	...	2	...	1	...	3 ^d
Rhinovirus	7 (17)	16 (39)	...	3 (27)	7 (13)	19 (37)
Influenza virus	1 (2)	2 (5)	1 (2)	2 (4)
Parainfluenzavirus	...	2 (5)	1 (9)	1 (9)	1 (2)	3 (6)
RSV	1 (2)	3 (7)	...	3 (27)	1 (2)	6 (12)
Human coronavirus	...	2 (5)	...	2 (18)	...	4 (8)
Adenovirus	...	1 (2)	1 (2)
Enterovirus	1 (2)	1 (2)	1 (2)	1 (2)

NOTE. LRTI, lower respiratory tract illness; RSV, respiratory syncytial virus; URTI, upper respiratory tract illness.

^a $P < .0001$.

^b $P = .008$.

^c $P < .0001$.

^d Three patients had a dual infection with a respiratory virus (RSV with rhinovirus, rhinovirus with adenovirus, or parainfluenza virus with rhinovirus).

these patients developed an URTI due to the initially detected virus (rhinovirus) ≤ 1 month after the initial collection. Prolonged asymptomatic excretion of rhinovirus was observed in 2 other patients (for 2 months for one patient and for 3 months for the other).

DISCUSSION

Our prospective study shows that respiratory viruses can frequently be detected in adults who undergo SCT. We found an incidence of URTI or LRTI associated with a respiratory virus of 21% by conventional viral culture and of 63% by real-time RT-PCR. In a previous retrospective study, we also observed a significant increase in the incidence of respiratory virus infection among immunocompromised patients when a nested RT-PCR was compared with viral culture [17]. Reported incidences of respiratory virus infections in adults who undergo SCT who have acute URTI and LRTI range in the literature from 3.5% to 36%. In these studies, viral culture or direct immunofluorescence examination of nose-throat swab, nasopharyngeal aspirate, or BAL fluid specimens was used [1–3].

Our data clearly demonstrate that URTI is associated with the detection of respiratory viruses. In contrast with other studies [1–3, 24], we did not observe that SCT recipients with an URTI had progression to an LRTI. Although we also showed that LRTI is often associated with the presence of respiratory viruses, all patients with an LRTI had clinical features of pneumonia without having made obvious complaints of a viral URTI previously, indicating that, in immunocompromised patients, respiratory viruses may cause LRTI that is not clinically pre-

ceded by an URTI. The rather low overall mortality rate in our study is in accordance with recent studies of immunocompromised patients with respiratory virus-associated respiratory tract illness [2, 3, 8, 25, 26], whereas, in earlier literature, a mortality rate of 16%–26% has been reported [1, 4, 6].

The negative results in the detection of respiratory viruses in nose-throat and BAL fluid specimens by viral culture, compared with real-time RT-PCR, may be due to several reasons. The sampling methods used (combined nose-throat swab specimens instead of nasal wash specimens) may have influenced the results of viral culture, but probably only for the detection of RSV [27]. All of the SCT recipients in our study were adults, who are commonly believed to shed less virus than are children, which may result in lower rates of detection of respiratory virus by viral culture or rapid diagnostic tests, such as direct antigen detection [28]. Also, some viruses, such as coronaviruses, are difficult to culture in a routine laboratory [22]. Rhinoviruses were identified both by viral culture and by real-time RT-PCR as the major cause of URTI and were also detected in 3 patients with an LRTI (once in combination with a PIV). This outcome is not surprising, because in immunocompetent adults, rhinoviruses account for 30%–50% of the average 2–4 episodes of the common cold per year, and they are well known to occasionally cause LRTI in older adults and neonates [29, 30]. Rhinoviruses have been described before as causative pathogens of LRTI in immunocompromised patients [9, 17, 31], either as the sole pathogen or as a copathogen with bacteria or other respiratory viruses, although 2 recent studies that prospectively investigated the incidence of respiratory virus infections in pa-

Table 4. Differences between patients who received allogeneic stem cell transplants (SCTs) from a sibling donor, those who received SCTs from a voluntary, unrelated donor (VUD), and those who received autologous SCTs.

Variable	Allogeneic SCT recipients (n = 37)	VUD SCT recipients (n = 17)	Autologous SCT recipients (n = 18)
RTI	23 (62)	7 (41)	10 (56)
Episodes of RTI			
All	31	9	12
Respiratory virus detected by viral culture	5 (16)	4 (44)	2 (17)
Respiratory virus detected by real-time RT-PCR	15 (48)	7 (78)	7 (58)
Neutropenia during episode	2 (6)	3 (33)	1 (8)
Receipt of immunosuppressive therapy during episode	20 (65)	3 (33)	NA
GVHD during episode	13 (42)	2 (22)	NA

NOTE. Data are no. (%) of patients, unless otherwise indicated. None of the differences between the types of transplant were significant. GVHD, graft-versus-host disease; NA, not applicable; RTI, respiratory tract illness.

^a Defined as a neutrophil count of $<0.5 \times 10^9$ cells/L.

tients with hematological cancer observed no or only a very low number of rhinovirus infections in cases of both URTI and LRTI [2, 24]. Rapid detection of rhinovirus infection could be of importance, especially in patients with LRTI, because it has been suggested that the antiviral drug pleconaril could be effective for the treatment of severe picornavirus infection, including infection due to rhinoviruses in SCT recipients [14].

Of interest is the number of human coronaviruses that we detected by real-time RT-PCR. Human coronavirus types 229E and OC43 are major causes of the common cold, but they are notoriously difficult to culture. These viruses, however, may sometimes cause pneumonia in both immunocompetent and immunocompromised patients [17, 30, 32]. We found that coronavirus was the probable cause of illness in 8% of episodes of respiratory tract illness. Last year, a new human coronavirus was discovered to have been the causative pathogen of an outbreak of severe acute respiratory syndrome, and recently, another new coronavirus was described in association with LRTI [33, 34]. These findings underscore the potential of coronaviruses as a cause of LRTI.

Adenovirus was detected in only 3 surveillance samples or follow-up samples. No respiratory tract illnesses due to adenovirus were detected in our study population. Adenoviruses have been recognized as serious pathogens in pediatric SCT recipients [35], but these viruses can also be a threat to adult SCT recipients [36]. It is thought that most adenovirus infections in SCT recipients are caused by reactivation of infection, but adenovirus can also cause acute upper respiratory disease by spread of aerosols. Although we were especially interested in adenoviruses that had recently been obtained from the environment and that had caused acute respiratory tract illness, one cannot determine whether samples obtained from the respiratory tract of SCT recipients will reveal recently obtained

adenovirus infection or reactivated infection with latent adenoviruses from the lymphoepithelial tissue of the nasopharynx.

To establish the diagnostic value of real-time RT-PCR, we also analyzed nose-throat swab specimens obtained during periods when SCT recipients did not have complaints of respiratory tract illness ("surveillance samples"). By real-time RT-PCR, we found respiratory viruses in 9% of these samples, of which 88% were rhinoviruses. A recent case-control study on acute respiratory infections in patients in general practices in The Netherlands showed that the incidence of rhinovirus among healthy control subjects was 11% [37]. These results show that rhinoviruses can be shed both by symptom-free immunocompromised patients and by healthy persons over a longer period of time, but also that interpretation of the value of PCR for the detection of rhinoviruses may be complicated.

Table 5. Findings for 259 surveillance nose-throat samples analyzed with viral culture and real-time RT-PCR in a study of respiratory tract illness.

Finding	No. of viral cultures (n = 3)	Real-time RT-PCR (n = 24)
Rhinovirus	2	21 ^a
RSV
Human coronavirus	...	2
Adenovirus	1	3
Influenza virus
Parainfluenza virus
Enterovirus

NOTE. Two samples contained 2 respiratory viruses: adenovirus with rhinovirus and coronavirus with rhinovirus. RSV, respiratory syncytial virus.

^a $P < .0001$.

In summary, the present study has shown that respiratory viruses are a major cause of respiratory tract disease in SCT recipients. We have demonstrated that real-time RT-PCR is much more sensitive than viral culture for detection of respiratory virus infection in SCT recipients, as well as in cases of LRTI. Rhinovirus caused the majority of URTIs, but one must be aware that rhinoviruses can also be detected occasionally in asymptomatic patients, suggesting that persistent shedding of respiratory viruses can occur in immunocompromised patients.

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