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Molecular Characterization of a Respiratory Syncytial Virus Outbreak in a Hematology Unit in Heidelberg, Germany

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In 2011 and 2012, a large outbreak of respiratory syncytial virus (RSV) infections affecting 57 laboratory-confirmed patients occurred in an adult hematology unit in Heidelberg, Germany. During the outbreak investigation, we performed molecular genotyping of RSV strains to differentiate between single versus multiple introductions of the virus into the unit. Furthermore, we assessed the time of viral shedding of consecutive samples from the patients in order to better understand the possible impact of prolonged shedding for outbreak control management. We used subtype-specific reverse transcription-PCR on nasopharyngeal and bronchoalveolar specimens for routine diagnostics and for measuring the viral shedding period. Samples of 47 RSV-infected patients involved in the outbreak were genotyped by sequence analysis and compared to samples from RSV-infected hospitalized children representing the timing of the annual RSV epidemic in the community. Molecular investigation of the virus strains from clinical samples revealed a unique cluster with identical nucleotide sequences of RSV type A (RSV A outbreak strain) for 41 patients, while 3 patients were infected with different RSV A (nonoutbreak) strains and three other patients with RSV type B. Outbreak strains were identified in samples from November 2011 until January 2012, while nonoutbreak strains were from samples coinciding with the community epidemic in February and March 2012. Median duration of viral shedding time was 24.5 days (range, 1 to 168 days) with no difference between outbreak and nonoutbreak strains ($P = 0.45$). Our investigation suggests a single introduction of the RSV A outbreak strain into the unit that spread among the immunocompromised patients. Prolonged viral shedding may have contributed to nosocomial transmission and should be taken into account in the infection control management of RSV outbreaks in settings with heavily immunosuppressed patients.

Respiratory syncytial virus (RSV) is a frequent cause of lower respiratory tract infection (LRTI) worldwide. The virus is stable for hours in the environment. Main routes of transmission are through droplets and by direct contact (1, 2). Strain variation is thought to contribute to its ability to cause frequent reinfections (3, 4), enabling RSV to remain present at high levels in the population (5). In immunocompromised patients, RSV may cause severe illness with prolonged virus shedding, especially among hematopoietic stem cell transplant (HSCT) recipients (6). Progression of RSV infection from upper respiratory tract infection (URTI) to LRTI is associated with an increased risk of death in these patients (7), resulting in a 7 to 70% case fatality rate among patients with hematological disorders (8–11).

Several RSV outbreaks among hospitalized hematology patients have been reported in the past (12–15). In one study involving bone marrow transplant patients, it was found that nosocomial infection with RSV occurred in almost half of all patients on the ward posttransplant (16). Hence, the establishment of new strategies for control of RSV has become an important task especially in the areas of prevention, diagnosis, and development of an effective vaccine.

Viral strains are separated into two major groups based on genetic and antigenic variability. Several lineages within groups A and B cocirculate simultaneously in the population (17) and their relative proportions may differ between epidemics, although group A viruses tend to predominate. Sequencing of the variable regions of the G protein gene has been used widely in further subdividing the two groups into genotypes and facilitated differentiation between RSV isolates. Eight RSV subgroup A genotypes have been described so far, GA1 to GA7 and South Africa A1, or SAA1 (18, 19). Studies on RSV strains show an accumulation of amino acid changes over the years, suggesting antigenic drift-based immunity-mediated selection (20, 21). Molecular characterization of RSV has the potential to aid in the identification of infection chains. However, its application in RSV outbreak situations has rarely been reported so far.

In our study, we performed molecular characterization of RSV strains involved in an outbreak of hospitalized hematology patients in order to differentiate between single versus multiple introductions of the virus into the unit. Furthermore, we assessed the duration of viral shedding to better understand the possible impact of prolonged shedding for outbreak control management.

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MATERIALS AND METHODS

Outbreak setting and management. The hematology unit of the Heidelberg University Hospital comprises four wards on two floors with 62 patient beds; eight rooms have the facility for positive- or negative-pressure isolation of patients. The major focus of this unit is autologous and allogeneic blood stem cell transplantation. All transplant patients underwent a transplant conditioning with reduced intensity. In December 2011, sporadic cases of RSV-infected patients were detected, and in early January the number of RSV-positive patients increased substantially in this unit. Patients with laboratory-confirmed RSV infection were isolated. Patients with respiratory symptoms were screened for influenza virus and RSV in nasopharyngeal samples by PCR. These patients were isolated while awaiting the laboratory result. Despite these precautions, additional patients became infected with RSV. During the outbreak, infection control measures were intensified, with particular emphasis on hand disinfection, barrier nursing, isolation of infected patients, and stopping access to the unit by staff and visitors with respiratory tract infection. Barrier measures were reinforced, and disposable gloves, gowns, and filtering face piece class 2 (FFP-2) masks had to be used by all personnel during contact with patients. In addition, all inpatients and all medical personnel were screened for RSV. Patients who had been in contact with RSV-infected patients were isolated in a single room and were released from isolation if they tested negative after 1 week, equivalent to the maximum duration of the incubation period. All RSV-infected inpatients were transferred to a single dedicated ward (cohort isolation). At the peak of the outbreak, new admissions were suspended.

Sample collection. Consecutive nasopharyngeal swabs were collected from all patients in the unit, and bronchoalveolar lavage (BAL) fluid samples were available from patients with pneumonia. Screening for RSV was performed on all patients in the unit twice every week starting in week 2 of 2012. A previously infected patient was considered to have cleared the infection when he/she was asymptomatic and had two nasopharyngeal samples taken a week apart that tested negative for RSV. A cross-sectional screening of all hospital personnel having direct contact with patients from this unit was performed in week 3 of 2012. Further testing of personnel was done when they had respiratory symptoms. Environmental samples of the door handle, sink, faucet, bed, and table were taken once in one RSV-positive patient's room. The samples were either processed within 2 h or frozen at −20°C.

Thirty of 121 RSV-positive nasopharyngeal specimens from children hospitalized in the Department of Pediatrics, University Hospital Heidelberg, during the winter of 2011 to 2012 (convenience samples) were sequenced. These RSV-infected children were hospitalized in the separate department of pediatrics and were not related to the ongoing outbreak in adults in the hematology unit. All frozen stored respiratory specimens from July to December 2011 from hematological patients of the outbreak unit that were not tested for RSV yet were retrospectively subjected to RSV analysis.

Real-time PCR. RNA was extracted from respiratory specimens using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Amplification and detection of viral RNA was performed with the RealStar RSV real-time reverse transcription-PCR (RT-PCR) kit (Altona Diagnostics, Hamburg, Germany) on a LightCycler 480 instrument II (Roche, Mannheim, Germany) according to the manufacturer's instructions. This assay distinguished RSV subtype A from RSV subtype B.

Sequencing and phylogenetic analysis. For a subset of 47 patients out of 57 RSV-infected patients, enough sample material was available for phylogenetic analysis. For phylogenetic analysis, extracted RNA was reverse transcribed using random hexamer primers. Subsequently, the second variable region of the G gene was amplified from cDNA using primers as described previously (18). Resulting PCR products of the expected size (≈480 bp for RSV A and ≈520 bp for RSV B) were sequenced completely in both directions using BigDye Terminator chemistry version 1.1 and the Prism 3130xl instrument (Applied Biosystems, Darmstadt, Germany).

Overlapping sequences were assembled using the SEQMAN II software of the Lasergene package (DNAstar, Madison, WI). Multiple alignments from RSV A and RSV B nucleotide sequences were carried out with the MegAlign software of the Lasergene package. A phylogenetic tree was constructed in MEGA version 5.05 using the maximum-likelihood method and the Tamura-Nei algorithm (22, 23). Representative reference sequences from RSV A and RSV B genotypes were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and included in the phylogenetic analysis. The statistical significance of the tree topology was assessed by bootstrapping with 1,000 replicates.

Statistical analysis. Analysis of the duration of viral shedding was done among all RSV-infected patients with samples available from at least two consecutive weeks. Duration of viral shedding was defined as the time between the first and last laboratory-confirmed RSV-positive sample. We calculated the medians (and interquartile intervals) of all patients. To compare the length of the viral shedding period of the outbreak and the nonoutbreak strains, we used the Wilcoxon rank sum test. We used Stata version 11 (Stata Corp., College Station, TX, USA) for all statistical analyses.

Nucleotide sequence accession numbers. RSV G gene nucleotide sequences were deposited in GenBank under accession numbers JX967562 (outbreak), JX967571 (P40), JX967563 (P41), JX967564 (P45), JX967572 (P47), JX967565 (C5), JX967566 (C11), JX967567 (C13), JX967568 (C15), JX967569 (C19), JX967570 (C21), JX967573 (C22), JX967574 (C27), and JX967575 (C29).

RESULTS

Descriptive epidemiology and laboratory results. In November and December 2011, four cases of RSV-infected patients in the unit were detected by PCR and interpreted as sporadic cases without any relationship between these cases at that time. In the first week of January 2012, four patients with RSV infection were detected, more than ever before in a single week. Further investigations disclosed an on-going outbreak of RSV with a total of 57 RSV-infected patients. Screening of all inpatients was performed in the second week of January, as well as the screening of all personnel having direct contact with these patients. During this screening, 13 of 61 (21.3%) patients tested RSV positive, while only 1 staff member out of 209 (0.5%) tested positive for RSV subtype A. In the following weeks, five more staff members tested RSV positive (four RSV subtype A and one RSV subtype B) with mild symptoms. None of them tested positive in a second swab taken 1 week (7 ± 1 days) later. Out of five environmental samples from one RSV-positive patient room, i.e., from bed, table, door handle, sink, and faucet, the door handle and sink tested positive.

The peak of the outbreak was seen in week 2 of January 2012, with 13 newly diagnosed RSV-infected patients, corresponding to 23% of RSV-infected patients during the overall outbreak (Fig. 1). In order to better define the start of the outbreak, all available specimens from July to December 2011 from patients of this unit were analyzed retrospectively and revealed another eight unrecognized RSV-infected patients for whom fungal pneumonia had been suspected. Despite rigorous infection control measures during the outbreak, more RSV infections in the unit were observed from week 7 to week 10, with a peak in week 9, coinciding with an RSV epidemic in the community among pediatric patients (patients C1 to C121). These 121 RSV-infected children were hospitalized in the separate Department of Pediatrics, University Hospital Heidelberg, and were not related to the ongoing outbreak in adults in the hematology unit. Molecular analysis of the RSV strains detected up to week 5 of 2012 revealed a unique RSV strain (RSV A outbreak strain), suggesting a single introduction into the

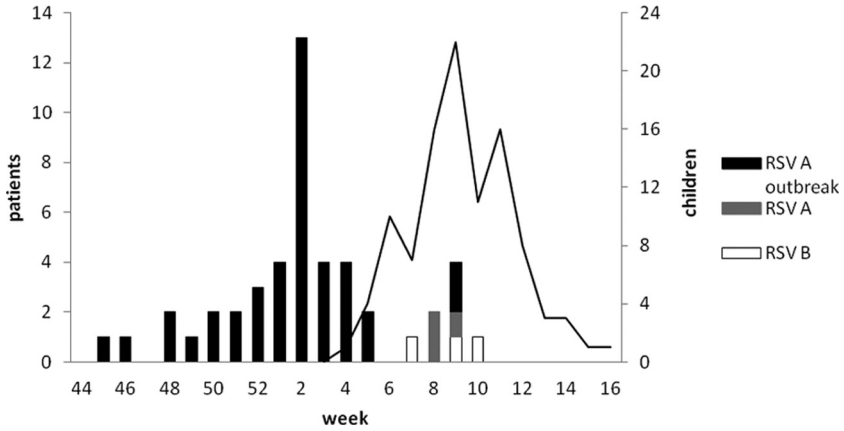

FIG 1 Cases of RSV in the hematology unit (columns) and in non-outbreak-related pediatric patients (line) by week of laboratory confirmation.

hematology unit and subsequent nosocomial transmission (Fig. 1). In late February, a mixture of RSV A outbreak strain, RSV A nonoutbreak strains, and RSV B strains in the hematology unit was observed, and these different strains corresponded to RSV strains circulating in the community in the same time period. Of 121 RSV-positive samples from the separate department of pediatrics, subtype-specific PCR assays revealed 80 RSV subtype A (66%) and 41 RSV subtype B (34%) samples. Retrospective analysis revealed that the hospital RSV outbreak was already contained in early February, while further observed RSV infections between week 7 and 10 corresponded with new introductions of RSV strains circulating in the community at that time. The hospital outbreak preceded the community epidemic by about 3 months.

Clinical outcome. In the subset of 47 further analyzed RSV-infected patients, 24 were male with a mean age of 56 years. There were four patients with autoimmune disorders and 43 with hematologic disorders. Twenty-nine were HSCT recipients (14 allogeneic, 14 autologous, and 1 allogeneic and autologous transplantation), and 14 had an underlying pulmonary disease. Two patients had no signs of respiratory infection, 7 patients had URTI, 38 patients showed signs and symptoms of LRTI, and 14 RSV-infected patients with pneumonia died despite intensive care unit admission and supportive ventilation. Twelve of 14 fatal cases were infected with the RSV A outbreak strain, one patient was infected with an RSV A nonoutbreak strain, and one patient was infected with RSV B. Oral ribavirin was given as treatment to 30 patients and immunoglobulins to 14 patients; palivizumab was not used during the outbreak. No preemptive treatment was administered.

Phylogenetic analysis and genotype distribution pattern. RSV G gene sequences of RSV-positive samples from patients P1 to P47 and from 30 randomly chosen patients of the department of pediatrics were analyzed, and a sequence alignment and phylogenetic tree were established (Fig. 2 and 3). Forty-one out of 47 patients (P1 to P39, P43, and P44) and 4 pediatric community patients (C1, C2, C9, and C16) displayed an identical RSV genotype GA2 sequence (RSV A outbreak strain). RSV strains of patients P41, P42, and P45 were also genotyped as RSV GA2 but were different from the outbreak strain, while strains from patients P40, P46, and P47 and pediatric cases C22, C27, and C29 belonged to the RSV B BA IV genotype. For 38 of 47 (80%) patients two or more independent samples have been sequenced, always resulting in absolutely identical sequences of the analyzed RSV G gene region.

Viral shedding. Beginning in January 2012, all RSV-infected patients were monitored prospectively in order to determine the duration of viral shedding. Stored respiratory samples from 2011 of these patients that had not been analyzed for RSV were tested retrospectively for RSV in order to determine the time point at which RSV infection started. The duration of viral shedding was calculated from first to last RSV-positive sample for each patient, including gaps of several weeks in a few cases. Samples before and after gaps were sequenced and always revealed the identical RSV strain. Results for all RSV-infected patients with samples available from at least two consecutive weeks are shown in Fig. 4. Thirty-five cases with two or more sequential RSV-positive specimens and a positivity period of 7 days or longer were identified. The median time of RSV shedding of all patients was 24.5 days (range, 1 to 168 days) without a significant difference between the RSV A outbreak strain and RSV A nonoutbreak or RSV B strains (25.5 versus 18.5 days; $P = 0.45$). Three patients (P12, P23, and P40) were lost to follow-up for prolonged viral shedding analysis, and six patients (P4, P5, P9, P10, P38, and P41) died while still shedding RSV at time of death. However, 26 of 35 (74%) long-term shedding patients cleared viral infection, as demonstrated by RSV-negative follow-up samples. Patient P39, who was diagnosed with chronic lymphatic leukemia (CLL) and who had received allogeneic transplantation, was persistently infected for 24 weeks.

DISCUSSION

This study describes the molecular analysis of RSV strains detected during an outbreak in hematology patients in Heidelberg, Germany, in 2011 and 2012. An identical RSV A outbreak strain was detected for all patients involved in the outbreak that preceded the community epidemic by about 3 months. Further introductions of different RSV strains, a mixture of the RSV A outbreak strain, RSV A, and RSV B, was observed during the community epidemic from mid-February to mid-March. The affected immunocompromised patients shed the virus for 25 days on average with no difference between the RSV A outbreak strain and the other strains.

Twelve RSV infections occurred in the hematology unit before the outbreak was recognized in the beginning of January 2012. At that time, infections of a few patients were considered sporadic

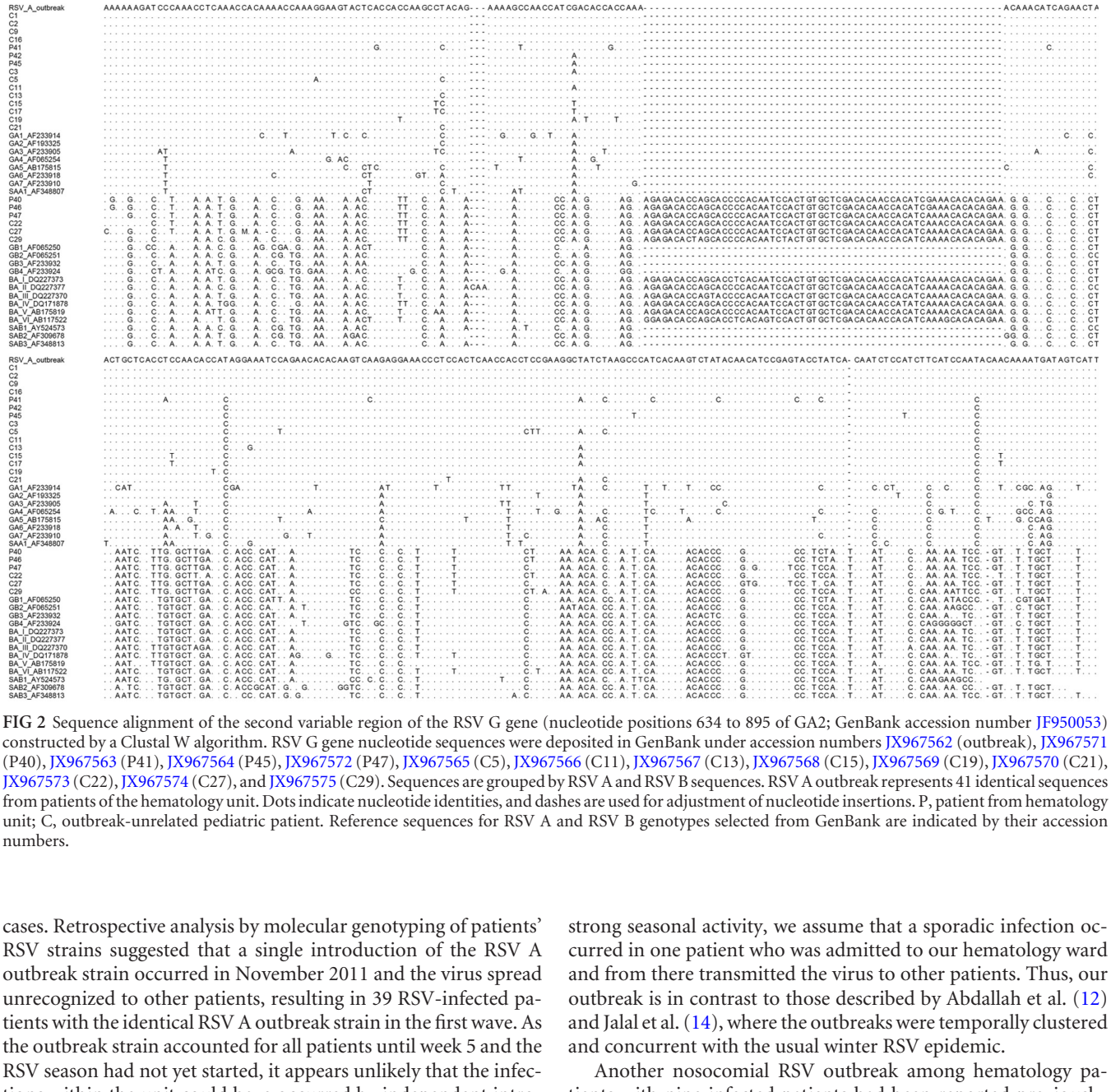

FIG 2 Sequence alignment of the second variable region of the RSV G gene (nucleotide positions 634 to 895 of GA2; GenBank accession number JF950053) constructed by a Clustal W algorithm. RSV G gene nucleotide sequences were deposited in GenBank under accession numbers JX967562 (outbreak), JX967571 (P40), JX967563 (P41), JX967564 (P45), JX967572 (P47), JX967565 (C5), JX967566 (C11), JX967567 (C13), JX967568 (C15), JX967569 (C19), JX967570 (C21), JX967573 (C22), JX967574 (C27), and JX967575 (C29). Sequences are grouped by RSV A and RSV B sequences. RSV A outbreak represents 41 identical sequences from patients of the hematology unit. Dots indicate nucleotide identities, and dashes are used for adjustment of nucleotide insertions. P, patient from hematology unit; C, outbreak-unrelated pediatric patient. Reference sequences for RSV A and RSV B genotypes selected from GenBank are indicated by their accession numbers.

cases. Retrospective analysis by molecular genotyping of patients' RSV strains suggested that a single introduction of the RSV A outbreak strain occurred in November 2011 and the virus spread unrecognized to other patients, resulting in 39 RSV-infected patients with the identical RSV A outbreak strain in the first wave. As the outbreak strain accounted for all patients until week 5 and the RSV season had not yet started, it appears unlikely that the infections within the unit could have occurred by independent introductions of transmission from the community. The laboratory evidence strongly supports the hypothesis that it was introduced once into the unit and then spread nosocomially. This is further supported by the European pattern in seasonal activity of RSV, in which an early season (beginning in October/November) with strong RSV activity is followed by a weaker late season (beginning in February/March) in a regular biennial rhythm (6). According to that rule, the winter of 2011/2012 was predicted as a late, weak RSV season. As the outbreak strain accounted for all patients until week 5 in 2012 and the RSV season had not yet started in the community, it is highly unlikely that the infections in the unit from November until week 5/2012 could have occurred by independent introductions of transmission from the community. Since RSV infections can take place throughout the year despite a strong seasonal activity, we assume that a sporadic infection occurred in one patient who was admitted to our hematology ward and from there transmitted the virus to other patients. Thus, our outbreak is in contrast to those described by Abdallah et al. (12) and Jalal et al. (14), where the outbreaks were temporally clustered and concurrent with the usual winter RSV epidemic.

Another nosocomial RSV outbreak among hematology patients with nine infected patients had been reported previously; eight of these patients revealed an identical RSV strain (24). Similar to the second peak of RSV infections in our hematology unit, three different RSV strains were found in another outbreak of RSV infections involving seven patients of an adult leukemia/lymphoma ward (25). Visser et al. (26) reported an RSV outbreak in a kangaroo mother care unit in South Africa with 19 RSV-infected preterm pediatric patients and identified eight separate introductions.

Respiratory infections in heavily immunosuppressed adults hospitalized in high-dependency hematology posttransplant units are particularly difficult to manage (27), as we experienced in our setting as well. Six of the fatal cases had undergone blood stem cell transplantation, three were autologous and three were allogeneic. All fatal cases had severe comorbidities, e.g., progressive leukemia

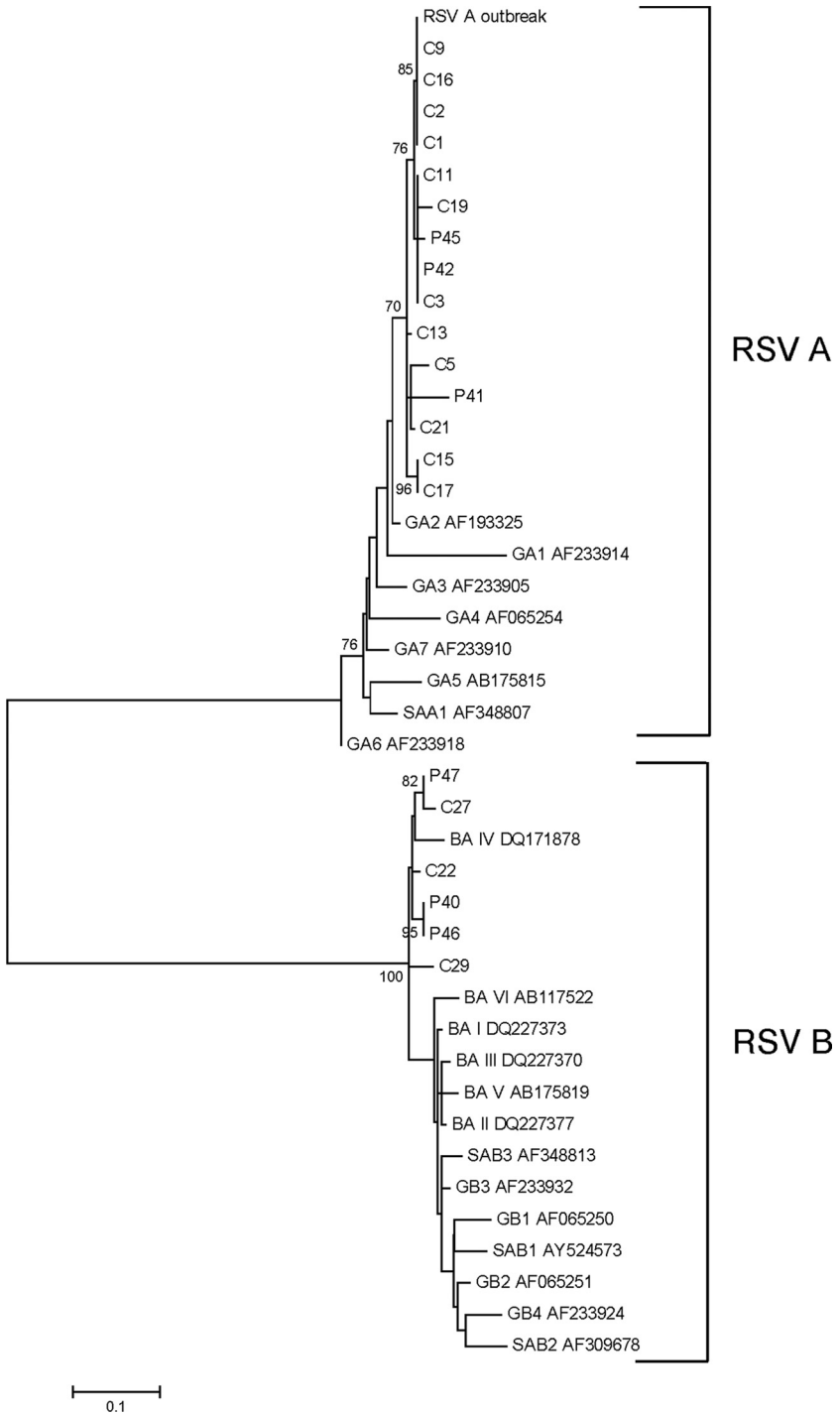

FIG 3 Phylogenetic tree of RSV A and RSV B sequences from the second variable region of the G gene constructed with MEGA version 5.05 using the maximum likelihood method. RSV A outbreak represents identical sequences from patients of the hematology unit. P, patients of the hematology unit; C, outbreak-unrelated pediatric patient. Reference sequences for RSV A and RSV B genotypes selected from GenBank are indicated by their accession numbers. The bar indicates 0.1 nucleotide substitutions per site. Bootstrap values greater than 60 are displayed on branch nodes.

or graft versus host disease or coinfections, like *Aspergillus*, *Pseudomonas*, or herpesviruses. Although RSV can cause fatal pneumonia, the contribution of RSV to the fatal outcome is difficult to assess and might be attributed to only some of the fatal RSV cases. The symptoms of respiratory virus infections are often nonspecific early on, viral shedding is prolonged, and outbreaks therefore are often recognized late. However, implementation of interventions is still recommended to stop the outbreak (14). Consequently, respiratory infections in immunocompromised patients need to be tested for RSV and other respiratory infectious agents

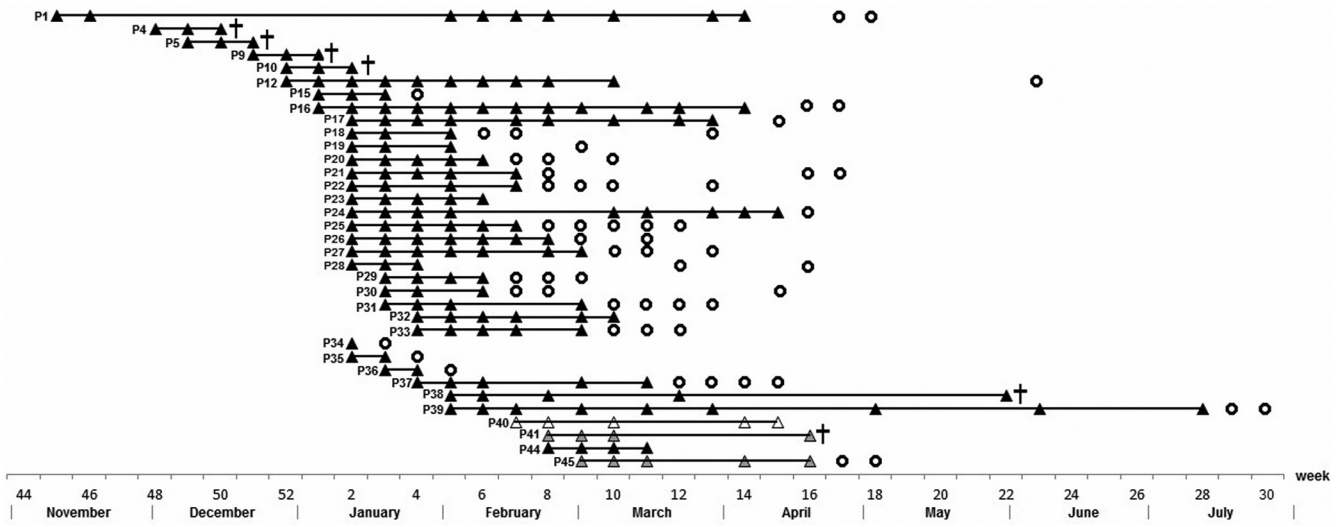

FIG 4 Representation of the shedding periods and temporal distribution of RSV infection for long-term RSV-infected hematology patients. The sampling dates of RSV-positive specimens are indicated with triangles. Black triangle, RSV A outbreak strain; gray triangle, RSV A nonoutbreak strain; white triangle, RSV B strain. Circles denote RSV-negative samples indicating clearance of infection. Thirty-five cases with two or more sequential RSV-positive specimens and a positivity period of 7 days or longer were identified. Patients P12, P23, and P40 were lost to follow-up, and patients P4, P5, P9, P10, P38, and P41 died (†) while still shedding RSV.

even outside the regular season. A broader screening for respiratory pathogens in HSCT recipients, i.e., influenza virus, RSV, and parainfluenza virus as a first-line and adenovirus, metapneumovirus, coronavirus, rhinovirus, and enterovirus as second-line agents has been recommended by several expert groups (28, 29).

The clinical and epidemiological significance of RSV variability still remains unclear. Identical genotypic features were shared by all strains in the first wave. For 80% of the patients, two or more independent samples were sequenced and revealed identical sequences, thus reinforcing the clonal robustness of the RSV A outbreak strain. The second wave was characterized by a mixture of RSV A outbreak strain, RSV A nonoutbreak strain, and RSV B strains, representing the pattern of RSV lineages in the community during the ongoing RSV season and indicating community-acquired infections. RSV GA2 lineages similar to the outbreak strain were simultaneously circulating in Germany prior to the winter of 2011/2012. RSV group A was dominant in Germany during the last decade (21), with cocirculation of genotypes GA2 and GA5. Viral factors associated with virulence and severity of disease are still not completely understood. The difference between groups A and B in clinical severity has been discussed (19), and RSV A might be associated with more severe clinical disease (30). It has been suggested that virus variants within group A are responsible for this discrepancy (31). In contrast, clinically more severe cases were found spread over the branches of the phylogenetic tree for RSV A and RSV B (19, 32). According to Martinello et al. (33), the severity of illness caused by subgroup A isolates does not differ from that caused by subgroup B isolates. However, the GA3 clade was associated with greater severity of illness compared to clades GA2 and GA4. In our setting, we did not recognize a significant difference in clinical presentation and fatality between the outbreak and the community epidemic. We could not verify this impression due to the small number of community-acquired infections during the second wave and the complex underlying illnesses of our patients.

Viral clearance of RSV in the respiratory tract occurs after 3 to 6 days in immunocompetent patients (34). However, persistence of viral shedding has received little attention in hospitalized immunosuppressed patients, and shedding in hematology patients was reported for 2 to 4 weeks (14, 24, 35), particularly in patients with lymphocytopenia (1). Complete viral clearance was correlated with lymphocyte reconstitution irrespective of receipt of antiviral medication (36). According to Khanna et al. (35), the duration of shedding is not associated with site of infection, severity of immunodeficiency, RSV subtype, or treatment regimen. Twenty of 35 patients with prolonged viral shedding were transplant patients, 8 patients with autologous and 12 patients with allogeneic transplants, thus prolonged viral shedding was found in patients with and without transplantation. In our study group, viral shedding was observed for a period of time similar to that reported previously and was up to 24 weeks in a single case. However, there was no obvious difference in viral shedding between the outbreak strain itself and strains of the community-acquired cases. Follow-up of shedding of RSV-infected patients was conducted on wards as well as in the outpatient facility and applied similar reinforced infection control measures as those described for inpatient wards. All outpatient and staff in the outpatient facility were screened the same as inpatients. Thus, nosocomial transmission in the outpatient facility seems unlikely. Since no circulation of the new introduced strains was observed, it can be assumed that the intensified infection control measures stopped further transmission for these strains on the ward. This also suggests that the undiscovered long-term viral shedding during the outbreak maintained the circulation on the wards, although personnel-to-patient (droplet or direct contact) and fomite transmission are possible transmission modes with unknown contributions. The question of whether long-term shedders excrete large enough doses to constantly spread the virus remains unclear.

In the present study, retrospective molecular investigation suggested the spread of a single RSV strain among patients on differ-

ent wards in the hematology unit over an extended period of time without a change in sequence. Further analysis revealed that two factors might have promoted the circulation of this strain in the unit. First, a low index of suspicion delayed the discovery of the responsible agent and hence infection control. Second, prolonged viral shedding could have contributed to maintaining the outbreak.

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