Epidemiological, Molecular, and Clinical Features of Enterovirus Respiratory Infections in French Children between 1999 and 2005[†]

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Enteroviruses (EVs) can induce nonspecific respiratory tract infections in children, but their epidemiological, virological, and clinical features remain to be assessed. In the present study, we analyzed 252 EV-related infection cases (median age of subjects, 5.1 years) diagnosed among 11,509 consecutive children visiting emergency departments within a 7-year period in the north of France. EV strains were isolated from nasopharyngeal samples by viral cell culture, identified by seroneutralization assay, and genetically compared by partial amplification and sequencing of the VP1 gene. The respiratory syndromes (79 [31%] of 252 EV infections) appeared as the second most common EV-induced pediatric pathology after meningitis (111 [44%] of 252 cases) (44 versus 31%, $P < 10^{-3}$), contributing to lower respiratory tract infection (LRTI) in 43 (54%) of 79 EV respiratory infection cases. Bronchiolitis was the most common EV-induced LRTI (34 [43%] of 79 cases, $P < 10^{-3}$) occurring more often in infants aged 1 to 12 months ($P = 0.0002$), with spring-fall seasonality. Viruses ECHO 11, 6, and 13 were the more frequently identified respiratory strains (24, 13, and 11%, respectively). The VP1 gene phylogenetic analysis showed the concomitant or successive circulation of genetically distinct EV respiratory strains (species A or B) during the same month or annual epidemic period. Our findings indicated that respiratory tract infections accounted for the 30% of EV-induced pediatric pathologies, contributing to LRTIs in 54% of these cases. Moreover, the concomitant or successive circulation of genetically distinct EV strains indicated the possibility of pediatric repeated respiratory infections within the same epidemic season.

Enteroviruses (EVs) (*Picornaviridae*) are among the most common viruses infecting human beings worldwide (13, 23, 25). Current taxonomy divides nonpolio human EV into four species (human EVs A to D), including a total of 89 serotypes (24, 38). Individual serotypes have different temporal patterns of circulation and can be associated with different clinical manifestations (24, 39). Although the majority of human EV infections remain asymptomatic, these viruses are associated with diverse clinical syndromes, ranging from minor febrile illness to severe and potentially fatal pathologies, including aseptic meningitis, encephalitis, myopericarditis, acute flaccid paralysis, and severe neonatal sepsis-like disease (24). Moreover, EV can induce nonspecific respiratory illnesses in infants or adults, including upper respiratory tract infections but also lower respiratory tract infections (LRTIs), resulting in bronchitis, bronchiolitis (37), and pneumonia (13). Different human EV strains—including EV 68 and 71, coxsackie A9, A21, B2 and B4 viruses, and echovirus 9, 11 and 22—have been isolated from nasopharyngeal samples, tracheal aspirates, bronchoalveolar lavages, or lung tissues by classical cell culture assays

and identified as the cause of severe or fatal viral bronchopneumonia (4, 6, 10, 11, 29, 32, 35). At present, our understanding of the epidemiology and clinical profile of EV pediatric respiratory infections is restricted to the prevalence and the epidemiological significance of EV respiratory infections as the cause of bronchiolitis or acute wheezing in cohorts of hospitalized infants (1, 22, 31, 33). In these reports, no serotyping identification or molecular comparative analysis of EV respiratory viral strains was performed, whereas recent molecular approaches can allow a reliable molecular characterization and a phylogenetic comparison of the EV strains by partial amplification and sequencing of the VP1 gene (30).

In the present study, we retrospectively analyzed the epidemiological and clinical features of EV-related respiratory pathologies diagnosed in a large cohort of French hospitalized and nonhospitalized children visiting emergency departments of the region Champagne Ardenne (France) between 1999 and 2005. Moreover, using partial amplification and sequencing of the VP1 gene, we carried out a phylogenetic comparative analysis of EV strains isolated from the nasopharyngeal samples of children with EV-related respiratory syndromes.

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MATERIALS AND METHODS

Study population. From January 1999 through December 2005, 11,509 consecutive children (mean age, 2.3 years; age range, 9 days to 15 years) attending one of the five referent pediatric emergency departments of the region Cham-

TABLE 1. Clinical features of 252 selected children with an EV infection diagnosed during the study period (1999 to 2005)

Patients subgroups ^a	Total no. (%)	Mean age in yr (SD)	No. of males/no. of females (ratio)	Clinical outcome (fatal/good)
Outpatients	35 (14)	4.4 (3.0)	23/12 (1.9)	0/35
Bronchiolitis	13 (37)	1.1 (1.0)	7/6 (1.2)	0/13
Flu-like illness	11 (31)	2.4 (3.0)	8/3 (2.7)	0/11
Febrile illness	6 (17)	3.2 (2.6)	3/3 (1.0)	0/6
Asthma attack	2 (6)	4.16 (2.5)	2/0	0/2
Rhinopharyngitis	1 (3)	3.2	1/0	0/1
Sore throat	1 (3)	2.4	1/0	0/1
Bronchitis	1 (3)	0.58	1/0	0/1
Inpatients	217 (86)	5.4 (3.8)	136/81 (1.7)	2/215
Meningitis	111 (51)	6.9 (3.5)	71/40 (1.8)	1/110
Febrile illness	56 (26)	4.4 (1.7)	33/23 (1.4)	0/56
Bronchiolitis	21 (10)	0.5 (0.4)	14/7 (2.0)	0/21
Sore throat	8 (4)	6.2 (3.8)	6/2 (3.0)	0/8
Flu-like illness	6 (3)	1.4 (2.1)	2/4 (0.5)	1/5
Asthma attack	4 (2)	2.8 (1.9)	4/0	0/4
Rhinopharyngitis	3 (1)	6.3 (7.2)	2/1 (2.0)	0/3
Pharyngitis	3 (1)	8.8 (4.2)	2/1 (2.0)	0/3
Rhinitis	3 (1)	1.3 (1.2)	2/1 (2.0)	0/3
Bronchitis	2 (1)	0.7 (0.7)	1/1 (1.0)	0/2
Total	252 (100)	5.1 (3.6)	159/93 (1.7)	2/250

^a Outpatients, nonhospitalized infants visiting emergency departments of the region Champagne Ardenne between 1999 and 2005; inpatients, hospitalized patients visiting emergency departments of region Champagne Ardenne between 1999 and 2005.

pagne Ardenne (France) were prospectively enrolled in a north French Caucasian pediatric cohort for the exploration of viral respiratory diseases. For each child, informed consent was obtained from their family or relatives. The present study was conducted by the university medical hospital of Reims (Champagne Ardenne, France) and was approved by the hospital's ethics committee. All children underwent general, neurological, and respiratory examinations by a pediatrician, who carried out a nasopharyngeal aspirate sampling for the detection of common respiratory human viral pathogens by classical cell culture and immunofluorescence assays (17, 19). Moreover, according to the clinical symptoms observed, cerebrospinal fluid, peripheral blood, throat, or urine samples were also concomitantly sampled and tested by standard culture assays for the detection of classical human bacterial or viral pathogens, by standardized reverse transcription-PCR (RT-PCR) assays for the detection of EV strains in cases of aseptic meningitis or by standardized PCR assays for the detection of *Mycoplasma* sp. or *Chlamydia pneumoniae* in respiratory tract samples (9, 18). Among these 11,509 consecutive children enrolled in the study cohort, 252 (median age, 5.1 years; age range, 14 days to 15 years; male/female ratio, 159/93) were retrospectively selected because (i) they were aged ≤ 15 years; (ii) EV was the unique infectious agent detected in their nasopharyngeal samples as determined by classical cell culture assays and then identified as the etiological cause of the clinical syndrome diagnosed at the time of emergency visit; and (iii) they were free of cystic fibrosis, bronchopulmonary dysplasia, congenital heart disease, and systemic glucocorticoid treatment or with a chronic genetic or acquired immunodepression.

During the present study, 61 children were excluded because they demonstrated mixed respiratory infections ($n = 33$) or because they were suffering from chronic genetic or acquired respiratory pathologies or immunological immunodepression ($n = 28$) (data not shown). Table 1 presents the clinical features based on discharge diagnosis for the selected population.

Nasopharyngeal sample collection. Nasopharyngeal secretions were collected from all of the studied patients using 2 ml of sterile physiological saline fluid with a disposable mucus extractor at time of hospital admission (2). Nasopharyngeal wash fluids were then diluted in 3 ml of virus transport medium (0.5% bovine serum albumin, 1,500 U of penicillin, 1 ng of streptomycin, minimal essential medium, and 4.76 mg of HEPES in 2 ml of tryptose phosphate broth) and divided into aliquots into two separate sterile tubes. One tube was directly used to perform immunofluorescence detection of viral antigens and cell culture detection assays; the second tube was immediately frozen and stored at -80°C prior to molecular assays (17, 20).

Classical cell culture assays for virus isolation. For each studied patient, 200 μl of nasopharyngeal secretion sample diluted in viral transport medium was

inoculated in duplicate onto 24-well plates covered with monolayers of continuous human diploid fibroblasts (MRC-5), rhesus monkey kidney (MA-104) and Madin-Darby cell kidney (MDCK) cells as previously described (17). Each plate was incubated for 8 days at 37°C in a 5% CO_2 :95% air atmosphere. The plates were examined daily under light microscopy to detect the presence of a cytopathic effect on the cell culture monolayers. Two subcultures were performed at days 8 and 16 for each well as described above. EV isolates were typed by the standard method neutralization with EV type-specific antisera (17, 19, 26).

RNA isolation. Total RNA was extracted from 140 μl of culture supernatants by using the QIAamp viral RNA minikit (Qiagen, Courtaboeuf, France), according to the manufacturer's recommendations. Nucleic acids were eluted in a final volume of 50 μl of diethyl pyrocarbonate-sterile water, as described by the manufacturer's recommendations. The nucleic acid concentration was estimated by spectrophotometric measurement (optical density) at 260 nm before the samples were divided into aliquots and stored at -80°C until used (17).

Phylogenetic comparison of partial EV VP1 capsid protein region. RT-PCR amplification and sequencing of a part of the VP1 capsid gene were carried out as described previously (30). The sequences were manually corrected and aligned by using the computer program MEGA 3.1 (S. Kumar, K. Tamura, I. Jakobsen, and M. Nei [http://www.megasoftware.net]) with corresponding reference EV strains (GenBank accession numbers AY679736, AY208085, AB167989, AM159197, AF160024, AY875692, AM236930, AF295521, AF521340, AF521310, AB055923, DQ092796, AY227344, AF295445, AY208115, AB199314, AJ309245, AJ417364, and AY207635). The phylogenetic trees were built with the MEGA 3.1 program using the neighbor-joining method (34) as implemented in the MEGA computer program. During sequence comparisons, gaps, missing data and ambiguities in the sequences were ignored in pairwise comparisons. In the phylogenetic inference, pairwise genetic distances were calculated by using the Kimura two-parameter model of sequence evolution to account for multiple nucleotide substitutions (27). The reliability of the phylogenetic topologies (branching patterns) was determined by the bootstrap resampling test with 1,000 replicates (15, 27). VP1 sequences obtained from the study are available in the EMBL database under accession numbers AM492321 to AM492504.

Statistical analyses. Quantitative data are presented as means, standard deviation, and range. Qualitative data are presented as the number of observations and percentages. Chi-square tests with or without Yates' correction and analysis of variance tests were carried out when necessary to compare quantitative or qualitative data. The results were considered as statistically significant for two-sided P values of <0.05 . The statistical analysis was performed with the SAS software version 8.2 (SAS Institute, Cary, NC).

RESULTS

Virological and clinical features of the study population. Of 11,509 consecutive children enrolled in the study cohort, 2,444 (21%) were positive for the isolation of a human viral strain in their nasopharyngeal sample (Fig. 1A). Of these 2,444 children, 285 (11.6%) demonstrated the presence of an infectious EV strain isolated by classical cell culture assays, with annual rates of EV isolation ranging from 3 to 31% during the 7-year study period (Fig. 1). Of the 285 subjects from which an infectious EV strain had been isolated from a nasopharyngeal aspirate, 252 (88%) were selected because the detection of other common human viral or bacterial pathogens in their nasopharyngeal tracts had remained negative by classical culture or molecular assays at the time of emergency visiting (results not shown). Of these 252 EV-positive children, 111 (44%) had aseptic meningitis, 79 (31%) had upper or lower respiratory tract infections, and 62 (25%) had febrile illness (44% versus 31% versus 25% [chi-square test, $P < 10^{-3}$]; 31% versus 25% [chi-square test, $P = 0.11$]) (Table 1).

Annual and seasonal variations of EV infections. Compared to the total number of the diagnosed EV pediatric infections, the rates of EV-related respiratory syndromes ranged from 5 to 60% during the 7-year study period (Fig. 2). These rates appeared to be significantly lower in years 2000 (chi-square test, $P = 0.0002$) and 2005 (chi-square test, $P = 0.0002$), during

FIG. 1. Positive respiratory viral findings in the nasopharyngeal samples of 11,509 consecutive children attending one of the five referent pediatric emergency departments of the region Champagne Ardenne (France) from 1999 through 2005. (A) Distribution (%) of the positive viral isolation of human respiratory strains by classical cell culture assays in nasopharyngeal samples of 2,444 (21%) of 11,509 children during the 7-year of the study period. (B) Distribution of the annual rates of EV-positive isolation by classical cell culture assays among the 2,444 positive nasopharyngeal samples.

which epidemics of aseptic meningitis occurred in the region Champagne Ardenne (France) (Fig. 2). From 1999 to 2005, all of the EV-related respiratory infections were diagnosed from February to December. Interestingly, a peak of EV respiratory infection cases occurred in June 2000, 2001, and 2005 and in July 2002. EV respiratory infections had prominent spring-fall seasonality, with June-July period accounting for 47% of all EV infection reports (Fig. 2).

Virological characteristics of EV strains isolated from the respiratory tract samples of the selected children. Frequencies, ranks, and number of years reported for all of the EV strains isolated from the nasopharyngeal samples of children with respiratory and nonrespiratory tract infections are indicated in the Table 2. Echovirus 11, echovirus 6, echovirus 30, echovirus 13, and coxsackievirus B2 accounted, respectively, for 24, 13, 11, 11, and 11% of all EV respiratory syndromes

FIG. 2. Number of EV-related infections in Champagne Ardenne between 1999 and 2005. *, Years wherein the rates of EV-related respiratory infections appeared to be significantly lower than those of other EV-induced infections (chi-square test stratified according to the years of the study period; $P < 0.001$).

TABLE 2. Frequencies, ranks, and number of years reported for individual EV serotypes

EV serotype	EV-induced respiratory infections		EV-induced nonrespiratory infections ^a			No. of yr in which serotypes were reported	
	Overall rank	Diagnosed cases with identified serotype (<i>n</i> = 79)	Overall rank	Diagnosed cases with identified serotype (<i>n</i> = 173)			
		No.		%	No.		%
Echovirus 11	1	19	24.0	5	6	3.8	6
Echovirus 6	2	10	12.7	3	21	12.1	4
Echovirus 30	3–5	9	11.4	1	67	38.7	6
Echovirus 13	3–5	9	11.4	2	40	23.1	1
Coxsackievirus B2	3–5	9	11.4	6–7	5	2.9	3
Echovirus 7	6	6	7.6				3
Coxsackievirus B4	7–8	4	5.0	11–13	2	1.2	2
Coxsackievirus A16	7–8	4	5.0	14–17	1	0.6	4
Echovirus 3	9	3	3.8	11–13	2	1.2	3
Coxsackievirus B5	10	2	2.5	4	9	5.2	6
Echovirus 18	11–14	1	1.3	8	4	2.3	2
Coxsackievirus A9	11–14	1	1.3	14–17	1	0.6	2
Echovirus 5	11–14	1	1.3				1
Enterovirus 71	11–14	1	1.3				1
Echovirus 9				6–7	5	2.9	2
Coxsackievirus B1				9–10	3	1.7	3
Echovirus 16				9–10	3	1.7	2
Echovirus 25				11–13	2	1.2	2
Echovirus 17				14–17	1	0.6	1
Echovirus 20				14–17	1	0.6	1

^a EV-induced nonrespiratory infections included meningitis and febrile illness.

(Table 2). Echovirus 7, EV 71, coxsackievirus A16, echovirus 11, coxsackievirus B4, coxsackievirus B2, echovirus 3, and coxsackievirus A9 were isolated and identified in 100% (6 of 6 isolates), 100% (1 of 1 isolates), 83% (4 of 5 isolates), 76% (19 of 25 isolates), 66% (4 of 6 isolates), 64% (9 of 14 isolates), 60% (3 of 5 isolates), and 50% (1 of 2 isolates), respectively, of samples from infants with respiratory syndromes, indicating a dominant respiratory tropism for these EV isolates (Table 2).

Phylogenetic comparison of partial VP1 capsid protein region of EV respiratory strains. A phylogenetic tree on partial VP1 sequences of 77 of 79 EV strains isolated from nasopharyngeal samples of children with respiratory pathologies was built, allowing identification of temporal trends and patterns of circulation of respiratory species, serotypes, and strains (Fig. 3). This phylogenetic approach demonstrated the concomitant or successive circulation of species A and B human respiratory EVs within a single French geographical area (Fig. 3). During each year of the study period (1999 to 2005), a cocirculation of genetically distinct EV respiratory serotypes occurred between February and December. Moreover, in the two clusters of strains corresponding to echovirus 7 and echovirus 11 serotypes, we observed the cocirculation of genetically distinct subgroups of strains (bootstrap values of >60%) during the same annual epidemic period (Fig. 3) (22). Finally, for each EV serotype identified as related to both respiratory and neurological pathologies in children, various nucleotide or amino acid phylogenetic analyses of VP1 gene sequences were carried out, and their results did not identify the existence of distinct subgroups of strains that might have temporarily acquired a specific respiratory or neurological tropism (not shown).

Clinical features of EV-induced respiratory infections. The signs and symptoms of the 79 children with different EV-induced respiratory infections are reported in the Table 3.

Three patients demonstrated a secondary bacterial respiratory infection developed during hospitalization and inducing a fatal cardiorespiratory distress in one case (Table 3). Among the 79 children with a documented EV respiratory infection, 43 (54%) had from LRTIs and 36 (46%) had upper respiratory tract infections (54 versus 46%; chi-square test, $P = 0.27$). During the study period, we observed that bronchiolitis was the most frequent EV-related respiratory pathology diagnosed (43% of 79 cases) compared to flu-like illness (21% of cases), sore throat (11% of cases), or asthma attack (8% of cases) (43 versus 21 versus 11 versus 8%; chi-square test, $P < 10^{-3}$) (Table 3). EV-induced bronchiolitis occurred significantly more often in infants aged 1 to 12 months than in children in other age groups (73.5 versus 26.5%; chi-square test with Yates' correction, $P = 0.0002$). No EV serotype could be associated with a specific respiratory clinical manifestation (analysis of variance test, $P > 0.5$).

DISCUSSION

Our retrospective study assessed important epidemiological and clinical features of EV-related respiratory pathologies in a representative North French pediatric population (Fig. 1). Our findings indicated that EVs were respiratory pathogens with an epidemic behavior and that EV respiratory strain substantially contributed to LRTI (17% of children with EV infections), leading to hospitalization in 62% of these children (Table 1). All specimens of the present study were prospectively tested by classical cell culture and seroneutralization assays, demonstrating the infectivity of the EV strains detected in the nasopharynxes of infants at the time of the clinical diagnosis (16, 21). It is clear that our global rates of EV respiratory infections might be significantly enhanced by using RT-PCR detection

TABLE 3. Clinical signs, symptoms, and virological features of 79 pediatric patients with an EV-induced respiratory infection diagnosed during the study period (1999 to 2005)

Signs and symptoms	Total no. of patients (%)	Mean age in yr (SD)	No. of males/ no. of females	Multiple infection	Clinical outcome (fatal/good)	EV VP1 genotyping (no of strains) ^a
Bronchiolitis	34 (43)	0.7 (0.7)	21/13	<i>Streptococcus pneumoniae</i>	0/34	Cox B2 (5), Cox B4 (2), Cox B5 (1), cox A16 (3), Echo 3 (2), Echo 6 (5), Echo 7 (4), Echo 11 (3), Echo 13 (3), Echo 18 (1), Echo 30 (5)
Flu-like illness	17 (21)	2.1 (2.6)	10/7	<i>Moraxella catarrhalis</i>	1/16	Cox B2 (2), Cox B4 (2), Cox A9 (1), Ent 71 (1), Echo 3 (1), Echo 6 (2), Echo 11 (5), Echo 13 (1), Echo 30 (2)
Sore throat	9 (11)	5.8 (3.7)	7/2		0/9	Echo 5 (1), Echo 7 (1), Echo 6 (1), Echo 11 (4), Echo 13 (1), Echo 30 (1)
Asthma attack	6 (8)	3.6 (2.0)	6/0		0/6	Cox B2 (1), Cox A16 (1), Echo 6 (1), Echo 11 (1), Echo 13 (2)
Rhinopharyngitis	4 (5)	5.3 (5.4)	3/1		0/4	Cox B2 (1), Echo 6 (1), Echo 11 (2)
Pharyngitis	3 (4)	8.8 (4.2)	2/1		0/3	Echo 13 (2), Echo 30 (1)
Bronchitis	3 (4)	0.7 (0.5)	2/1	<i>Haemophilus influenzae</i>	0/3	Echo 7 (1), Echo 11 (2)
Rhinitis	3 (4)	1.3 (1.2)	1/2		0/3	Cox B5 (1), Echo 11 (2)
Total	79 (100)	2.4 (3.1)	52/27		1/78	

^a Cox, coxsackievirus; Echo, echovirus.

assays in the nasopharyngeal aspirates of study children, allowing the detection of low levels of cultivable and noncultivable human EV strains; however, this molecular approach might have identified asymptomatic respiratory picornavirus shedding or past or future respiratory symptomatic infections (28). Moreover, our molecular features obtained by partial sequencing of the VP1 gene of the isolates demonstrated that several individual respiratory strains had specific temporal trends and patterns of circulation (Fig. 3) but that no EV serotype could be associated with a specific respiratory clinical manifestation. Finally, the EV-induced respiratory pathologies appeared as the second most common EV-induced pediatric pathology after meningitis, contributing to LRTI with spring-fall seasonality.

The present study determined that among French immunocompetent children aged ≤15 years, respiratory diseases were the second most common EV-induced pediatric pathology after aseptic meningitis (Table 1). Bronchiolitis appeared significantly as the most frequently EV induced respiratory syndrome (43% of 79 EV respiratory cases), and comparison between age groups showed that this pathology occurred more frequently in infants aged 1 to 12 months. Recent studies published by our group (21) and others (1, 22) have shown that picornaviruses, specifically EVs, were identified as the third etiological cause of bronchiolitis in infants after human respiratory syncytial virus (HRSV) and rhinoviruses, with prevalence detection rates ranging from 9 to 25% of hospitalized infants with bronchiolitis or acute wheezing. In these reports,

EV-induced bronchiolitis or acute wheezing appeared to be detected more frequently in children aged 6 months to 2 years (1, 21, 22). Our findings are in agreement with these previous studies and argue for the significant role EV respiratory strains as the etiological cause of bronchiolitis in infants aged less than 12 months (Tables 1 and 3). Whatever the exact prevalence of respiratory picornavirus infection as a cause of bronchiolitis remains to be determined in longitudinal prospective studies, testing the presence of all common viral pathogens in the respiratory tract of study infants before and after the development of respiratory symptoms.

Our findings indicated that EV respiratory diseases could be diagnosed from February to December, encompassing a part of the classical epidemic period of viral respiratory infections in French children (16, 21). These data suggested the possibility of mixed respiratory infections with EVs associated with other common respiratory viruses (1, 22). During the present study, we only identified 31 cases (13% of 285 cases) of mixed viral infection with EV in association with HRSV, human rhinovirus, or *Mycoplasma pneumoniae*. This detection of mixed respiratory infections might be significantly enhanced by the use of real-time RT-PCR or PCR assays for the detection of respiratory pathogens, which are known to significantly increase the sensitivity levels of virus or bacteria detection and to allow the detection of noncultivable or new respiratory viruses as human coronavirus, metapneumovirus, or bocavirus (3, 12). Interestingly, EV respiratory strains were characterized by substantial fluctuations in levels of circulation over 84 months,

FIG. 3. Phylogenetic tree based on partial VP1 sequences (348 pb) of 77 human EV isolates identified as the viral cause of the respiratory syndrome diagnosed in French infants between 1999 and 2005. The numbers correspond, respectively, to the month and the year of isolation (set as "month-year" in the tree) of EV strains from pediatric nasopharyngeal samples. Solid boxes indicate the cases of a cocirculation of distinct EV respiratory tropism species during the same month of the same annual epidemic season. Dotted rectangles indicate examples of cocirculation of distinct EV respiratory serotypes within the same species during the same month of the same annual epidemic season. Bootstraps in boldface indicate examples of cocirculation of phylogenetically distinct subgroups belonging to the same EV strain (bootstraps > 60%) during the same month of the same annual epidemic season.

including large peaks, which was consistent with an epidemic pattern of circulation (Fig. 2). Moreover, we observed that the annual rates of the EV respiratory infections were significantly decreased in years 2000 and 2005 and that this phenomenon was concomitant with the emergence of echovirus 13 and 30 strains, respectively, which were responsible for two aseptic meningitis outbreaks (Table 2) (5, 14). These findings suggested that the annual rates of EV respiratory infections might be significantly decreased by the emergence of EV neurotropic strains responsible for aseptic meningitis outbreaks.

In the present investigation, the phylogenetic comparison of partial VP1 capsid protein region confirmed the identification of the serotypes obtained by micro-seroneutralization and allowed us to identify temporal trends and patterns of circulation of EV respiratory serotypes and strains. This phylogenetic approach demonstrated the concomitant or successive circulation of distinct species, serotypes, or subgroups of EV strains within the same month or the same annual epidemic period (Fig. 3). Taken together, these findings indicated the possibility of successive or repeated infections by distinct EV respiratory strains during the same or successive annual epidemic periods in children (Table 2 and Fig. 3). Moreover, these molecular findings suggested the possibility of viral genetic recombination within species A and B human EVs circulating in a single geographical area and infecting concomitantly infants with a symptomatic or asymptomatic respiratory picornavirus shedding (36).

Our data found that EV respiratory diseases cannot be distinguished from HRSV- and influenza virus-induced respiratory syndromes based on clinical findings in children (Table 3) (7, 8). However, EV-related respiratory diseases might be less severe with no cases of pneumonia, no admission to an intensive care unit, and a low death rate during the study period (Tables 1 and 3). Nevertheless, EV respiratory infections were associated with a substantial clinical and economical impact, as shown by a median hospital stay of 3.5 days (not shown) and by the observation that 20% of all patients with EV-induced respiratory infections were hospitalized with the risk of nosocomial transmission of EV strains to other patients and with the risk of nosocomial infection by other viral or bacterial agents in these children. No specific underlying diseases had been identified among children with EV-associated respiratory diseases (data not shown). Whatever the clinical impact of various underlying diseases on the severity and clinical outcome of EV-induced respiratory pathologies is remains to be determined in larger prospective studies, specifically studies in infants aged less than 12 months.

Taken together, our results indicate that EVs are important etiologic agents of childhood lower respiratory tract diseases and that these viral agents can be isolated from the nasopharyngeal tracts of infants with respiratory symptoms. However, like other human picornaviruses, EVs can be isolated by cell culture systems or detected by RT-PCR assays in the nasopharynxes of infants without EV-related respiratory symptoms (28). Therefore, it would be of major interest to assess the EV genomic RNA load in respiratory samples from infants with or without respiratory symptoms by quantitative real-time RT-PCR systems. Using such approaches in further prospective studies would allow researchers to determine the significant clinical threshold of EV-RNA values quantified in the nasopharyngeal samples of infants with EV-related respiratory dis-

eases. Moreover, the use of such quantitative EV RNA detection systems would improve the management of infant and adult patients with EV-related respiratory diseases by reducing unnecessary diagnostic and therapeutic interventions and by preventing nosocomial respiratory viral infections. These further quantitative molecular assays would be also valuable particularly with regard to disease control during the EV annual epidemic season.

In conclusion, we determined that respiratory diseases were the second most common EV-induced pathology after aseptic meningitis in infants, contributing substantially to LRTIs in infants aged 1 to 12 months. Moreover, the successive or concomitant circulation of genetically distinct EV strains indicates the possibility of pediatric repeated respiratory infections during the same epidemic season and suggests the possibility of genetic recombination within species A and B human respiratory EV strains. The further development of valuable EV quantitative real-time RT-PCR assays associated with a genotyping RT-PCR assay will allow the rapid and valuable etiologic diagnosis of EV childhood respiratory infections and help to prevent nosocomial transmission of these viruses in pediatric departments and also help to control the emergence of new EV respiratory strains.

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