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The added value of diagnostics to characterize age-specific patterns of respiratory viral infections and coinfections and to detect emerging threats

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Abstract

Background Pandemic restrictions caused variation in respiratory virus circulation until the winter of 2022/23. The aim of this study was to monitor respiratory virus cases in the 2023/24 epidemic season.

Methods Children and adults attending Sapienza University Hospital for acute respiratory infections (October 2023-June 2024) were tested for respiratory viruses via molecular methods.

Results Of the 1121 patients included, 880 (78%) were positive for rhinovirus (HRV, 32%), Influenza A (IAV, 29%), and respiratory syncytial virus (RSV, 28%). RSV is more common in infants, and IAV is more common in adults, whereas HRV is more common in children aged 1–5 years. IAV, RSV and HRV cocirculate in winter; HRV cases also occur in spring, along with Influenza B (IBV) and other viruses. Despite circulating in the same weeks, the number of observed coinfections was much lower than that predicted for IAV and RSV ($p<.0001$) and lower also for the IAV/IBV, IBV/RSV and RSV/HRV pairs ($p<.0001$, $p=.0059$, $p=.015$, respectively). IAV and RSV cocirculated with different patterns in different age groups. In fact, in children aged 1–5 years, the RSV peak preceded that of IAV, whereas in older age groups, the RSV peak occurred toward the end of IAV circulation. Sequencing of HRV/EV cases in spring revealed 25 HRV genotypes and two EV-C105 cases.

Conclusions Respiratory viruses can cause age-specific seasonal peaks that are modulated by viral interference phenomena. Molecular diagnostic data should be integrated with surveillance programs to characterize seasonal circulation patterns of common respiratory viruses and to rapidly detect the next pandemic threat.

Keywords Respiratory virus diagnostics, Viral coinfections, Viral interference, Molecular epidemiology, Enterovirus-C105

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Introduction

Severe acute respiratory infections (ARIs) caused by respiratory viruses remain a major global health problem, despite advances in primary and secondary prevention, with few options for antiviral therapy. In addition to SARS-CoV-2, viruses such as influenza A (IAV) and respiratory syncytial virus (RSV) can cause ARIs with significant morbidity and mortality [1]. Prior to the COVID-19 pandemic in temperate climates, IAV and RSV showed winter seasonality influenced by meteorological factors [2–4], whereas other respiratory viruses caused more infections in the fall and spring [5]. However, during the prepandemic period, the emergence of new viruses/genotypes, such as IAV H1N1 2009 [6–8] and the RSV ON1 genotype [9], anticipated or delayed other virus epidemic peaks compared with historical time series due to a lack of herd immunity and/or viral interference [10].

The COVID-19 pandemic has caused important changes in the seasonality of respiratory viruses and in hospitalization trends [11]. The circulation of almost all respiratory viruses was halted by pandemic restrictions in the first half of 2020, and the number of cases of ARIs decreased dramatically; only human rhinoviruses (HRVs) appeared to circulate in the fall–winter of 2020/21 [11]. Since the relaxation of restrictions in 2021, RSV was the first to reemerge and caused an intense epidemic in many countries around the world in summer–autumn 2021 [12]. Influenza and other respiratory viruses do not show the same epidemiological trend and do not circulate again until fall-winter 2022/23 [11]. This season was characterized by the resurgence of IAV and the circulation of RSV, which increased again, together with intense circulation of SARS-CoV-2; these three viruses caused a "tripledemic" with a severe impact on health services [13]. Other respiratory viruses have also gradually returned to cause infections, allowing for the possibility of coinfections in the same host [11].

The profound changes in the major causes of respiratory viral infections observed in recent years have reinforced the concept that routine etiologic diagnosis can lead to optimized clinical management of patients and integrate syndromic surveillance [11]. The main objective of this study was to document the circulation of the main respiratory viruses after the perturbations observed during and after the pandemic period, using and integrating the routine molecular diagnostics performed on ARI cases from patients of all ages since the beginning of the fall–winter season (2023/24). A secondary objective was to understand how and when different viruses cocirculate in the same population, despite the documented phenomenon of viral interference [10]. The distributions of the main respiratory viruses (IAV, IBV, RSV and HRV) were analyzed by sex and age, and the frequency of coinfection was compared. The overall distribution of positive cases by week of occurrence and by age group was also analyzed. Since the number of hospitalizations for ARIs did not decrease until June 2024, this study further characterized the HRV genotypes responsible for this unusual peak.

Materials and methods

Patients and samples

Patients of all ages presenting with respiratory symptoms to the Pediatric and Adult Emergency Departments (EDs) of Sapienza University Hospital or to other departments of the same hospital are initially triaged with SARS-CoV-2 rapid antigen tests. The SARS-CoV-2 negative cases are then evaluated by the attending physician, for the presence of ARI, defined according to the European Respiratory Virus Surveillance Guidelines [14] as the sudden onset of at least one of the common respiratory symptoms such as cough, sore throat, shortness of breath, coryza, and the clinician's judgment of an infectious disease. Respiratory specimens from ARI cases are sent to the Microbiology and Virology Laboratory of Sapienza University Hospital, Policlinico Umberto I, for the diagnosis of respiratory pathogens.

Patients were enrolled from October 2023 until the end of the respiratory season, 20 June 2024. Samples from the same patient within three weeks were not included, to avoid double counting of a single case in the event of persistent positivity. Adult and pediatric cases with a clinical/microbiologic diagnosis of bacterial pneumonia, obtained by common biochemical blood markers and/or cultural methods, and samples positive for atypical respiratory bacteria (*Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumoniae*, *Bordetella pertussis*) were excluded. Finally, because most patients with ARI are initially tested with SARS-CoV-2 rapid antigen tests and, if positive for SARS-CoV-2, are not further tested with the multiplex respiratory panels but follow other procedures for public health and infection control reasons, the SARS-CoV-2 positive cases were not included in this study.

The institutional review board and ethics committee of Sapienza University approved the study protocol for pediatric patients (Prot. 107/12) and for adult patients (Prot. 0966/2023) and waived the need for informed consent because patient demographics were retrospectively extracted from the diagnostic records and anonymized, and no clinical data were obtained.

Detection of respiratory viruses

Approximately half of the enrolled pediatric ARI cases were tested via the QIAstat-Dx® Respiratory SARS-CoV-2 Panel (Qiagen), a fully automated syndromic assay that detects 23 respiratory pathogens: IAV, IBV, RSV, human metapneumovirus (hMPV), HRV/enterovirus

(both detected but not differentiated), human coronavirus (hCoV) 229E, HKU1, NL63, OC43, SARS-CoV-2, parainfluenza virus (PIV) 1–4, adenovirus (AdV), bocavirus (BoV), *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumoniae*, *Bordetella pertussis*. The remaining pediatric samples and most adult nasal or nasopharyngeal samples were tested with the rapid Xpert® Xpress CoV-2/Flu/RSV plus (Cepheid) molecular test, which can distinguish between IAV and IBV. To integrate the number of respiratory viruses detected by the syndromic assay with those tested with the rapid molecular test, an aliquot of the diagnostic sample was preserved for the detection of HRV/enterovirus (EV), hMPV, hCoVs 229E, HKU1, NL63 and OC43, PIV 1–3, AdV, and BoV via custom-made qualitative PCR methods previously published [15]. In a subset of samples, HRV/EV genotyping was performed via Sanger sequencing of the conserved 5' UTR tract [16] and/or the VP4/2 coding region [17].

Statistical analysis

The chi-square test was used to test for significant differences in categorical variables; Student's t test or the Mann–Whitney test was used to compare quantitative variables.

The rate of respiratory viruses by age was analyzed by stratifying patients into five age groups: infants (< 1 year), children (1–5 years), older children (6–17 years), adults (18–65 years), and older adults (> 65 years).

Seasonal onset for a respiratory virus was defined as the first 2 consecutive weeks in which > 10% of respiratory samples tested positive for that respiratory virus.

The observed and expected coinfections of the more frequently detected respiratory viruses were compared via the method recently described by Wu et al. [18]. Briefly, the expected number of coinfections for a specific virus pair was calculated as the product of the incidence of virus 1 and the incidence of virus 2 multiplied by the total number of samples tested and then compared with the observed coinfections via chi-square tests.

Results

Respiratory virus detection rates

A total of 1324 respiratory specimens were tested at the Microbiology and Virology Laboratory of Sapienza University Hospital from October 1, 2023, to June 20, 2024. Repeated samples from the same patient were excluded to have a single sample per infection case. Moreover, patients who were positive for atypical bacteria, had a diagnosis of bacterial pneumonia, or were positive for SARS-CoV-2 were excluded. These criteria resulted in the inclusion of 1121 patients with ARIs, as shown in Fig. 1; of these, slightly more than half (595/1121: 53.1%) were pediatric patients (< 18 y). Overall, 241/1121 (21.5%) patients were negative for respiratory viruses, 880/1121 (78.5%) were positive for any respiratory virus, and 159/880 (18.1%) patients were infected with two or more different viruses. No sex difference was found between those negative and positive for respiratory viruses (Table 1). The virus-positive individuals were significantly younger than the virus-negative individuals were, and viral infections were significantly more common at a young age (Table 1).

Table 2 shows the total number of samples that met the inclusion criteria and were positive for each respiratory virus tested. The vast majority of virus-positive patients (785/880: 89.2%) were infected with one of the four respiratory viruses, namely, IAV (N = 257/880: 29.2%), IBV (N = 71/880: 8.1%), RSV (N = 249/880: 28.3%), or HRV (N = 285/880: 32.4%), in single infection or coinfection. For the other respiratory viruses, the number of positive cases, including coinfections, was 50 or less (Table 2): positivity rates for hMPV, AdV, hCoVs, PIV, and BoV ranged from 5.7 to 2.4% of positive cases.

The distribution of these cases was similar by sex but differed significantly by median age (Table 2). In fact, positive cases were distributed differently by age group (p <.0001). Specifically, IAV was much more prevalent in both adults and older adults (p <.0001); pediatric cases of IAV were also numerous, with the exception of infants < one year of age. IBV infected older children/adolescents more than other age groups did (p <.0001). As expected, RSV caused far more infections in infants (p <.0001); however, almost 10% of the RSV-positive patients were elderly individuals (≥ 65 years). HRV-positive cases were also distributed differently by age (p =.001); cases were more common in children aged 1–5 years than in infants (Table 2). All other respiratory viruses were more common in infants/children, except hCoVs, which were evenly distributed by age (Table 2).

Respiratory virus circulation by calendar week

In October 2023, approximately half of the samples tested were virus negative; HRV positive samples accounted for approximately 40% of the total cases tested. The start of the season for IAV and RSV was recorded at weeks 44–45/23, and the number of detections then increased rapidly; the seasonal peak of detection was almost simultaneous for IAV and RSV at the beginning of the year and at weeks 52/23 and 1/24, after which the number of positive cases slowly decreased for both viruses (Fig. 2). IAV and RSV cases reached very low levels in weeks 10–13, when IBV cases peaked; in turn, IBV cases seemed to decrease as HRV cases increased in April. The incidence of HRV-positive cases remained high in May and the first three weeks of June. Overall, the number of cases of ARIs was particularly high in the spring, which was also the

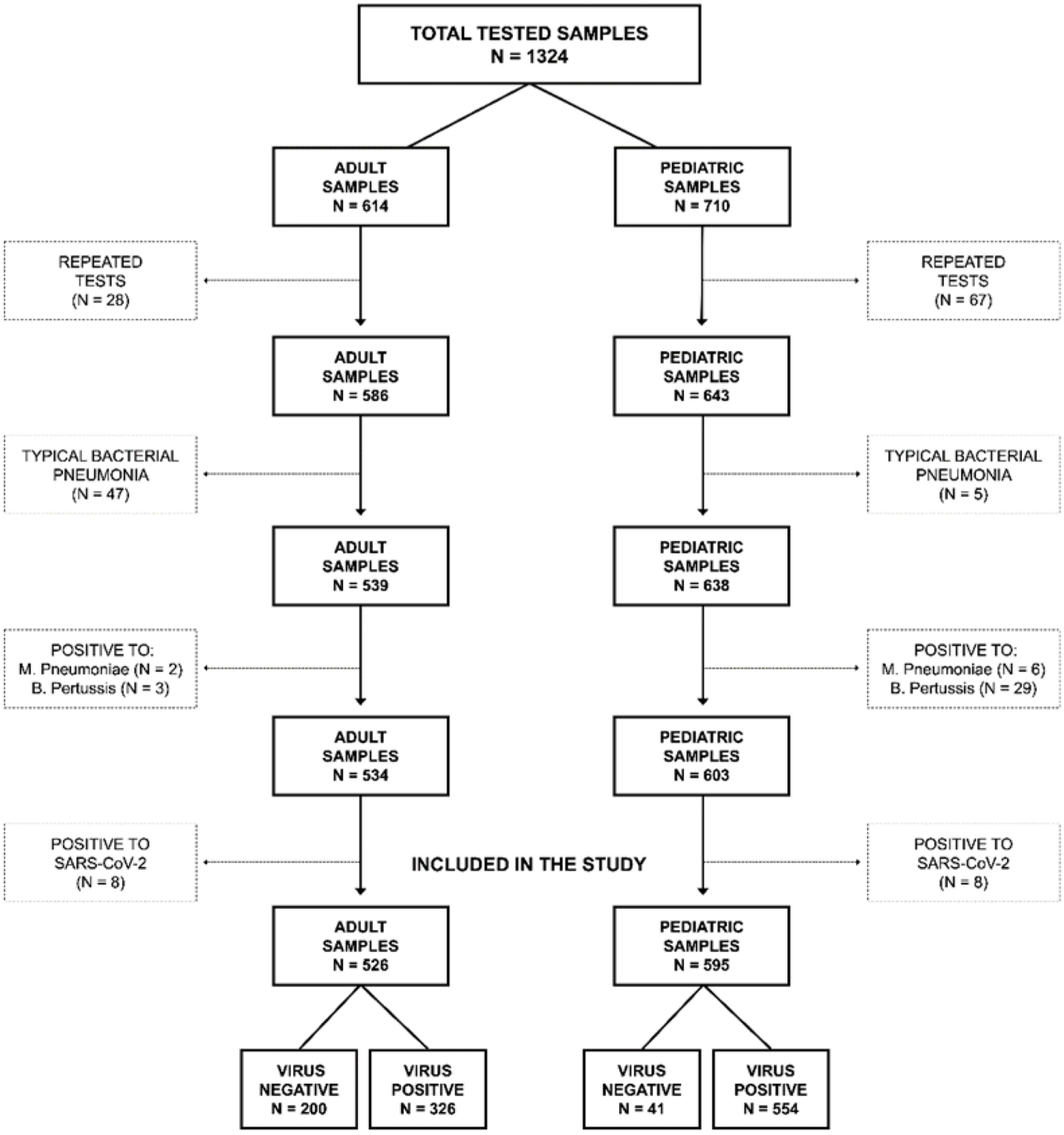

Fig. 1 Flowchart showing the selection of cases included in the study and those excluded for the various exclusion criteria

peak circulation period for hMPV, AdV, hCoV, PIV, and BoV (Fig. 2).

Respiratory virus coinfection rates

Notably, the two major respiratory pathogens, IAV and RSV, had the lowest coinfection rates (18.7% and 21.7%, respectively), followed by IBV (25.3%) (Table 3). Conversely, the coinfection rate for HRV was as high as 42.4%. The occurrence of HRV coinfections was 87 of 155 cases (56.1%) during the winter weeks (including 48–52/2023 and 1–11/2024) and 34/130 cases (26.1%) during the fall/spring weeks (Fig. 2, $p<.0001$). Therefore, HRV was more frequently detected in coinfections during the winter months, when seasonal peaks of IAV and RSV occurred, than during the fall and spring, when HRV circulated with other respiratory viruses (Fig. 2).

We subsequently sought to estimate whether the observed rates of coinfection differed from those that

Table 1 Demographic data of patients positive and negative for respiratory viruses

Patients		Virus negative (*N* = 241)	Virus positive (*N* = 880)	*p* value
Female sex[a] (%)		107 (44.4%)	425 (48.3%)	0.308
Median age in years (IQR)[b]		66 (0–96)	4 (0-101)	< 0.0001
Pediatric cases (< 18 y)		41	554	< 0.0001
Patients' Age group	< 1 year	16	295	< 0.0001
	1–5 years	18	167	
	6–17 years	7	92	
	18–65 years	75	137	
	> 65 years	125	189	

[a] Data on sex assigned at birth; [b]IQR: interquartile range

Table 2 Distribution by sex and age of the different respiratory viruses among positive cases. Data are shown for the total cases (including coinfections) positive for influenza A virus (IAV), influenza B virus (IBV), respiratory syncytial virus (RSV), rhinovirus/enterovirus (HRV), human metapneumovirus (hMPV), adenovirus (AdV), human coronavirus (hCoV) 229E, HKU1, NL63, OC43 together, parainfluenza viruses (PIV) 1–4 together, and bocavirus (BoV)

Positive cases	IAV *N* = 257	IBV *N* = 71	RSV *N* = 249	HRV *N* = 285	hMPV *N* = 50	AdV *N* = 47	hCoV *N* = 46	PIV *N* = 29	BoV *N* = 21	*p* value
Female sex [a] (%)	142 (55.2)	28 (39.4)	124 (49.8)	133 (46.7)	21 (42)	21 (46.7)	24 (52.2)	9 (31.0)	7 (33.3)	0.114*
Median age in years (IQR) [b]	62 (55)	6.5 (13)	0 (2)	6.5 (57)	1.5 (63)	9 (60)	31 (65)	1 (5)	1 (7)	< 0.0001**
< 1 year	14 (5.4)	7 (9.8)	170 (68.3)	100 (35.1)	21 (42.0)	13 (27.7)	12 (26.1)	22 (75.9)	7 (33.3)	< 0.0001**
1–5 years	48 (18.7)	12 (16.9)	39 (15.7)	68 (23.9)	15 (30.0)	17 (36,2)	6 (13.0)	3 (10.3)	11 (52.4)	
6–17 years	19 (7.4)	38 (53.5)	4 (1.6)	37 (13.0)	5 (10.0)	4 (8.5)	8 (17.4)	2 (6.9)	2 (9.5)	
18–65 years	72 (28.0)	9 (12.7)	12 (4.8)	37 (13.0)	2 (4.0)	6 (12.8)	9 (19.6)	1 (3.4)	0	
> 65 years	104 (40.5)	5 (7.0)	24 (9.6)	43 (15.1)	7 (14.0)	7 (14.9)	11 (23.9)	1 (3.4)	1 (4.8)	
p value*** (column)	< 0.001	< 0.001	< 0.001	0.001	0.032	0.050	0.336	0.001	0.001	

[a] Data on sex assigned at birth; [b] IQR: interquartile range;

* p value calculated for single infection cases; ** p value relative to the comparison of the age distribution among all positive cases (including coinfections); *** p value calculated for the case distribution by age (including coinfections) for each virus

would be expected by chance. Among the 765 samples meeting the inclusion criteria tested during the winter, 237 (31%) were positive for IAV, 32 (4.2%) were positive for IBV, 224 (29.3%) were positive for RSV, and 147 (19.2%) were positive for HRV. On the basis of these rates, the expected number of coinfections was calculated as described in the Methods section, resulting in 69 IAV/RSV cases, 45 IAV/HRV cases, and 43 RSV/HRV cases. The observed cases were then compared with the expected cases, which yielded the following results: the observed coinfections were significantly lower than expected between IAV and RSV (8 vs. 69, $p < .0001$) and lower than expected for the RSV/HRV pair (24 vs. 43, $p = .015$). In contrast, the difference between the expected and observed cases was not statistically significant for the IAV/HRV pair (32 vs. 45, $p = .105$). No IAV or IBV coinfections were detected during the winter, which was significantly different from the predicted number of 10 coinfections ($p < .0001$). The observed coinfections of IBV and RSV during the winter months were less common than expected (1 vs. 9; $p = .0059$), whereas for the pair IBV/HRV, the observed coinfections were not significantly different from those expected (5 vs. 6; $p = .740$).

Respiratory virus biweekly circulation by age group

While the circulation of IAV and RSV appeared to be simultaneous when considering cases of all ages (Fig. 2), the very low coinfection rate observed suggested a negative association between IAV and RSV. In this context, an age-specific picture of the seasonal patterns of the four main respiratory viruses revealed interesting differences (Fig. 3).

In infants < 1 year of age, RSV cases ($N = 170$) outnumbered IAV cases ($N = 14$), as expected in this age group. RSV detection began in mid-November (weeks 44–45/23), and from weeks 46–47/23 to weeks 06–07/24, RSV-positive cases accounted for approximately 80% of the cases tested. Thereafter, a decrease in the prevalence of RSV-positive cases was observed, with an increase in the number of HRV-positive cases (Fig. 3A). In children up to 5 years of age, the first cases of RSV and IAV were detected in November; subsequently, there was a peak of RSV-positive cases, but then the number of RSV-positive cases decreased in favor of the IAV peak (Fig. 3B). In the other age groups, far more IAV- than RSV-positive cases were detected (Table 2; Fig. 3C-E); interestingly, in the older age groups, the RSV peak occurred at weeks

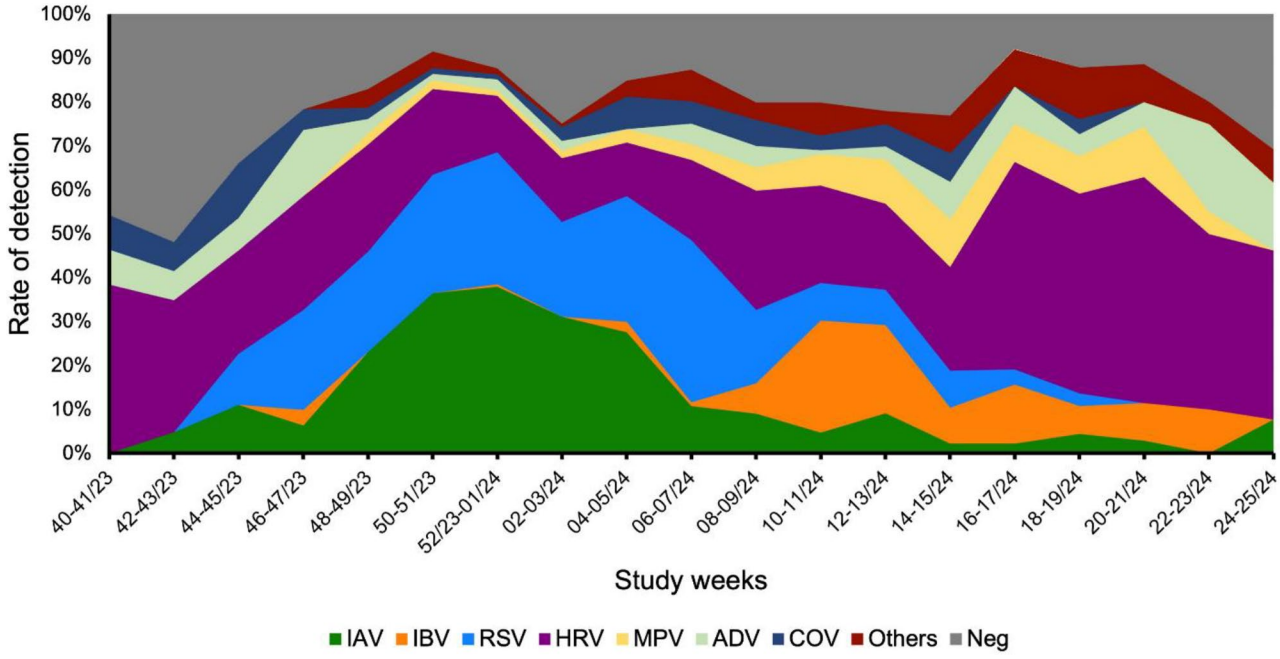

Fig. 2 Biweekly percentage of virus positive and negative samples relative to the total number of respiratory samples tested. The percentages of cases positive for influenza A virus (IAV), influenza B virus (IBV), respiratory syncytial virus (RSV), rhinovirus/enterovirus (HRV), human metapneumovirus (hMPV), and adenovirus (AdV), human coronavirus (hCoV) 229E, HKU1, NL63, and OC43 together, parainfluenza virus (PIV) 1–4, and bocavirus (BoV) grouped as others, and negative cases among the total number of tested samples were calculated biweekly and plotted on the Y-axis. The calendar weeks are plotted on the X-axis

Table 3 Distribution of the different respiratory virus coinfection cases. The total numbers of cases in coinfection are shown for influenza A virus (IAV), influenza B virus (IBV), respiratory syncytial virus (RSV), rhinovirus/enterovirus (HRV), human metapneumovirus (hMPV), adenovirus (AdV), human coronavirus (hCoV) 229E, HKU1, NL63, OC43, parainfluenza viruses (PIV) 1–4, and bocavirus (BoV). The number of cases for the specific coinfection pair is shown only for the more frequently detected viruses, IAV, IBV, RSV and HRV, with all other viruses

Positive cases	**IAV *N*=257**	**IBV *N*=71**	**RSV *N*=249**	**HRV *N*=285**	**hMPV *N*=50**	**AdV *N*=47**	**hCoV *N*=46**	**PIV *N*=29**	**BoV *N*=21**	
N (%) of cases in coinfection[a]	48 (18.7)	18 (25.3)	54 (21.7)	121 (42.4)	22 (44)	17 (36.2)	24 (52.2)	15 (51.7)	12 (53.2)	<0.0001
Specific coinfection pair[a]										
	IAV	**IBV**	**RSV**	**HRV**	**hMPV**	**AdV**	**hCoV**	**PIV**	**BoV**	
IAV *N*=48		0	8	33	7	0	3	0	1	<0.0001
IBV *N*=18	0		1	15	0	0	1	0	1	
RSV *N*=54	8	0		30	2	6	7	1	1	
HRV *N*=121	33	15	30		11	9	10	10	9	

[a] The total number of viruses in coinfections (row total counts) exceeds the total number of coinfected patients because more than two viruses may be present in a patient

6–7/24 (the first half of February) toward the end of IAV circulation.

Overall, the occurrence of IBV cases followed the peak of IAV and RSV cases (Fig. 2), and the age distributions of the infections were quite different. The incidence of IBV infection is relatively low in infants and elderly individuals. In contrast, IBV infection was more prevalent in children aged 1–5 years and reached its highest prevalence in older children (6–17 years).

The occurrence of HRV-positive cases was mainly observed in infants and children up to 5 years of age (35% and 24% of HRV cases, respectively), and these cases circulated throughout the study period. However, it caused more cases as a single pathogen and was the predominant respiratory virus circulating in the fall and spring, which is consistent with the seasonal patterns more commonly described for HRV. In addition, the number of HRV-positive cases, particularly in children, during the late spring period was somewhat anomalous compared with data previously collected during the spring (our unpublished data). This prompted us to characterize the genotypes of HRVs circulating in spring.

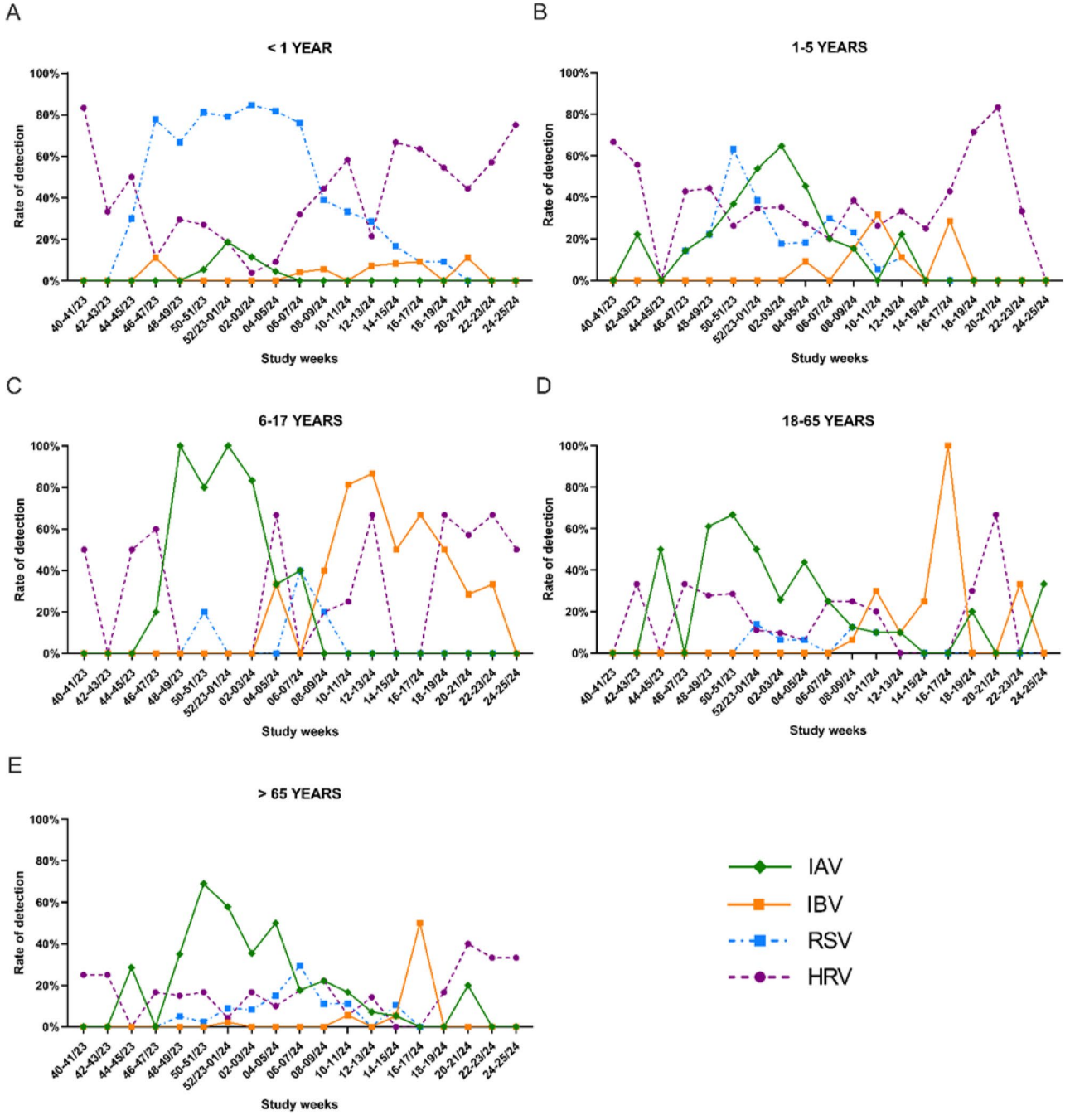

Fig. 3 Biweekly percentage of samples positive for IAV, IBV, RSV and HRV by age group. The percentages of influenza A virus (IAV)-, influenza B virus (IBV)-, respiratory syncytial virus (RSV)-, and rhinovirus/enterovirus (HRV)-positive cases among the total tested cases were calculated biweekly by age group and plotted on the Y-axis. Calendar weeks are plotted on the X-axis

Sequence analysis of the HRVs circulating in spring

Of the 93 HRV-positive samples detected in spring (from weeks 12–13 to weeks 24–25), approximately half had sufficient remaining aliquots that were retrospectively subjected to PCR for genotyping, as detailed in the Methods section. Among these, 38 were successfully genotyped; the demographic data and species and genotype distributions of the sequenced HRV-positive cases are presented in Table 4. Among the 38 HRV cases, 25 were identified as HRV-A (65.8%), two as HRV-B (5.3%), and nine as HRV-C (23.7%). Among the HRV-A cases, 16 distinct genotypes were identified, with at least one occurrence, four genotypes were detected twice, and two genotypes were identified in three cases. Among the HRV-C cases, seven different genotypes were identified, with two genotypes detected twice. Furthermore,

Table 4 Characterization of rhinovirus (HRV)- and enterovirus (EV)-positive cases detected in spring. Patient data, species distributions and genotype assignments are shown for 38 HRV/EV-positive cases that were sequenced

Positive cases		HRV-A *n* = 25	HRV-B *n* = 2	HRV-C *n* = 9	EV *n* = 2
Female sex[a] (%)		10 (40.0)	1 (50.0)	4 (44.4)	2 (100)
Pediatric cases (< 18 y)		21 (84.0)	1 (50.0)	8 (88.9)	2 (100)
Patients' Age group	< 1 year	8	0	2	0
	1–5 years	6	0	2	2
	6–17 years	7	1	4	0
	18–65 years	3	0	1	0
	> 65 years	1	1	0	0
HRV genotype detected (N) [b]		1, 7, 9, 12(2), 15, 16(3), 20(2), 24, 40(2), 58(2), 63, 71,80, 82(3), 89, 102(2)	4, 70	5, 19, 25, 27, 48(2), 56(2), NT	EV-C105(2)

[a] Data on sex assigned at birth; [b] The number of occurrences of each genotype is given in parentheses when more than one. One case was not typed (NT) because the sequence had < 90% similarity to the classified HRV-C genotypes

two cases (5.3%) were classified as EV-C105, as the sequences obtained from the conserved 5' UTR tract [16] and from the VP4/2 coding region [17] exhibited high similarity to the strain Pavia/9095 (NCBI accession number KM880100), which was identified more than 10 years ago in northern Italy [19]. These EV-C105 infections occurred at weeks 17 and 21 in two female pediatric patients aged 2 and 4 years, respectively.

Discussion

The COVID-19 pandemic underscores the critical importance of diagnosing the causative agents of ARI to improve the clinical management of patients but also for prevention and control, given the potential to identify novel or mutated viruses that may challenge the healthcare system and cause severe outbreaks. To evaluate the potential utility of a comprehensive characterization of respiratory infections other than SARS-CoV-2, this study integrated the routine molecular diagnosis performed on patients with ARIs from October 2023 to June 2024 with laboratory-developed qualitative PCR or real-time PCR methods and Sanger sequencing. We used these data to observe how interactions between respiratory viruses influenced the age-specific seasonal peak of the major respiratory viruses in this third postpandemic season.

This study included more than 1,100 pediatric and adult patients presenting with symptoms of ARI to a large hospital in Rome, Italy, nearly 80% of whom tested positive for a respiratory virus.

Overall, the most commonly detected virus was HRV, which is in agreement with recent literature reporting a high prevalence of HRV infections in hospitalized patients of all ages [20–23]. The widespread use of multiplex testing for respiratory viruses has increased the detection of HRV, often with a high rate of cases in coinfection, but its etiologic role in severe infections is no longer questioned [24, 25].

IAV and RSV caused a similar number of cases and were the most prevalent viruses in the more vulnerable age groups, with RSV being the predominant virus in infants and IAV in elderly individuals, as in the pre-COVID-19 period [1, 2].

IBV was the fourth most common virus and mainly infected children and adolescents. This indicates the resurgence of typical IBV circulation in contrast to the 2022/23 season, during which, in contrast to IAV, IBV cases were sporadic in Italy [26]. Indeed, IBV appeared to be affected by pandemic restrictions more than IAV has been, with the B/Yamagata lineage becoming undetectable since 2020 [27].

Additionally, all other respiratory viruses included in the diagnostic panels (hMPV, AdV, hCoVs, PIV, BoV) were identified, with positivity rates ranging from 4.5 to 2% of the total patients.

Given these observed rates, it appears that all respiratory viruses returned to normal circulation during this third postpandemic season. In fact, although a similar ARI surveillance program was not performed in our center prior to the onset of the pandemic, other surveillance studies performed by our group reported similar positivity rates in infants hospitalized for bronchiolitis in Rome [15] and, limited to RSV and hMPV, in patients of all ages hospitalized for ARI in Italy [28].

No surveillance studies on ARIs have yet been published for the 2023/24 epidemic season as of September 2024, but the European Mortality Monitoring Network (EuroMOMO) recently conducted an evaluation of all-cause mortality for the winter of 2023/24 [29]. The results indicate a notable increase from week 48/23 to week 6/24, with the peak number of cases occurring at week 51/23, which was attributed to infections with SARS-CoV-2, influenza, or RSV [29]. In Italy, the number of weeks with the most pronounced increases in excess mortality was 51/23, 52/23, and 1/24 [29], which corresponds with the peak incidence of IAV and RSV in our study.

Notably, IAV and RSV, despite circulating in the same weeks, had the lowest rate of coinfection among themselves and with the other respiratory viruses tested in this study. In contrast, the coinfection rate of HRV was significantly greater than that of IAV, IBV and RSV and comparable to that of the other respiratory viruses. Notably, the HRV coinfection rate was significantly greater during the winter, when the HRV circulated concurrently with IAV and RSV.

Recent studies have evaluated the expected versus observed incidence of respiratory virus coinfection via various statistical and mathematical techniques [18, 30–31] to determine whether viruses can potentially interfere with each other. In addition, the mechanisms of viral interference have been evaluated via in vitro and in vivo experimental models [10].

We calculated the expected versus observed incidence of coinfection among the four respiratory viruses more frequently detected during the winter, using the method implemented by Wu et al. [18]. Confections occurred less frequently than expected by chance for the pairs IAV/IBV, IAV/RSV IBV/RSV, and RSV/HRV, whereas the frequency was similar to that expected for IAV/HRV and IBV/HRV coinfections. These data suggest that IAV and IBV interfere with each other and with RSV but not HRV.

In accordance with our findings, previous studies reported low frequencies of IAV and IBV coinfection in patients [32, 33]; however, the latter study revealed that IAV/IBV coinfection was associated with increased clinical severity [33]. In vitro experiments have shown that IBV strongly suppresses IAV replication in cell culture [34], but IAV increased IBV infectivity in a fluorescent virus model [35].

IBV circulation generally occurs after the seasonal peak of the IAV epidemic, and its pathogenicity tends to be lower than that of IAV [36]. Recently, IBV infection was reported to cause a decreased risk of noninfluenza respiratory virus infection in children [37]; our analysis suggests that IBV and RSV but not IBV or HRV interfere with this risk.

Consistent with our findings, replication interference between influenza viruses and RSV has been demonstrated in different experimental models [38–41]; IAV may prevent subsequent infection with RSV [38, 39], and prior RSV infection decreases the efficiency of subsequent IAV infection [40, 41]. Furthermore, low rates of IAV/RSV coinfection have been reported in children [42]. Similarly, interference between RSV and HRV has previously been suggested by documenting a lower than expected rate of coinfection in infants [43, 44].

Our analysis did not suggest strong interference phenomena between IAV and HRV in the winter, when both circulated abundantly. In contrast, large studies [18, 23, 31, 45] reported significantly lower than expected IAV/HRV codetections. However, in these studies, which were conducted in the prepandemic period, HRV circulation was more common in the fall and spring months and did not overlap much with IAV. In contrast, in our study, HRV circulated abundantly even during the winter, when IAV/HRV coinfections were most frequently detected. Cell culture experiments [18, 46] and results from a mouse model [47] revealed that previous HRV infection induced an interferon response capable of inhibiting IAV replication [18, 46]. It is quite possible that the opposite is not true, i.e., that a previous IAV infection is not capable of inhibiting HRV replication. In fact, epidemiological reports have identified an effect of HRV cases on IAV pandemic peaks [6] and not vice versa. In any case, the possibility of HRV coinfection with IAV or RSV during the winter months is high and should not be overlooked, but further studies are needed to clarify whether HRV coinfection could worsen the clinical course of influenza, pneumonia, and bronchiolitis.

To reconcile the observed viral cocirculation with the coinfection rates suggesting viral interference, we examined the age-specific biweekly distribution of the major respiratory viruses. Although the seasonal circulation of IAV and RSV and their epidemic peaks appeared to be similar overall, the age-specific infection patterns were quite different. In children up to 5 years of age, the RSV peak preceded that of IAV; conversely, in elderly individuals, the peak in RSV cases coincided with a decline in IAV cases. These patterns are consistent with the mutual ability of IAV and RSV to interfere and with the hypothesis that a trade-off exists that enables them to circulate in the same weeks while causing infections at different ages. Moreover, this would imply that the occurrence of RSV in older adults should not be assumed to be the same as that in pediatric patients but that a peak of RSV hospitalizations could be expected when IAV cases decline, i.e., weeks after the peak of pediatric cases. More data are needed to confirm that RSV infections may occur with age-specific seasonal peaks, as new preventive measures have been approved to prevent severe disease.

HRV cases were detected throughout the study period and in all age groups, especially in infants and children up to 5 years of age. After observing an increase in HRV/EV-positive cases in the spring, we further investigated these viruses by performing Sanger sequencing on residual diagnostic samples. Genetic characterization resulted in the identification of 25 HRV genotypes; the species distribution was similar to that reported in our previous study [48] and others [49, 50]. The large diversity of HRV genotypes identified rules out a nosocomial HRV outbreak and suggests that this surge in cases seems not to be caused by a few more virulent genotypes that survived the genetic bottleneck caused by pandemic restrictions. However, patients' clinical data were not available at the

time of the analysis, and we do not have information on the clinical course of these HRV-positive patients. We previously reported that HRV was the only respiratory virus that circulated in 2020 despite the lockdown measures implemented in Italy [51]; subsequently, we did not detect anomalous peaks of HRV hospitalizations in children from 2021 to 2023 (unpublished data). In contrast, a surge of HRV cases in pediatric ICU patients was observed in the UK after the first lockdown and until December 2022 [52]. Additionally, in the US, an increase in hospitalizations of pediatric patients with severe ARIs was observed in the late summer of 2022, with approximately 30% of cases positive for HRV/EV; of these, 17% were attributed to EV-D68 [53]. Among the HRV/EV-positive samples sequenced, two (5%) were identified as EV-C105 strains with the highest similarity to those detected in northern Italy in 2014 [19]. At present, we cannot exclude the possibility that other EV-C105 or other EV-positive samples will be among those positive for HRV/EV in spring 2024. The nonpolio enterovirus EV-C105 has been identified relatively recently [54] and is rarely detected in Europe [55, 56], but this infection may pose a risk of acute flaccid paralysis (AFP) in children [57]. Differentiation of EVs from the HRV is challenging with molecular diagnostic techniques, and genotype identification is not routinely performed. In Italy, enterovirus surveillance is routinely performed in wastewater and fecal samples from hospitalized AFP patients; this surveillance could be strengthened by enabling tertiary diagnostic centers to rapidly identify EVs in respiratory samples from suspected cases to prevent outbreaks. This integrated surveillance is in line with the recent WHO working group proposal [58] to "develop well-coordinated mosaics of multiple fit-for-purpose surveillance approaches" for the detection and assessment of respiratory viruses with epidemic and pandemic potential.

This study has limitations. First, the study design is limited to the period from October to June, 20 whereas a full year of surveillance could potentially add data to this study. The original design of the study was to enroll cases during the respiratory season, which generally lasts from October to April in Italy. However, persistent respiratory virus circulation was observed in April and the study was extended until week 25 of 2024, when very few specimens were positive. We believe that further extension of surveillance into the summer season would not significantly change the observed patterns of respiratory virus infections and co-infections. A second limitation, inherent to molecular testing, is that molecular multiplex assays detect the viral/bacterial genome and not the presence of the infectious pathogen. This limitation can be particularly relevant when coinfections are detected, as one of the viruses may be unrelated to the patient's clinical symptoms. Finally, due to the design of the study and the nature of the ethical approval, only laboratory records were analyzed and it was not possible to obtain clinical data.

In conclusion, this study characterized the circulation patterns of respiratory viruses in both pediatric and adult patients in the third postpandemic season and documented the return of all respiratory viruses to circulation in their usual seasonality. By analyzing coinfection rates and age-specific patterns, we demonstrated how IAV and RSV managed to cocirculate in the community during the same weeks, targeting hosts of different ages and thus avoiding mutual interference. Although its circulation was almost stopped by pandemic restrictions, HRV also circulated abundantly in 2023/24, causing many coinfections in winter and an unusual peak of cases in late spring, in which we identified several different HRV genotypes and two EV-C105 cases.

Routine molecular diagnostics should be implemented and integrated with virological, epidemiological and clinical data in new surveillance approaches that can help rapidly alert public health decision-makers in the event of intense and/or severe outbreaks.

Abbreviations

ARI	Acute respiratory infection
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
COVID-19	coronavirus disease-2019
IAV	influenza A virus
IBV	influenza B virus
RSV	respiratory syncytial virus
HRV	rhinovirus
hMPV	human metapneumovirus
AdV	adenovirus
hCoV	human coronavirus 229E, HKU1, NL63, and OC43 together
PIV	parainfluenza virus
BoV	bocavirus
EV	enterovirus
ED	Emergency Department
PCR	polymerase chain reaction
UTR	untranslated region
VP	viral protein
AFP	acute flaccid paralysis

Author contributions

AP: Designed the study and conducted data analysis; wrote the original draft of the manuscript; OT. GD'E and FM: Contributed to the study methodology and supervised data collection; MF, FF, AD'A, and RC: Performed sample collection, molecular diagnosis and sequencing; CS and RF: Performed statistical analysis, reviewed and edited the manuscript; PR and LC: Performed data analysis, prepare tables and figures GC, LP and GG: Collected patients' demographic data and performed data analysis; GA: Contributed to the study methodology, Funding acquisition, reviewed and edited the manuscript.

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Data availability

The data of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the principles of the Declaration of Helsinki. The institutional review board and ethics committee of Sapienza University approved the study protocol for pediatric patients (Prot. 107/12) and for adult patients (Prot. 0966/2023), and waived the need for informed consent because patient demographics were retrospectively extracted from the diagnostic records and anonymized, and no clinical data were obtained.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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