Le Infezioni in Medicina, n. 3, 311-320, 2025
doi: 10.53854/liim-3303-8

Back to normality? Respiratory viruses, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Bordetella pertussis* trends: local epidemiological update after the COVID-19 storm

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Article received 10 July 2025 and accepted 9 August 2025

SUMMARY

Background: The COVID-19 pandemic deeply impacted the epidemiology of respiratory viruses and bacteria, including *Mycoplasma pneumoniae* (MP), *Chlamydophila pneumoniae (CP)*, and *Bordetella pertussis* (BP). We have retrospectively examined the circulation patterns of major non-culturable respiratory pathogens that cause acute respiratory infections (ARIs) over four years (2021-2024), encompassing both the pandemic and post-pandemic phases.

Methods: The study was conducted in the five hospitals of the Friuli Venezia Giulia region, in northeastern Italy from January 2021 to December 2024. A total of 11,208 respiratory samples from adult and pediatric patients displaying symptoms of ARIs, but negative for SARS-CoV-2, in accordance with our testing protocols, were tested for the following pathogens: influenza A and B (FLU A-B), adenovirus (ADV), coronaviruses (COV) 229E, NL63, OC43, bocavirus (BOV), enterovirus (EV), metapneumovirus (MPV), rhinovirus (RV), parainfluenza types 1–4 (PIV 1–4), respiratory syncytial virus A and B (RSV A/B), MP, CP, BP, and *B. parapertussis* (BPP).

Results: The number of tests increased from 1,076 in 2021 to 4,377 in 2024. Overall positivity rates rose as follows: 27.4% (295/1,076) in 2021, 33.6% (714/2,125) in 2022, 41.3% (1,500/3,631) in 2023, and 47.2% (2,067/4,377) in 2024. RV, ADV, BOV, COV, PIV 1-4, MPV, and RSV A/B gradually returned to pre-pandemic circulation levels. FLU A-B, not detected in 2021, re-emerged in 2022. MP, BP, and CP, nearly absent in 2021 and 2022, reappeared in 2023.

Conclusion: This retrospective study assessed the circulation of respiratory viruses, MP, CP and BP in our geographical area, observing their gradual and asynchronous re-emergence following the COVID-19 pandemic. Strengthening advanced molecular microbiological diagnostics within clinical and epidemiological settings is crucial for supporting new surveillance models and promoting the judicious use of antibiotics.

Keywords: Respiratory viruses, acute respiratory infections (ARIs), *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Bordetella pertussis*, molecular epidemiology.

INTRODUCTION

Acute Respiratory Infections (ARIs) are a major cause of morbidity worldwide. They can be caused by a range of pathogens, including viruses and the so-called atypical bacteria such as *Mycoplasma pneumoniae* (MP), *Chlamydophila pneumoniae* (CP), and *Legionella pneumophila* (LP). Among viral agents, the most prevalent include influenza viruses (FLU A-B), respiratory syncytial viruses (RSV), and parainfluenza viruses (PIV). In addition, *Bordetella pertussis* (BP), though less frequent, is responsible for the characteristic clinical syndrome known as whooping cough [1].

While viruses are the primary cause of upper and lower respiratory tract infections, mixed infections involving both viral and bacterial pathogens are well documented, posing diagnostic and treatment challenges, as clinical features often fail to differentiate between viral and bacterial etiologies.

The COVID-19 pandemic has significantly changed the epidemiology of respiratory infections, impacting viral seasonality and altering hospitalization patterns. Respiratory viral infections -especially those caused by influenza viruses and RSV - declined significantly at the onset of the pandemic and fluctuated with subsequent waves of SARS-CoV-2. From the beginning, the widespread adoption of Non-Pharmacological Interventions (NPIs), including social distancing, mask use, and lockdowns, helped curb the spread of the virus [2]. These measures not only limited SARS-CoV-2 transmission but also greatly disrupted the circulation of other common respiratory viruses by breaking their transmission chains. As a result, the 2020-2021 season witnessed a dramatic decline in respiratory viral infections, with some pathogens, like influenza viruses and RSV, nearly disappearing in certain areas, including ours [3, 4]. The subsequent lifting of restrictions led to an unusual and asynchronous resurgence of respiratory infections, resulting in significant shifts in seasonality and age distribution for several viruses, including RSV, FLU A-B, rhinovirus (RV), seasonal coronaviruses (COV), PIV, adenovirus (ADV), and metapneumovirus (MPV) [5-7]. These epidemiological shifts highlight the need for a systematic and organized approach to rapid multiplex molecular testing. This approach enables the simultaneous detection of multiple pathogens associated with a given syndrome, supporting more timely and accurate diagnosis and treatment decisions. Early viral identification is also vital to prevent healthcare-associated outbreaks, provided that appropriate containment measures are implemented [8]. Meanwhile, molecular testing is essential in ruling out bacterial causes, enhancing targeted antimicrobial stewardship, and minimizing unnecessary antibiotic use [9, 10].

Thus, data collected from molecular syndromic epidemiology plays a crucial role in developing control and prevention strategies. This includes guiding vaccine design, establishing early detection programs, and promoting responsible antibiotic use [9, 10]. In this context, we aimed to examine the circulation patterns of major non-culturable respiratory pathogens associated with ARIs from 2021 to 2024, covering both the pandemic and post-pandemic phases of COVID-19. SARS-CoV-2 infections were excluded from the study due to changes in testing protocols. Initially, molecular testing was utilized, but it shifted to rapid antigen testing, which became the primary method. Therefore, individuals confirmed with SARS-CoV-2 via antigen tests were not included in the study, as no further respiratory testing was performed on those cases [2]. This study has been conceived as a natural extension of our previous work [3].

MATERIALS AND METHODS

We analyzed 11,208 respiratory samples (nasopharyngeal swabs or bronchoalveolar lavage samples) collected between January 2021 and December 2024 from adult and pediatric patients showing symptoms of ARIs at the five hospitals (one hub and four spoke hospitals) of the Friuli Occidentale Health Authority, which serves approximately 310,000 residents in northeastern Italy. Clinical suspicion of ARI was based on the presence of one or more of the following symptoms, as assessed by the attending physician: cough, fever, dyspnea, expectoration, sore throat, sneezing, or radiological evidence of pneumonia (detected by chest ultrasound or chest X-ray). To ensure data

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accuracy, repeated samples from the same patient within a single infection episode were excluded, with only one sample retained per episode.
All specimens were analyzed using the Allplex respiratory panel (Seegene®, Seoul, Republic of Korea), a fully automated multiplex real-time polymerase chain reaction (PCR) syndromic assay capable of detecting the following respiratory pathogens: FLU A-B, ADV, COV (229E, NL63, OC43), Bocavirus (BOV), Enterovirus (EV), MPV, RV, PIV (types 1-4), RSV A/B, MP, CP, BP, and *B. parapertussis* (BPP). For each sample, multiplex reverse transcription (RT) PCR was performed. The analyses were conducted within 24 hours of sample collection.
LP was excluded from the analysis because its environmental mode of transmission - via aerosolized water - fundamentally differs from the person-to-person transmission characteristic of the other respiratory pathogens studied.
SARS-CoV-2 was also excluded for methodological reasons. Over the course of the study, screening for COVID-19 shifted from molecular testing to rapid POCT antigen tests, which became the main method used in clinical practice. Furthermore, patients with suspected ARIs were systematically screened for SARS-CoV-2 using POCT antigen tests. If positive, no further respiratory tests were performed, and thus, these cases were excluded from the study cohort.
Demographic data were obtained from the healthcare authority's electronic information system. Patients were categorized into four age groups: infants and young children (0-5 years), school-aged children and adolescents (6-17 years), adults (18-65 years), and older adults (over 65 years).
Ethical approval was waived as the study involved secondary use of fully anonymized data collected during routine clinical care. All data were managed in accordance with Italian privacy regulations and the ethical principles outlined in the Declaration of Helsinki.
We analyzed the overall detection rates, counts, and seasonality of each pathogen. Categorical variables are shown as absolute numbers and percentages, while continuous variables are reported as medians and interquartile ranges (IQRs) due to the non-normal data distribution.

RESULTS

During the study period from January 2021 to December 2024, a total of 11,208 respiratory samples were analyzed, with 4,576 (40.8%) testing positive. Table 1 summarizes the demographic characteristics of the tested population. Among the positive cases, 52% were male (n=2,359). Adults accounted for 75% of the positive cases, with older adults (over 65 years) representing the largest subgroup at 53.3% (n=2,980). Children under 18 years made up the remaining 25%.
The number of tests steadily increased over the four-year study period across all age groups, rising from 1,076 tests in 2021 to 4,377 in 2024, representing a 307% increase (data are shown for the total cases, including coinfections). This upward trend was especially significant among adult and elderly populations: among adults (18-65 years), tests grew from 238 in 2021 to 1,048 in 2024, a 340% increase; for older adults (over 65 years), tests increased from 578 in 2021 to 2,006 in 2024, a 247% rise.
Over the four-year period, overall positivity rates increased from 27.4% (295/1,076 tests) in 2021 to 33.6% (714/2,125) in 2022, 41.3% (1,500/3,631) in 2023, and 47.2% (2,067/4,377) in 2024. Year-to-year pairwise comparisons using chi-square tests confirmed that each yearly increase in pos-

Table 1 - Demographic characteristics and distribution of sample requests.

Patients' characteristics (n=11,208)	*Age group*	*Number (%)*	*Gender M/F*	*Median age (IQR)*	*Hospital ward (n=10,892)*					*Outpatients (n=316)*
					ED	*ICU*	*Med*	*Surg*	*Ped*	
Children (n=2,808)	0-5 y	1865 (16.6)	995/870	1 (0-3)	1868	1	2	0	436	52
	6-17 y	943 (8.4)	510/433	11 (8-14)						
Adults (n=8,400)	18-65 y	2420 (21.6)	1360/1060	52 (38-59)	2481	914	4348	516	0	214
	> 65 y	5980 (53.3)	3076/2904	82 (75-88)						

Notes: ED = Emergency Department, ICU = Intensive Care Unit, Med = Medical ward (internal medicine, pneumology unit, nephrology unit, neurology unit), Surg = Surgical wards (general surgery, orthopaedics, gynaecology, otolaryngology, urology), Ped = pediatric wards.

itivity was statistically significant ($p<0.001$ for each comparison). Age-stratified trends showed notable differences in positivity rates and testing volumes (Figure 1). Children aged 0-5 years had the highest positivity rates, ranging from 79.1% in 2021 to 83.1% in 2024, with relatively stable year-to-year values despite nearly a 3.5-fold increase in testing volume (from 211 to 750 tests). Conversely, school-aged children (6-17 years) experienced a sharp rise in positivity from 34.7% in 2021 to 55.9% in 2022, followed by slower increases to 62.3% in 2023 and 67.0% in 2024, alongside more than a 10-fold increase in testing volumes (from 49 to 573 tests). Adults aged 18–65 saw a steady increase in positivity from 13.9% in 2021 to 39.7% in 2024. Testing volume in this group grew by over 340% (from 238 to 1,048 tests), representing the largest relative growth across all age groups. Older adults (>65 years) also experienced a consistent rise in positivity, from 13.5% to 32.1%, with testing volume nearly quadrupling (from 578 to 2,006 tests), reflecting the highest absolute increase (Figure 1).

Figure 2 shows the testing and positivity rates in EDs for both adult and pediatric patients. The number of tests increased over the study period: 292 in 2021, 482 in 2022, 1,413 in 2023, and 2,162 in 2024, compared to pre-pandemic levels of 177 tests in 2018, 632 in 2019, and 550 in 2020 – unpublished data. Positivity rates initially rose from 40% (similar to the average of 40% in 2018-2020, unpublished data) in 2021, then stabilized at 54% in 2022 and 56% in both 2023 and 2024.

In Figure 3, we present the annual total numbers and positivity rates for each pathogen. Notably, RV, ADV, BOV, COV, PIV 1-4, MPV, and RSV A/B have gradually returned to pre-pandemic circulation levels [3]. FLU A-B, which disappeared entirely in 2020 and 2021, re-emerged in 2022 (positivity rate: 15%) and then showed a decline in percentage from 2022 onward (6% in 2024). The percentage of positive samples for FLU A-B, which accounted for 15-20% of the pre-pandemic virus (data not shown) in our area, has decreased in recent years despite a significant increase in sample numbers. This could be due to increased focus on respiratory viruses following the pandemic and the resulting strengthening of flu monitoring, infection control measures, and vaccination efforts. EV surpassed the 3% positivity rate in 2024,

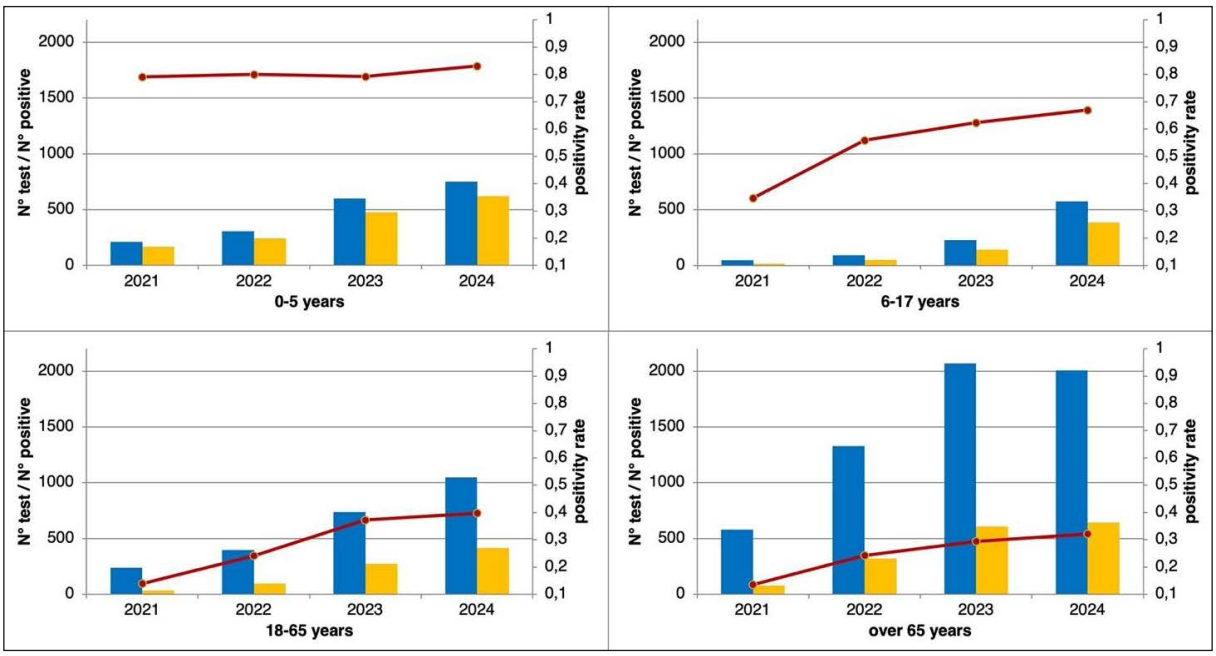


Figure 1 - Age-stratified temporal trends in respiratory testing and positivity from 2021 to 2024.
Notes: Blue bars: total number of tests performed per year; yellow bars: positive specimens. The red line (right y-axis) denotes the positivity rate.

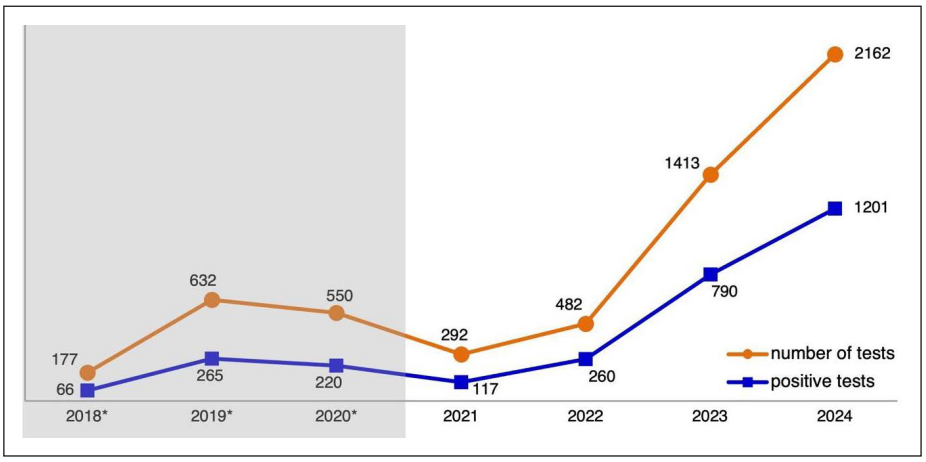
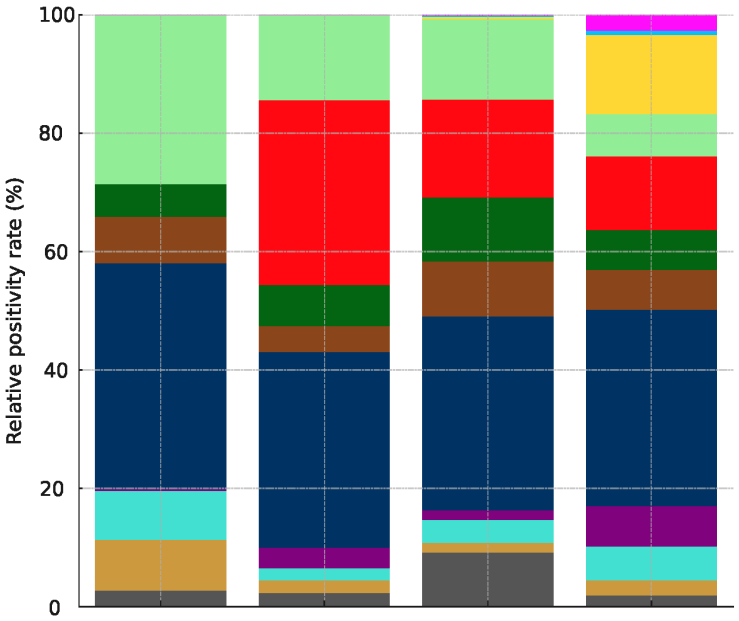


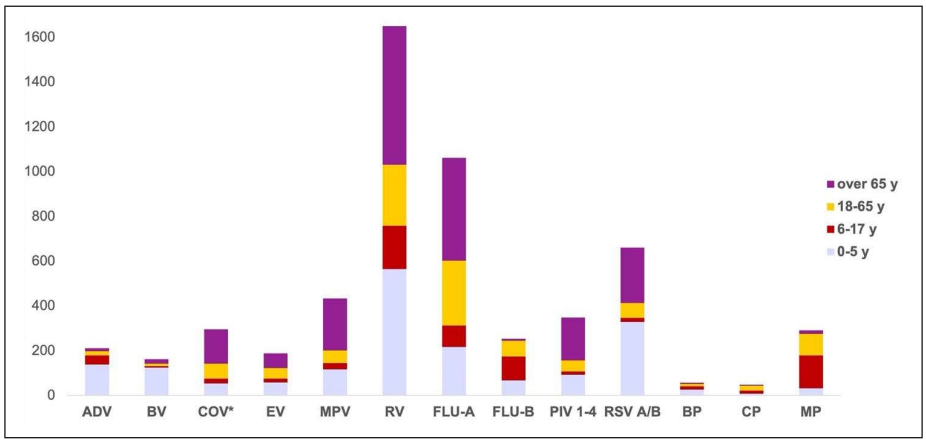
Figure 2
Trends in testing and positive rates for respiratory pathogens (viruses and atypical bacteria) in EDs.
Notes: Grey background indicates unpublished data preceding the study period.



	2021	2022	2023	2024
BP	0 (0.00%)	0 (0.00%)	2 (0.05%)	53 (1.21%)
CP	0 (0.00%)	0 (0.00%)	2 (0.05%)	14 (0.32%)
MP	0 (0.00%)	0 (0.00%)	6 (0.17%)	264 (6.03%)
RSV A-B	83 (7.71%)	102 (4.80%)	200 (5.51%)	142 (3.24%)
FLU A-B	0 (0.00%)	222 (10.45%)	242 (6.67%)	248 (5.66%)
MPV	16 (1.49%)	49 (2.30%)	159 (4.38%)	133 (3.04%)
PIV 1-4	23 (2.14%)	31 (1.46%)	137 (3.77%)	134 (3.06%)
RV	111 (10.32%)	235 (11.05%)	482 (13.27%)	659 (15.05%)
EV	1 (0.09%)	25 (1.18%)	24 (0.66%)	135 (3.08%)
COV (not SarsCov-2)	24 (2.23%)	14 (0.66%)	57 (1.57%)	114 (2.60%)
BOV	25 (2.32%)	15 (0.71%)	24 (0.66%)	51 (1.16%)
ADV	8 (0.74%)	17 (0.80%)	135 (3.72%)	39 (0.89%)

Figure 3
The table shows, for each year, the absolute number of positive detections for each target and, in brackets, the corresponding percentage relative to the total number of tests performed. The bars represent the relative distribution of each pathogen among all positive samples.

Figure 4
Age distribution of total positive specimens for each pathogen, 2021-2024.



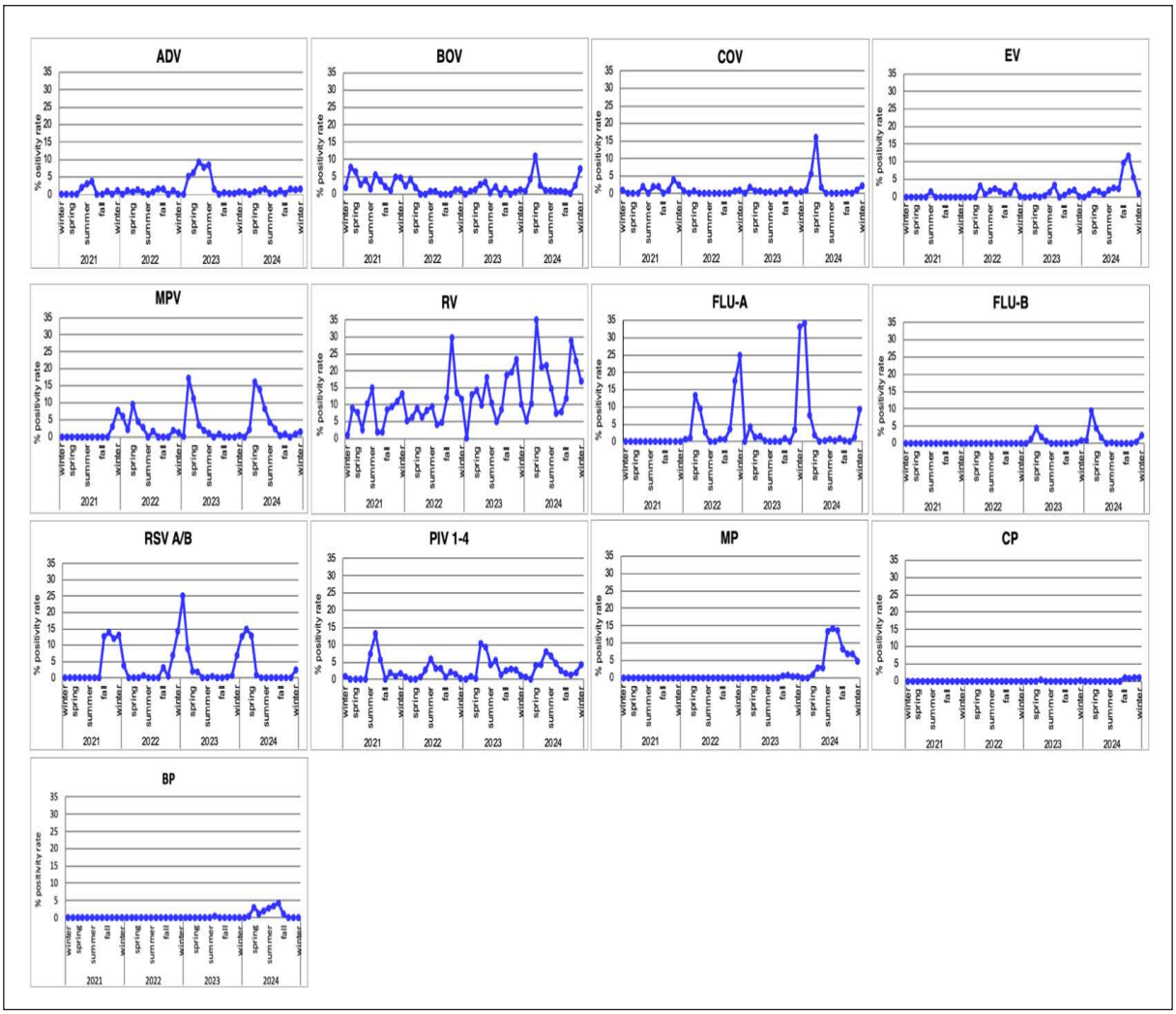


Figure 5 - Seasonal microorganism circulation, 2021–2024. Each dot represents a month.

which was notably higher than pre-COVID levels, where it consistently stayed below 1% [3]. MP, BP, and CP, which were nearly absent in our local epidemiology during 2021 and 2022, showed a significant increase in circulation starting in 2023 (we did not find BPP-positive samples).
The distribution of viruses by age revealed distinct patterns, with RSV A/B, BOV, and ADV showing a strong prevalence in the 0-5 age group. In contrast, viruses such as MPV, seasonal COV, and PIV 1-4 were detected more frequently in the elderly, especially among those over 65 years old. Influenza exhibited a more balanced distribution, though there was a noticeable increase in the older patient group. Regarding the circulation of bacteria, with the exception of BP, which was more common in the 0-5 age group, CP and MP were predominantly found in the 6-17 and 18-65 age groups. These results reflect age-specific susceptibility and exposure patterns (Figure 4).
Figure 5 displays the trends in positivity rates for each analyzed pathogen from 2021 to 2024, organized by four seasons. It highlights shifts and recovery patterns, especially in the years following the pandemic. In our region, many respiratory viruses, including FLU A-B and RSV A/B, typically have higher incidence during the winter months, while ADV, MPV, and PIV 1-4 peak in spring and summer. Some viruses, such as BOV and RV, circulate throughout the year but at markedly different rates.

DISCUSSION

Our four-year retrospective analysis has enabled us to assess the resurgence of respiratory viruses, MP, CP and BP in our region, in a post-pandemic scenario. It is worth mentioning that our study, similar to other investigations, does not provide an exact measure of SARS-CoV-2 spread. After five years of the pandemic, with SARS-CoV-2 now endemic, the widespread use of POCT and self-testing has made systematic and timely epidemiological surveillance by microbiology laboratories nearly impossible [11]. Antigen tests tend to be less sensitive, and new mutations can further decrease their ability to detect variants. Molecular testing remains essential for monitoring new variants and tracking the virus's evolution. After a pandemic, this paradox is quite evident [12]. Still, increased testing is partly driven by heightened awareness and sensitivity among healthcare professional - an aspect that can be viewed as a positive legacy of COVID-19 [3, 13-15].
From this perspective, EDs have been pivotal in this phase, by serving as the largest single source of samples and acting as the main access point for patients with ARIs. EDs include a diverse patient population reflecting the community's pathogen spread, and are a key component of syndromic diagnostic efforts in the post-pandemic era. To enhance the etiological diagnosis of acute respiratory infections (ARIs), we started using standardized screening protocols in the emergency departments (EDs) from late 2020. These protocols included uniform case definitions, triage checklists, and testing algorithms. Over the course of the study, the number of tests performed increased significantly - by more than 300%, as shown in Figure 2. The consistent positivity rates indicate that our testing effectively identifies respiratory infections, facilitating prompt clinical interventions, reducing unnecessary antibiotic use, and supporting targeted infection control measures [8, 9, 14]. Since 2022, our ED positivity rates have exceeded 50%, which is higher than the rates reported in similar settings, typically ranging from 31.5% to 40% [15, 16].
The rise in positivity rates since implementing these measures can be linked to targeted testing of high-risk cases identified through our standardized protocols. These protocols focus on patients with key epidemiological risk factors and clinical features consistent with ARIs.
The increased use of syndromic testing has also greatly improved the identification and diagnosis of some previously underdiagnosed infectious diseases, such as MP, CP, and BP, providing a better understanding of the actual prevalence and the connection between various microorganisms and specific syndromes [9, 16-18].
The noted reemergence of MP, CP and BP in 2024 within our population reflects a broader global pattern of post-pandemic resurgence in atypical respiratory pathogens. Historically, MP has followed epidemic cycles every 1 to 4 years; however, its circulation was notably disrupted by non-pharmaceutical interventions (NPIs) during the COVID-19 pandemic. The sharp increase we observed in late 2023 and throughout 2024 appears to deviate from the expected timeline, mirroring similar trends reported in Europe, Asia,

and North America, which suggests a global synchronization of delayed transmission dynamics [6, 18-23]. Similarly, the epidemiology of BP shows a cyclical pattern, with epidemic peaks every 3–5 years, and we observed a rise in BP cases in 2024, aligning with international data documenting an age shift and increased pertussis cases following the easing of COVID-related restrictions [24-26].

Although CP detection remained at low absolute levels in our cohort, we observed an upward trend since 2023 and continuing in 2025, with a 1.6% positivity rate in the first three months (unpublished data). Our findings match recent reports of increased circulation in France and other areas, suggesting a return to normal endemic levels or a partial rebound in a population that had reduced exposure during the pandemic [27].

For intracellular pathogens, molecular diagnostics offer benefits that extend beyond epidemiological surveillance and directly impact empirical treatment approaches. An internal 2024 survey of general practitioners' prescriptions (unpublished) showed that 91% of empirical regimens lacked coverage for atypical bacteria. Considering this and the increasing incidence of these bacteria in our populations, we implemented targeted educational sessions for both hospital-based and community healthcare providers to update first-line empirical treatment, based on the assumption that ED positivity rates reflect community-level circulation.

Following the relaxation of COVID-19 containment measures, we also noted a resurgence of enterovirus D68 (EV-D68) infections, in line with its typical seasonal pattern previously observed across Europe.

The advent and widespread adoption of rapid molecular diagnostics for ARIs have produced substantial clinical and epidemiological benefits. In our EDs, increased detection of MP, CP and BP bacteria has prompted updates to empiric antibiotic protocols. Additionally, real-time viral identification enables more precise use of antivirals (e.g., oseltamivir for influenza), helping to avoid unnecessary antibiotic prescriptions. Beyond individual case management, these tools significantly enhance microbiological surveillance by enabling early pathogen detection, supporting epidemic prediction, and facilitating the monitoring of endemic respiratory pathogens.

This study has several limitations. First, it is a single-center retrospective analysis based on routinely collected diagnostic data. Although the study benefits from a large sample size, the data primarily come from patients who visited the EDs with acute respiratory symptoms or from hospitalized patients who developed symptoms requiring respiratory pathogen testing. As a result, the dataset may under-represent viral spread within the community. Additionally, cases positive for SARS-CoV-2 were excluded because most COVID-19 diagnostic tests were conducted using POCT and were not sent to the central laboratory. Thus, we were not able to assess co-infections involving SARS-CoV-2 and other respiratory viruses. Finally, we did not examine the presence or impact of co-infections, whether viral or bacterial, which could provide further insight into transmission dynamics and diagnostic results in this post-pandemic period, where such co-infections are increasingly reported [9, 30]. Therefore, this could be an interesting area for future research.

Another limitation we share with Di Pietra et al. is the change in testing strategy before and after the pandemic. Prior to 2021, molecular testing was mainly reserved for patients suspected of having influenza (FLU A-B). Since 2021, however, all patients presenting with symptoms of ARIs have been systematically tested using multiplex PCR panels. This shift in clinical approach has significantly increased the number of tests performed and may have influenced positivity rates, potentially limiting direct comparison between those periods [29, 30].

Nevertheless, the observed trends in pathogen detection match other epidemiological data, suggesting that our findings accurately reflect broader community transmission patterns and may impact diagnostic and treatment choices in our area.

It is well known that the COVID-19 pandemic has significantly affected the epidemiology of most respiratory viruses, as well as the circulation of MP, CP, and BP. In our study, we highlight that ARIs caused by respiratory viruses, which dramatically decreased during the pandemic, are now showing a resurgence and gradually returning to pre-pandemic levels. In contrast, MP, CP and BP have exhibited an upward trend above pre-pandemic levels [3]. One might ask if the idea of "back to normality" still applies. We would argue that improved testing protocols and increased awareness of the importance of an etio-

logic diagnosis should lead us to experience a new "normality," but data are still evolving. The pandemic has emphasized the importance of ongoing surveillance of circulating respiratory pathogens to detect any significant variations from seasonal epidemic expectations in real time.

In conclusion, enhancing the integration of advanced molecular microbiological diagnostics with clinical and epidemiological data is crucial to support new surveillance models and encourage the rational use of antibiotics. Ongoing, year-round monitoring of respiratory pathogen circulation is vital to significantly enhance antimicrobial stewardship programs and efforts to track respiratory infections.

Competing interests

No conflict of interest must be declared for any of the authors.

Funding

No fund was used for this study.

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