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RESEARCH ARTICLE

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Viral pathogens associated with acute respiratory illness in hospitalized adults and elderly from Zagreb, Croatia, 2016 to 2018

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Abstract

Aims: To investigate the viral etiology of acute respiratory infection (ARI) in hospitalized adults and elderly patients in Croatia, compare the prevalence of detected viruses, and to determine clinical characteristics and seasonal occurrence of investigated infections.

Methods: From January 2016 to June 2018, a total of 182 adult patients presented with symptoms of ARI and admitted to the hospital were tested for 15 respiratory viruses by multiplex reverse-transcription polymerase chain reaction. Clinical data were collected by retrospective analysis of the patient's chart.

Results: A virus was identified in 106 (58.5%) of the patients. The most commonly detected virus was influenza virus (41.5%), followed by respiratory syncytial virus (13.8%), human metapneumovirus (13.0%), parainfluenza viruses (12.2%), rhinoviruses (11.4%), adenovirus and coronaviruses with equal frequencies (3.3%), and enterovirus (1.6%). The serum level of C-reactive protein and white blood cell count were significantly lower in patients with respiratory viruses identified when compared with those in whom no virus was detected ($P < 0.001$ and $P = 0.007$, respectively). There were no differences in clinical symptoms according to the type of the detected virus, except for more frequent illness exposure recall for influenza infection ($P = 0.010$). Influenza, parainfluenza, and pneumoviruses were detected mostly in winter months, while rhinoviruses in autumn and spring.

Conclusions: In addition to influenza, pneumoviruses, rhinoviruses, and parainfluenza viruses play an important role in etiology of ARIs in adults. Fast and accurate laboratory diagnosis for respiratory viruses in routine practice is needed for clinicians optimally manage patients with ARI and potentially avoid the unnecessary use of antimicrobial drugs.

KEYWORDS

human metapneumovirus, influenza, multiplex reverse-transcription polymerase chain reaction, respiratory syncytial virus

1 | INTRODUCTION

Acute respiratory infections (ARIs) are the most common infections in humans of all ages. The disease burden from ARIs is substantial and thus their prevention and treatment are a priority for public health agencies.[1] Moreover, current data regarding patterns of unnecessary antimicrobial use in adult patients showed that ARIs are among most frequent indications for antimicrobial use.[2,3]

Children and older adults are the most vulnerable groups of the population, and ARIs are the most common cause of their hospitalization worldwide.[4,5] Older adults are at increased risk of morbidity and mortality due to ARIs because of coexisting chronic diseases and immune senescence.[6] Although bacteria, fungi, and parasites can cause ARIs, the majority of infections are caused by viruses. The most commonly diagnosed causative viral agents of ARIs are: influenza viruses (Flu) type A and B, adenoviruses (AdV), respiratory syncytial virus (RSV) and parainfluenza viruses (PIVs) types 1 to 4. Additionally, in the last 15 years, newly discovered respiratory viruses have been identified including human metapneumovirus (HMPV), human bocavirus (HBoV), coronaviruses NL63 (HcoV-NL63) and HKU1 (HcoV-HKU1), new human enterovirus (HEV), human parechovirus (HPeV), and human rhinovirus (HRV) strains.[7] Influenza is a well-recognized cause of ARIs in adults[8,9] but substantial adult disease is also caused by other respiratory viruses,[10] particularly RSV and HMPV.[11-14] Both viruses belong to the new family *Pneumoviridae* within order *Mononegavirales*,[15] and have similar clinical features.[16,17] The clinical and epidemiological characteristics of ARIs in children caused by RSV and HMPV in Croatia are well described.[18-20] In contrast, for adult populations in Croatia, data are limited primarily on influenza viruses due to laboratory use of direct methods (isolation, antigen detection, or molecular methods) for routine diagnosis of respiratory viruses. Influenza diagnosis is performed in the Croatian Institute of Public Health, acting as a National Influenza Centre, that collects samples from several local, regional, and national institutions for the purpose of the influenza surveillance. Therefore, this Centre performs diagnosis of influenza for our hospitals with the results available to clinicians within 24 to 72 hours, depending on the time of sampling. The diagnosis of other respiratory viruses has been neglected and their incidence and role in the etiology of ARI in adults in Croatia is unknown. Also, in the past the burden of noninfluenza respiratory viruses was underestimated due the insensitivity of older microbiologic tests and the inability to get certain types of clinical specimens.[21] New molecular techniques for laboratory diagnosis of respiratory viruses, particularly multiplex polymerase chain reaction (PCR), have enabled quick, sensitive, and specific, simultaneous detection of the most common respiratory viruses, making it easier for clinicians to determine etiologic diagnosis of ARIs. However, these molecular diagnostics for respiratory viruses are not available for routine patient care in Croatia. Since the epidemiology of viral infections may vary world-wide, local epidemiological data on viral ARIs will best inform public health authorities to develop efficient prevention measures and strategies. Also, these data could help to promote the introduction of molecular laboratory diagnostics for respiratory viruses in routine medical care procedure in Croatia by improving public and physician awareness of noninfluenza viral disease. The aim of this study was to investigate the viral etiology of ARIs in hospitalized adults in Croatia using multiplex PCR, to compare the prevalence of detected viruses, as well as determine the clinical characteristics and seasonal occurrence of investigated infections.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

The study was conducted at the Dr Fran Mihaljevic University Hospital for Infectious Diseases serving the capital city and central region of the northern part of Croatia with population of around 1 100 000 inhabitants (approximately one quarter of all population in Croatia). Patients consecutively approached to the hospital with symptoms of ARIs during study period (from 1 January 2016 until 30 June 2018) were tested for 15 respiratory viruses.

Inclusion criteria were: (1) age greater than or equal to 18 years, (2) acute febrile illness (>37.0°C) lasting for less than or equal to 7 days, (3) a diagnosis of respiratory tract infection, including, upper respiratory tract infection, bronchitis, or pneumonia, and (4) need for hospitalization (onward [≥1 day] or day hospital [for more than 6 but less than 24 hours]).

In addition to inclusion criteria, exclusion criteria were: (1) presumed bacterial respiratory infection, including, otitis, sinusitis, and bacterial pneumonia, (2) need for ICU admission, (3) healthcare-associated infection, and (4) ambulatory-treated patients.

Nasopharyngeal and pharyngeal-flocked swabs from each patient were collected, combined, and placed in viral transport medium (UTM, Copan, Italy). Specimens were immediately transported to the Molecular microbiology laboratory at Public Health Institute where were stored at −80°C until tested. The results of virology testing were released to the physicians periodically, approximately once in a week. As a part of routine care, nasopharyngeal, pharyngeal swabs, sputum culture, and blood cultures were taken from hospitalized patients and submitted for bacterial diagnostics using standard cultivation methods. In addition, upon clinical decision, antigen detection method in urine sample was used for *Legionella* and serology for *Mycoplasma pneumoniae* infection diagnosis, respectively. Demographic, clinical illness data, antimicrobial use, and results of routine bacterial studies were collected by a retrospective review of patient charts. Patients with samples positive on bacteriology testing were subsequently excluded from the analysis. Written informed consent was obtained from the patients. The study was approved by the Ethics Committee of the Teaching Institute of Public Health "Dr Andrija Stampar," and conducted as part of the project of Croatian Science Foundation titled "New and neglected respiratory viruses in vulnerable groups of patients."

2.2 | Laboratory testing

To isolate viral DNA and RNA from viral transport medium, 200 μL were extracted according to the manufacturer's protocol using

QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Multiplex reverse transcription polymerase chain reaction (RT-PCR) for 15 respiratory viruses using Seeplex RV15 detection kit (Seegene Inc, Seoul, Korea) was performed. Briefly, multiplex PCR and complementary DNA (cDNA) synthesis as one-step reaction was performed and set up in three different tubes with three sets of primers (A set contained primers for simultaneously amplification target sequences of AdV, HCoV 229E/NI63, PIV-1, PIV-2, PIV-3, and PCR internal control to check for the presence of substances that may interfere with amplification; B set contained primers for target amplification for HCoV OC43, HRV groups A/B/C, RSV type A and B, Flu A, and PCR internal control; and C set contained primers for HBoV, Flu B, HMPV, PIV-4, HEV, and whole process control [human RNase P was included throughout the entire process as a control from nucleic acid extraction to amplification]). Amplification was performed on thermal cycler GeneAmp 9700 PCR System (Applied Biosystems, Foster City, CA). Detection of PCR products was done by microchip electrophoresis on the MCE-202 MultiNA device (Shimadzu, Kyoto, Japan), including, analysis of software showing results in the form of electropherograms and virtual image gels.[20]

2.3 | Statistical analysis

Normality of data was tested using Kolmogorov-Smirnov test. Multiple comparisons between the groups were tested using χ^2 and Kruskal-Wallis' test with post hoc Dunn's testing when appropriate. A value of $P < 0.05$ was set. In addition, P was corrected to < 0.001 for post hoc comparisons between multiple groups. Statistical software on which all calculations were made was "R."

3 | RESULTS

During the study period 893 patients consecutively approached to the hospital with symptoms of ARIs and 182 (20%) were tested for 15 respiratory viruses. Most subjects who did not qualify had been ill longer than 7 days, were considered to have bacterial infection by the treating physician or refused to participate. Viruses were identified in 106 of 182 (58.5%) of the patients. The most commonly detected virus was Flu A and B (51 of 123; 41.5%), followed by RSV (17 of 123; 13.8%), then HMPV (16 of 123; 13.0%), PIV types 1 to 4 (15 of 123; 12.2%), HRV (14 of 123; 11.4%), AdV and HCoV with equal frequencies (4 of 123; 3.3%), and HEV (2 of 123; 1.6%). The HBoV was not detected (Figure 1). The most commonly detected PIVs was PIV-4 (7 of 123; 5.7%) followed by PIV-1 (5 of 123; 4.1%), PIV-3 (2 of 123; 1.6%), and PIV-2 (1 of 123; 0.8%). A single virus was detected in 92 (86.7%) patients, while codetection of two and three viruses in 11 (10.4%) and 3 (2.8%) patients, respectively, thus making a total of 123 virus detected. The most common dual codetection recorded was detection of Flu A/B with some other respiratory virus (8 of 11; 73%), mostly with PIVs (four of eight); other dual combinations detected were HMPV with AdV, RSV type B with HRV, and CoV OC43 with PIV type 4. Triple codetections were as follows: RSV type A with HEV and PIV type 4; Flu A with PIV types 2 and 4; RSV type B, HMPV, and HRV (Table 1).

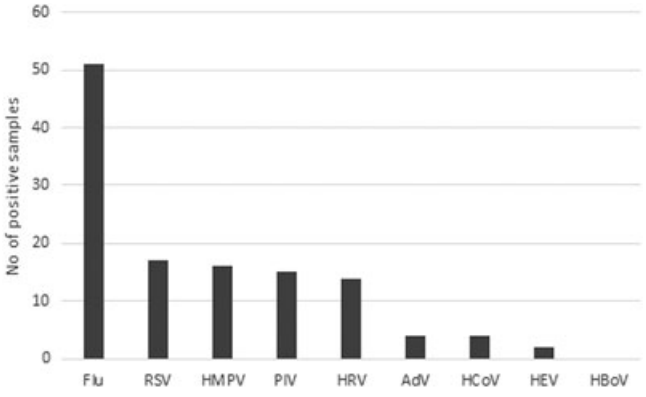

FIGURE 1 Frequency of detected viruses in samples with positive multiplex polymerase chain reaction for respiratory virus. AdV, adenovirus; Flu, influenza virus types A and B; HBoV, human bocavirus 1/2/3/4; HCoV, human coronavirus OC43 and 229E/NL63; HEV, human enterovirus; HMPV, human metapneumovirus; HRV, human rhinovirus A/B/C; PIV, parainfluenza virus types 1 to 4; RSV, respiratory syncytial virus

Median age of tested patients was 66 years ranged from 19 to 94 years, while median age of respiratory virus–positive patients was 65.5 years, ranged from 21 to 92. The age of all tested as well as positive patients was not normally distributed (Kolmogorov-Smirnov test; $P < 0.001$). In addition, the distribution of age was the same for patients with no respiratory virus detection, those with single virus detection and those with multiple virus detection (Kruskal-Wallis' test $P = 0.709$). Distribution of tested patients by age, with proportion of those positive for respiratory virus is presented in Figure S1. Overall there were 59 (32%) females and 123 (68%) males tested, of whom 59% of females and 57% of males were positive for respiratory virus. All respiratory virus–positive patients were hospitalized; 71 of 106 (67.0%) patients with a respiratory virus detected were hospitalized onward, with mean length of stay of 11.0 ± 4.9 days, while others were treated in our day hospital. More than two-thirds of respiratory virus–positive patients (73 of 106; 68.9%) had one or more comorbidities (ie, hypertension [50.7%], heart diseases [20.5%], diabetes mellitus [15.1%], urinary tract infection [11%], hepatal disorder [11%], mental disorders [9.6%], chronic pulmonary disease [8.2%], or malignant disease [5.5%]). Almost one-fifth of the tested patients (35 of 182; 19.2%), as well as those positive for any respiratory virus (21 of 106; 19.8%) were vaccinated against influenza for current year, and almost half of subjects with viral infections (50 of 106; 47.2%) recalled contact with persons with ARI or suspected/proven influenza. There were eight bacterial infections diagnosed in the respiratory tract of tested patients (8 of 182; 4,4%) caused by *Streptococcus pneumonaie* (six patients), *Legionella* spp. (one patient), and *Acinetobacter baumannii* (one patient); three of eight with bacterial infection (all *S. pneumoniae*) in respiratory virus positive and five in respiratory virus negative (2.8% vs 6.6%). Demographic, epidemiological, and clinical data of patients according to the detected virus are presented in Table 2. There were no

TABLE 1 Viruses involved in multiple detection

	Flu A/B	RSV A/B	HMPV	PIV 1	PIV2	PIV 3	PIV 4	HRV	AdV	HCoV	HEV	Total number of detected viruses
Patient 1	●			●								2
Patient 2	●						●					2
Patient 3	●						●					2
Patient 4	●						●					2
Patient 5	●	●										2
Patient 6	●							●				2
Patient 7	●										●	2
Patient 8	●								●			2
Patient 9		●						●				2
Patient 10			●						●			2
Patient 11							●			●		2
Patient 12	●				●		●					3
Patient 13		●					●				●	3
Patient 14		●	●					●				3
Total	9	4	2	1	1	0	6	3	2	1	2	31

Abbreviations: AdV, adenovirus; Flu, influenza virus types A and B; HBoV, human bocavirus 1/2/3/4; HCoV, human coronavirus OC43 and 229E/NL63; HEV, human enterovirus; HMPV, human metapneumovirus; HRV, human rhinovirus A/B/C; PIV, parainfluenza virus types 1 to 4; RSV, respiratory syncytial virus.

significant differences between Flu infected, RSV infected, HMPV infected, other virus infected, and those with multiple virus detection patients according to the age, type of hospitalization, average hospital stays, comorbidity, and Flu vaccination status. Flu-infected patients had significantly more frequent ill contacts compared with those infected with other respiratory viruses ($P = 0.010$) (Table 2).

To evaluate laboratory characteristics of viral infections, the serum level of C-reactive protein (CRP) and white blood cell (WBC) count were compared for those who were infected with single virus, those who were infected with two or more viruses, and those in who no virus was detected (Figure S2a and S2b). Those that were infected with single virus had significantly lower CRP levels compared to the patients with no viral respiratory infection proved ($P < 0.001$). There were no differences in CRP levels between patients with single and multiple virus detection, and those with multiple virus detection and no virus detection ($P = 0.108$ and 0.265, respectively). Those with no proven viral respiratory infection had higher WBC count compared with the those with single virus infection ($P < 0.001$), while there were no differences in WBC count between patients with single and multiple virus infection or multiple virus infection and no virus detection ($P = 0.948$ and 0.031, respectively). CRP levels and WBC counts were also compared in groups of patients according to the type of detected virus (Figure S3a and S3b). There were no differences observed in CRP levels between Flu A/B-, RSV-, HMPV-, PIV-, and HRV-infected patients ($P = 0.192$). There were observed difference in WBC counts between those five groups ($P < 0.001$), more precisely, for RSV-infected patients WBC counts were higher compared with the PIV-infected patients ($P < 0.001$).

The most prominent symptom in patients with proven viral etiology was cough (88 of 106; 83%), followed by fever greater than 38°C (77 of 106; 72.6%), chills/shivering (62 of 106; 58.5%) and vomiting (21 of 106; 19.8%). Overall, clinical diagnosis of lower respiratory tract infection had 68 respiratory virus–positive patients (64.2%). Detailed clinical data considering sign, symptoms, and therapy in group of patients according to the type of detected viruses are shown in Table 2. There were no significant differences between Flu-infected, RSV-infected, HMPV-infected, HRV-infected, PIV-infected patients, and those with multiple virus detection patients in clinical symptoms (cough, vomiting, physiological and radiograph findings, oxygen saturation, and fever).

All patients received symptomatic treatment, such as fluid resuscitation, oxygen, antipyretics, and analgesics (acetaminophen or nonsteroidal anti-inflammatory drugs), decongestives, antitussives, expectorants, as clinically indicated. Patients presumed to have influenza (56 of 182), during the influenza season, were treated with oseltamivir (75 mg bid for 5 days). Antibiotic was prescribed if bacterial superinfection or complication was suspected. Among all patients with confirmed viral etiology, more than two-thirds (68 of 106; 65.1%) received antibiotic therapy, and 42 (39.6%) received oseltamivir. Additionally, there were no differences in type of treatment (oxygen, antibiotic, and oseltamivir) between groups with different virus detected (Table 2).

Seasonal occurrence of Flu A/B, RSV, HMPV, PIVs, and HRV are presented in Figure 2. FluA/B, RSV, HMPV, and PIVs occurred mostly in winter months. RSV, HMPV, and PIVs were also detected in spring following influenza season. HRV were mostly detected in autumn and spring.

4 | DISCUSSSION

This project enabled molecular testing for 15 respiratory viruses in hospitalized adults over a two- and half-year period in Croatia and presents the first comprehensive study on viral etiology of ARI in

TABLE 2 Epidemiological and clinical data of patients with positive multiplex polymerase chain reaction for respiratory viruses (N = 101)[a]

	Patients with monoinfection						
	Flu A/B positive, N (%)	RSV A/B positive, N (%)	HMPV positive, N (%)	HRV positive, N (%)	PIV 1-4 positive, N (%)	Patients with multiple virus detection	*P* value[b]
Total	42 (41.6)	13 (12.9)	14 (13.9)	11 (10.9)	7 (6.9)	14(13.2)	**<0.001**
Male	25 (59.5)	8 (61.5)	11(78.6)	11 (100)	5 (71.4)	10 (71.4)	0.171
Age (min-max), y[c]	56 (22-29)	69 (23-86)	61 (28-85)	70 (21-74)	68 (37-80)	63 (26-82)	0.647
Hospitalized onward	29 (69.0)	8 (61.5)	10 (71.4)	7 (63.4)	3 (42.9)	12 (85.7)	0.478
Hospital (min-max) stays/d[c]	10 (5-16)	12 (7-16)	11 (8-19)	13 (4-31)	10 (6-12)	10 (5-29)	0.270
Comorbidity	31 (73.8)	9 (69.2)	5(35.7)	8 (72.7)	5 (71.4)	11 (78.6)	0.142
Vaccinated for flu	6 (14.3)	3 (23.1)	4 (28.6)	3 (27.3)	0 (0)	3 (21.4)	0.580
Know ill contacts	28 (66.7)	4 (30.8)	8 (57.1)	3 (27.3)	1 (14.3)	4 (28.6)	**0.010**
Fever >38.0°C	35 (83.3)	8 (61.5)	11 (78.6)	5 (45.5)	4 (57.1)	12 (85.7)	0.119
Chills/shivering	30 (71.4)	4 (30.8)	8 (57.1)	5 (45.5)	3 (42.0)	9 (64.3)	0.122
Cough	36 (85.7)	13 (100)	11(78.6)	7 (63.3)	7 (100)	13 (92.9)	0.156
Vomiting	8 (19.0)	2 (15.4)	2 (14.3)	2 (18.2)	1 (14.3)	4 (28.6)	0.939
Pathological auscultatory finding	28 (66.7)	8 (61.5)	10 (71.4)	8 (72.7)	6 (85.7)	7 (50.0)	0.646
Pathological X-ray finding	19 (45.2)	6 (46.2)	6 (42.9)	5 (45.5)	2 (28.6)	8 (57.1)	0.899
Lower respiratory tract infection clinical diagnosis	23 (54.8)	8 (61.5)	11 (78.6)	7 (63.3)	4 (57.1)	10 (71.4)	0.725
Oxygen saturation n < 95%	18 (42.9)	4 (30.8)	3 (21.4)	5 (45.5)	2 (28.6)	5 (35.7)	0.722
Oxygen therapy	11 (26.2)	3 (23.1)	2 (14.3)	2 (18.2)	1 (14.3)	3 (21.4)	0.938
Oseltamivir therapy	20 (47.6)	4 (30.8)	7(50.0)	1 (9.1)	4 (57.1)	4 (28.6)	0.152
Antibiotic therapy	26 (61.9)	9 (69.2)	11(78.6)	6 (54.5)	7 (100)	11 (78.6)	0.215

[a]Coronaviruses and adenoviruses monoinfection was not included in analysis due to low frequencies of detection (three and two cases, respectively).
[b]χ^2 test except for age and hospital stays where the Kruskal-Wallis test is used; a value of $P < 0.05$ was considered significant and presented in bold.
[c]Median.

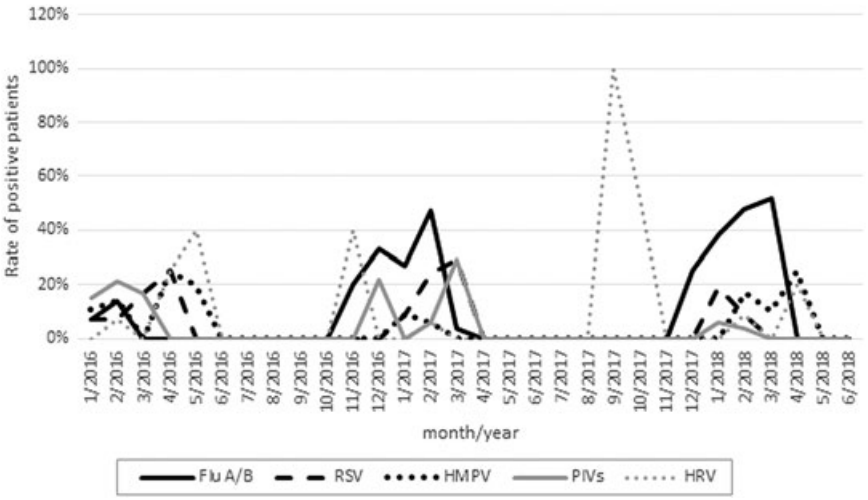

FIGURE 2 Seasonal occurrence of influenza, parainfluenza, pneumoviruses, and rhinoviruses in Croatia from January 2016 to June 2018. Flu A/B, influenza virus types A and B; HMPV, human metapneumovirus; HRV, human rhinovirus A/B/C; PIVs, parainfluenza virus types 1 to 4; RSV, respiratory syncytial virus

hospitalized adults in Croatia. The study not only demonstrates the frequency of viral infection in general but also highlights the burden of other respiratory viruses in addition to influenza in the Croatian population. Results of this study showed that the most common virus detected in older adults admitted to the hospital with symptoms of ARIs in Croatia is Flu types A and B which is consistent with many previously published studies.[8,22] However, the detection rate of influenza in this study (51 of 182; 28%) is much higher than in previously mentioned studies originated from the United States and Europe (11.6% and 9%, respectively). These results emphasize the epidemic and unpredictable nature of influenza but also potential opportunities in a country with low influenza vaccine uptake. The second, and the third most commonly detected viruses are the pneumoviruses, namely RSV and HMPV with detection frequencies of 9.3% (17 of 182) and 8.8% (16 of 182). Many studies around the globe have illustrated the importance of RSV as a cause of serious respiratory illness affecting older adults.[12,23,24] The high frequency of HMPV detection in adults, revealed in this study, is not as commonly reported when compared with the other studies.[22,25] However, one recent study that performed multiple imputation estimations of community-acquired pneumonia (CAP) with detections of specific respiratory viruses among 2259 adults hospitalized with CAP has revealed HMPV and RSV as the second, and third causative agents after Flu with detection frequencies of 3.9% and 3%, respectively.[26] Other recent studies using molecular diagnostics highlight the role of HRV as the most prevalent noninfluenza respiratory virus with detection rates of 11.5% in adults greater than 18 years.[27] In this study HRV was detected as the fifth most common virus after Flu, RSV, HMPV, and PIVs, with detection rate of 7.7% (14 of 182). HCoV and AdV in this study were detected with equal frequencies (4 of 182; 2.2%) and the low AdV frequency is similar to the other previously mentioned adult studies (1.6%),[25] whereas, the incidence of HCoV is lower than prior reports ranging from 3% to 14%.[28,29] HEV was rarely detected (2 of 182; 1%) and was always in codetection with another respiratory virus (Table 1), and HBoV was not detected during the entire study period. It should be noted that HBoV was detected in children during the same period at the same geographic region (Zagreb, Croatia) with frequencies of 6.2% (7 of 275) using the same molecular test.[30]

These data demonstrate the need for adult studies for different pathogens as the epidemiology can vary significantly.

This study also highlights the complex epidemiology of respiratory viruses when sensitive multiplex PCR assays are used for diagnosing respiratory viruses.

The clinical picture of patients infected with Flu A/B, RSV, HMPV, HRV, and PIVs in our study was very similar, specifically, there were no differences between these groups to help clinicians distinguish specific viral infection (Table 2 and Figure S3a and S3b) and support the concept that specific diagnosis of viral respiratory illness is impossible without a laboratory test. Although, patients with Flu A/B more often reported ill contacts than other patients, these features are not sufficiently specific to impact care. Of note, HMPV-infected patients had very similar features to those infected with Flu A/B and often received empiric treatment with oseltamivir. Another observation in our study was the high rate of antibiotics prescribed in patients with documented viral respiratory infection (up to 65%) and underscores the need for more judicious antibiotic use.

Older methods such as viral culture and antigen detection are available and routinely performed in Croatia for diagnosis of ARI in children. However, it is well known these types of assays do not perform well in adults due to low viral loads in respiratory secretions.[11,31] Additionally, if these insensitive tests are used in adults their use may perpetuate the misperception that noninfluenza viruses do not cause significant adult disease.

The development of molecular techniques that can detect minute quantities of nucleic have revolutionized the study of respiratory viral disease, especially in adults.[32] There are many respiratory viruses and investigations using PCR for individual viruses ("monoplex-PCR") are too time-consuming and elaborate for a clinical laboratory. Recently developed multiplex-PCR methods enable testing for many pathogens in parallel in a single analysis, and are commercially available.[33] Additionally, molecular techniques, especially molecular *point of care* tests, have enabled very fast detection (within few hours or even minutes) of respiratory viruses and can

provide clinicians with valuable information that may affect their decision on patient management.

Unfortunately, these methods are still expensive which makes them difficult to implement in daily practice. The cost of using multiplex testing for routine care is currently a subject of debate.[34,35] To be cost effective for routine care, testing should lead to change in management of patients. With exception of influenza where diagnosis leads to specific infection control measures and there is antiviral therapy currently available, the effect on patient management is less clear for the diagnosis other viruses. However, there are encouraging reports that if viral diagnosis is combined with proper clinical judgment and education, more rational use of antiviral drugs and antibiotics may result from the additional information.[36] The use of serum biomarkers such as procalcitonin or CRP to assess likelihood of concomitant bacterial infection may be complementary to viral testing and enhance rational antibiotic prescription.[37]

An additional observation that complicates etiological diagnosis is that multiple viruses may cocirculate as observed in our study where RSV, HMPV, and PIV seasons overlapped with Flu A/B season (Figure 2). Previously published long-term studies on pneumoviruses seasonality based on the monitoring in children[18,19] reported that pneumoviruses in Croatia show biannual cycles characterized by a large RSV winter season followed by a late spring outbreak of HMPV one year, and a winter HMPV outbreak and RSV spring outbreak the following year.[19] Large RSV epidemics in winter months appeared in the odd years, and large HMPV epidemics in occurred even years. The current study conducted in adults, did not confirm biennial RSV cycles, although conclusions about biennial occurrence is not possible in a 2.5-year study. Interestingly, high HMPV epidemic waves were still observed in even years (Figure 2). HRV mostly occurred in the spring and fall months although other studies reported its occurrence during colder months.[38] To the best of our knowledge this is the first report for HRV epidemiology in Croatia since laboratory diagnosis for HRV in Croatia was not possible until this study. Meta-analysis studies are being performed to make global estimates of seasonality and burden of disease and require the input specific viral activity data from different countries to make accurate estimates.[39] In addition, new adult vaccines for RSV are in active clinical development.[40] As new products become available, health ministries will need accurate local viral activity and seasonality data for optimal deployment.

There are several limitations of our study which includes low enrollment rate, allowance of clinicians to rule out possible bacterial infection, exclusion of persons in ICU (most severe disease), but also outpatients (mildest disease) which may have affected the viruses detected as well as the clinical features. Bacterial coinfection can be difficult to diagnose and conclusions about the frequency of this occurrence should be cautious. Lastly, our study was relatively short and future studies over multiple seasons are needed to generate comprehensive seasonality data.

5 | CONCLUSION

In conclusion, a variety of respiratory viruses are associated with serious illness leading to hospitalization in older Croatian adults. Implementation of rapid and sensitive diagnostics such as multiplex PCR that covers not only Flu but also other common respiratory viruses in clinics and public health laboratories could help clinicians and general practitioner's treatment decisions regarding antiviral agents and antibiotics. In addition, physician and public awareness of the importance of noninfluenza respiratory viruses will help policy on the use of new antivirals and vaccines for other viruses under development.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

RC, TT, ARF, JV, and SLJS contributed for design of the research; RC, TT, EH, and SLJS for data acquisition; and RC, TT, EH, and SLJS for data analysis. RC, TT, and SLJS were involved in data interpretation and drafting of the manuscript. JV and ARF carried out critical review of the draft. ARF, RC and SLJS were responsible for revision of initial draft and writing of the final version. All authors reviewed and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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