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Co-infection of Respiratory Syncytial Virus (RSV) and Influenza Virus in Iranian Pediatric Patients Hospitalized with Suspected COVID-19: A Comparative Analysis of Clinical Characteristics

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ABSTRACT

Background & Objective: Co-infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and either respiratory syncytial virus (RSV) or influenza virus may increase disease severity in children compared with single infections. This study investigated the frequency of SARS-CoV-2, RSV, and influenza virus infections and co-infections and compared the clinical and laboratory characteristics of affected patients.

Materials & Methods: Upper respiratory swab samples collected from 337 hospitalized pediatric patients. Multiplex real-time reverse transcription polymerase chain reaction (RT-PCR) was used to detect SARS-CoV-2, RSV, and influenza viruses. Clinical symptoms, characteristics, and laboratory findings were compared among virus-positive groups.

Results: Among the 337 hospitalized children, SARS-CoV-2, RSV, and influenza virus were detected in 18 (5.3%), 9 (2.7%), and 34 (10.1%) patients, respectively. Three cases of RSV/influenza virus co-infection and one case of SARS-CoV-2/influenza virus co-infection were identified. At hospital admission, fever was significantly more frequent among influenza- and SARS-CoV-2-positive patients than among RSV-positive patients (P=0.024). In addition, fatigue was reported significantly more frequently in the SARS-CoV-2-positive group than in the RSV- and influenza-positive groups (P=0.032).

Conclusion: Respiratory viral co-infections were uncommon in this cohort. All four patients with viral co-infections (one SARS-CoV-2/influenza and three RSV/influenza) presented with mild respiratory disease, similar to the majority of patients with single viral infections. These findings suggest that, in this cohort, respiratory viral co-infection was not associated with increased clinical severity.

Keywords: Severe acute respiratory syndrome coronavirus 2, Respiratory syncytial virus, Influenza virus, co-infection, Pediatric

1. Introduction

Acute respiratory infections (ARIs) are among the leading causes of morbidity and mortality in children worldwide, with a particularly substantial impact in low and middle-income countries. Approximately 4.5 million child deaths attributable to respiratory diseases are reported globally each year. Viruses are among the major causative pathogens of respiratory tract infections (1-2). In late 2019, an outbreak of pneumonia of unknown etiology was reported in Wuhan, China. Genome sequencing subsequently identified the causative pathogen as severe

acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19) (3-4). During the SARS-CoV-2 pandemic, concerns arose regarding its impact on the pediatric population and the potential for co-infection with other respiratory viruses, particularly during periods of high influenza and respiratory syncytial virus (RSV) activity. Influenza and RSV infections commonly present with symptoms such as rhinitis, headache, fever, sore throat, myalgia, cough, dyspnea, and radiographic evidence of pneumonia. These manifestations overlap considerably with those of SARS-CoV-2 infection, making differentiation based on clinical symptoms alone challenging (3,5–7). However, advances in molecular diagnostic techniques, including multiplex real-time reverse transcription polymerase chain reaction (RT-PCR), have enabled the simultaneous detection of multiple respiratory viruses with high specificity (8).

Multiple viral infections are frequently detected in children with respiratory tract infections (9). Although SARS-CoV-2 infection in children is generally associated with milder disease, a better prognosis, and lower mortality than in adults, co-infection with other respiratory viruses may contribute to acute respiratory illness and potentially life-threatening complications (10-11). Severe pediatric respiratory diseases, including pneumonia and bronchiolitis, have also been reported in the context of respiratory viral infections; however, the interactions between SARS-CoV-2 and other respiratory viruses remain incompletely understood (12–14). Therefore, distinguishing SARS-CoV-2 mono-infection from co-infection with other respiratory viruses is important for appropriate clinical management and infection-control measures. Although respiratory viral co-infections have been reported worldwide, data from the Middle East, particularly Iran, remain limited. Furthermore, comparative studies evaluating SARS-CoV-2 alongside common pediatric respiratory pathogens, such as RSV and influenza virus, are needed to better characterize their clinical and laboratory profiles. Therefore, this study was conducted during the second to fifth waves of the COVID-19 pandemic (April 2020–September 2021) at a tertiary care center in northern Iran. The primary objective was to compare the clinical and laboratory characteristics of SARS-CoV-2, RSV, and influenza virus infections, including co-infections, among hospitalized children suspected of having COVID-19. This study aimed to identify clinical and laboratory features that may help differentiate these respiratory viral infections in a region with limited published data.

2. Materials and Methods

2.1 Clinical samples and patients

The present study was performed during the second to fifth COVID-19 epidemic surge in Iran (April 2020 to September 2021). The study population consisted of 337 hospitalized pediatric patients presenting with suspected COVID-19 at Amirkola Children's Hospital, which is connected with Babol University of Medical Sciences. Combination oropharyngeal and nasopharyngeal swab samples were obtained for diagnostic assessment in accordance with established clinical standards (15). The protocols for identifying children suspected of having COVID-19 were established in accordance with the guidelines of the World Health Organization (WHO) (16). Swab samples were collected immediately after hospital admission using dry flocked swabs and were subsequently placed in viral transport medium (Pasteur Institute, Iran). The samples were then transported on ice to the molecular laboratory at Ayatollah Rohani Hospital, affiliated with Babol University of Medical Sciences. Swab samples were handled in a Class II biosafety cabinet according to standard laboratory procedures, divided into small-volume aliquots, and stored at −80°C until further analysis. Demographic and clinical characteristics, as well as paraclinical laboratory data, were obtained from the patients' medical records.

2.2 Viral Nucleic Acid Extraction and Multiplex rRT-PCR

Viral nucleic acid was extracted using the Behperp Viral Nucleic Acid Extraction Kit (BehGene Biotechnology, Shiraz/Fars, Iran) from 200 µL of swab-storage media by the manufacturer's protocols. Briefly, for virus dissociation and purification of viral nucleic acid, 200 µL of LB lysis buffer, 25 µL Proteinase K, and 6 µL of carrier RNA (2 µg/µL) were added to each swab-storage media containing microcentrifuge tube. Samples were subsequently incubated at 56ºC for 10 minutes until the virus particles were properly lysed. RNA cleanup was done using a mini spin column (silica matrix) according to the manufacturer's instructions. To rule out the possibility of contamination in DNA extraction, along with the tissue samples, negative controls (sterile microcentrifuge tubes containing only reaction mixtures) were also included. The samples were immediately subjected to real-time reverse transcription polymerase chain reaction (rRT-PCR) analysis after viral nucleic acid isolation. This process used the GA SARS, Flu & RSV One-step RT-PCR Kit (Geneova, Iran) and was conducted according to the manufacturer's guidelines. Six primer pairs and TaqMan probes for the SARS-COV-2 N1, N2, and ORF10 genes, the RSV L gene, and the influenza A/B M1 and M2 genes were included in the rRT-PCR Kit. A QIAquant 96 5plex Real-Time PCR device (Qiagen, Hilden, Germany) was used for the experiments. The rRT-PCR program included the following steps: A) cDNA synthesis at 53°C for 15 minutes, B) Holding at 95°C for 3 minutes, C) 46 cycles of PCR amplification (Denaturation, Annealing, Extension, and fluorescence measurement) at 60°C for 25 sec. For the SARS-CoV-2 N1, N2, and ORF10 genes, the reporter dye channel is set to FAM; for the human RNase-P internal control (IC) gene, it is set to HEX; for the influenza M1 and M2 genes, it is set to Texas Red; and for the RSV L gene, it is set to Cy5. A positive test result was defined as a cycle threshold (Ct) value of ≤40. As a non-template control (NTC), reaction mixtures devoid of an RNA template were included in every real-time PCR run. Additionally, a plasmid containing cloned target

sequences for influenza A & B, RSV, and SARS-CoV-2 genes was used as a positive control to ensure assay reliability and accuracy.

2.3 Statistical Analysis

The statistical analysis was conducted using SPSS version 22. Differences among groups were analyzed using the chi-square ($\chi 2$) test.

The Kolmogorov–Smirnov test was used to examine the normality of the variables. The effect of explanatory variables on the quantitative response was evaluated using a multivariate linear regression model. Statistical significance was set at $P<0.05$.

3. Result

3.1 Clinical and Demographic Features

A total of 337 hospitalized pediatric patients suspected of COVID-19 were included in this study.

The baseline demographic and clinical characteristics of the cohort are summarized in Table 1. The mean age of the patients was 3.7 ± 4.4 years (range: 1 month to 17 years), with the majority of the cohort (62.9%) being between 1 and 6 years of age.

A slight male predominance was observed (54.9%; male-to-female ratio of 1.21:1). The majority of patients (83.4%) had no underlying comorbidities. Among those with pre-existing conditions, seizures (4.2%) were the most common, followed by cerebral palsy (2.1%) and malignancy (1.8%).

In terms of disease severity, most patients (95.6%) presented with mild respiratory disease, while 4.4% were classified as severe.

No mortality was observed during hospitalization for any participant. Seasonal analysis revealed that 54.3% of the respiratory samples were collected during the winter season, compared to 45.7% in the fall.

Table 1. Demographic and Clinical Characteristics of the Study Population (N=337).

Variable	Category	n (%)
Age (years)	< 1	42 (12.5%)
	1 – 6	212 (62.9%)
	7 – 11	67 (19.9%)
	12 – 17	15 (4.5%)
	Mean ± SD	3.7 ± 4.4
Gender	Male	185 (54.9%)
	Female	152 (45.1%)
Season of Sample Collection	Winter	183 (54.3%)
	Fall	154 (45.7%)
Comorbidities	None	281 (83.4%)
	Seizures	14 (4.2%)
	Cerebral palsy	7 (2.1%)
	Malignancy	6 (1.8%)
	Metabolic disorders	4 (1.2%)
	Other*	25 (7.4%)
Disease Severity	Mild respiratory disease	322 (95.6%)
	Severe respiratory disease	15 (4.4%)
Outcome	Survived	337 (100%)
	Mortality	0 (0%)

Note: *Other comorbidities include congenital heart disease, asthma, immunodeficiency, and chronic kidney disease. SD: Standard Deviation.

3.2 SARS-CoV-2, Influenza and RSV detection

Among 337 hospitalized children with symptoms suspected of COVID-19, 18 (5.3%) showed a positive SARS-CoV-2 test. Within these positive cases were 11 (61.1%) males and 7 (38.9%) females. Most cases that tested positive for SARS-CoV-2 (66.7%) belonged to ≤6 years old. The SARS-CoV-2 positive cases comprised12 (66.6%) positive patients in the fall and6 (33.3%) in the winter. In SARS-CoV-2 positive subjects, the median Ct value was 25 (Ct range13-37). Regarding SARS-CoV-2 positive cases with comorbidities, only 1 hospitalized child with hyperammonemia was detected in our study.

Out of 337 specimens, 34 tested positive for the influenza virus, accounting for 10.1%. In the Influenza Positive group, 16 (47.1%) were males and 18 (52.9%) were female. The Majority of influenza- positive cases (85.3%) were in those aged≤6 years. The influenza- positive cases peaked in the fall (11 out of 34) and winter seasons (23 out of 34). The median Ct value in influenza virus-positive subjects was25.5 (Ct range 14-36). In influenza virus-positive cases with comorbidities, four hospitalized children (one case with seizure, one case with acute lymphocytic leukemia (ALL) and 2 cases with acute kidney injury (AKI) were detected in our study.

Moreover, out of 337 hospitalized pediatric patients suspected of having COVID-19, nine individuals (2.7%) were concurrently reported to be positive for RSV. Within this group of positive cases, the gender distribution was

noted to be four males (44.4%) and five females (55.6%). The Majority of the RSV-positive cases (66.7%) were ≤6 years old. The RSV-positive cases peaked in the winter season (eight out of nine positive cases). The median Ct value for samples that tested positive for RSV was 23(Ct range 21-32). No RSV-positive case with comorbidities was detected in our study.

3.3 Clinical features and laboratory findings of viral-positive cases

Table 2 compares laboratory results and clinical signs among hospitalized children with influenza, RSV, and SARS-CoV-2 infections. The data show that, in patients with SARS-CoV-2 and influenza, fever was the most common symptom observed upon hospital admission (P=0.024). At the same time, fatigue in SARS-CoV-2-positive cases was significantly higher than in influenza and RSV- positive patients(P=0.032). In addition, more than half of influenza-positive patients had cough, nausea, or vomiting symptoms, although they were not statistically significant (Table 2). On the day of the children's hospital admission, laboratory tests were performed.

The mean creatinine in patients infected with SARS-CoV-2 was greater than in influenza- and RSV-positive patients, but it was not statistically significant (0.086). In RSV-positive individuals, mean WBC and mean PMN were higher than in others, but they were not statistically significant (Table 2).

Table 2. Comparison of laboratory results and clinical symptoms in hospitalized children with RSV, influenza, and SARS-CoV-2

	Variables	SARS-CoV2 (N=17)	Influenzavirus (N=30)	RSV (N=6)	p-Value
Symptoms and signs	Fever	14 (82.4%)	28 (93.3%)	3 (50.0%)	**0.024**
	Cough	6 (35.3%)	16 (53.3%)	3 (50.0%)	0.487
	Shortness of Breath	2 (11.8%)	2(6.7%)	2 (33.3%)	0.170
	Tremor	3 (17.6%)	3 (10.0%)	0 (0%)	0.473
	Nasal Congestion	3 (17.6%)	8 (26.7%)	1 (16.7%)	0.725
	Sore Throat	3 (17.6%)	6 (20%)	0 (0%)	0.490
	Headache	1 (5.9%)	2(6.7%)	1 (16.7%)	0.665
	Fatigue	8 (47.1%)	4 (13.3%)	1 (16.7%)	**0.032**
	Nausea or Vomiting	6 (35.3%)	16 (53.3%)	3 (50.0%)	0.487
	Diarrhea	5(29.4%)	8 (26.7%)	1 (16.7%)	0.830
	Convulsion	1 (5.9%)	2 (6.7%)	0 (0%)	0.441
	Abdominal Pain	1 (5.9%)	4 (13.3%)	2 (33.3%)	0.233
Laboratory findings	mean rRT-PCR Ct Value	25± 8.77	25.71 ± 5.12	25.33 ± 4.15	-
	mean *WBC ($\times10^3/\mu L$)	7.20 ± 269	7.98 ± 4.31	10.53 ± 4.31	0.271
	mean *PMN ($\times10^3/\mu L$)	3.68 ± 1.91	3.67 ± 1.45	6.01 ± 3.43	0.309
	mean Lymphocyte ($\times10^3/\mu L$)	2.94 ± 1.51	3.65 ± 3.62	3.78 ± 1.52	0.343
	Hemoglobin (g / dL)	12.01 ± 1.79	11.06 ± 1.73	11.55 ± 1.09	0.169
	Platelet count (µL)	262411 ± 75070	271966 ± 111419	246500 ± 61714	0.914
	*CRP (mg/L)	27.88 ± 37	20.39 ± 28.7	26.6 ±34.35	0.752
	*ESR (mm/hr)	25.65 ± 15.9	31.13 ± 22.67	30.5 ±18.65	0.759
	Potassium (mEq/L)	4.21± 0.5	4.27 ± 0.29	4.09 ± 0.58	0.860
	mean Sodium (mEq/L)	134.74 ± 2.79	132.71 ± 3.01	133.45 ± 2.13	0.890
	Creatinine(mg/dl)	1.02 ± 1.56	0.66 ± 0.85	0.47 ± 0.11	0.086

Note: *WBC white blood cell, PMN polymorph nuclear leukocyte, CRP C-reactive protein, ESR erythrocyte sedimentation rate.

3.4 Viral co-infections

This study observed only one case of co-infection of influenza virus and SARS-CoV-2. In addition, influenza virus and RSV co-infections were detected in three cases. No case of SARS-CoV-2 and RSV co-infection was detected. Clinical and laboratory findings of four cases with viral co-infections were presented in Table 3. All of the co-infected patients had mild respiratory disease.

Table 3. Laboratory results and demographics of four patients with respiratory virus coinfection.

Case	1	2	3	4
Age	11Y	3Y	2Y	6Y
Gender	F	M	F	F
Respiratory Disease severity	Mild	Mild	Mild	Mild
SARS-CoV2 r-RT PCR Ct	_	_	_	37
Influenzar-RT PCR Ct	19	25	31	26
RSV r-RT PCR Ct	23	24	29	_
WBC (×109/L)	7.9	11	3.5	12.6
lymphocyte 10^3 / UL	1.8	5.9	1.8	1.3
ESR (mm/hr)	4	63	10	9
CRP (mg/L)	21	62	ND	39
Creatinine (mg/dl)	0.88	0.41	0.5	0.37
Potassium (mmol/L)	4	4.66	4.3	3.9
Sodium (mmol/L)	132	131	133	131

Note: Y years, M male, F female, WBC white blood cell, CRP C-reactive protein, ND not determine

4. Discussion

The Current investigation assesses the SARS-CoV-2, RSV and influenza virus infection rates among 337 Children hospitalized from April 2020 to September 2021 who suspected of having COVID-19. Additionally, we contrast the positive cases with respect to their test findings and medication characteristics at the time of hospitalization. The primary novelty of our investigation lies in its contribution of data from an underrepresented region and its comprehensive comparative design. Conducted in northern Iran during the second to fifth pandemic waves (April 2020-September 2021), our study captures a unique snapshot of viral interplay in a pediatric population before widespread immunity and changes in viral seasonality became established. Unlike many studies that focus solely on SARS-CoV-2 co-infections, we provide a direct comparison of clinical and laboratory features across SARS-CoV-2, RSV, and Influenza single infections, as well as their co-infections. This approach allows for a more nuanced understanding of disease presentation in a real-world clinical setting. While our finding that co-infection did not increase, disease severity aligns with some previous reports, confirming this in a distinct geographical and temporal context reinforces the generalizability of this conclusion and is a valuable contribution to the global body of evidence.

Respiratory infections are common in early childhood, with more than one-third of children experiencing an infection in their first year of life, some of whom require hospitalization.

Therefore, SARS-CoV-2 co-infection with other respiratory viruses, such as RSV or influenza, is a significant concern in pediatric patients. These co-infections may lead to synergistic effects, resulting in more severe symptoms, higher hospitalization rates, increased ICU admissions, and mortality **(6, 17)**.

In the present study, we detected four cases of viral co-infection, and their available medical symptoms, clinical characteristics, and laboratory findings were compared. A case report study from Germany demonstrated. SARS CoV-2 and influenza virus co-infection in 4-month-old infants with fever and cough symptoms.

The clinical presentation of the case was similar to our only SARS-CoV-2/influenza virus co-infection case **(18)**.

In another study in the USA, Co-infection between SARS-CoV-2 and influenza was documented in 6% (32 of 575) of hospitalized pediatrics in influenza seasons. Notably, Patients with co-infections of the influenza virus and SARS-CoV-2 required a greater percentage of invasive mechanical ventilation and continuous positive airway pressure, contrary to our study's results **(19)**.

Additionally, another study found that patients who have influenza and SARS-CoV-2 co-infection faced a higher risk of death compared to those with just one of the infections (either Influenza or SARS-CoV-2). That study indicated that SARS-CoV-2 and influenza viruses might work in concert in co-infected patients (14). Furthermore, a different study found no significant clinical differences between hospitalized children suffering from respiratory infections due to a single virus and those infected by multiple viruses. This observation aligns with our findings (17). Three cases of influenza virus and RSV co-infection were found in the current study. In contrast, the study in China did not report any co-infections from February 2020 to October 2021 in hospitalized pediatric patients. However, in December 2018, before the COVID-19 pandemic, 19 co-infections were found among the RSV and Influenza viruses. The average age of all patients in 2018–2019 was lower than that of 2020–2021; it should be mentioned (20). Although our study did not find anySARS-CoV-2 and RSV co-infection, a case report research in a 4-month- old girl co-infected with RSV and SARS-CoV-2 with cough and fever, bronchiolitis, and mild respiratory distress with hemodynamic stability (21). Another study included identification of 1974 children with RSV infection during RSV seasons from May 2021 to April 2022, and detected SARS-CoV-2 co-infection in 3% (60/1974) of patients. Children with co-detection had a median age of 1.8 years (22). In addition, another investigation identified (6/97) hospitalized infants with SARS-CoV-2 and RSV co-infection from December 1, 2020, to March 30, 2021, and these patients had no worse clinical expression than single-infected patients (23). In line with our study, the findings of a systematic review and meta-analysis show that children with respiratory infections who were infected with two or more viruses did not experience an increase in the severity of their illness (24). This systematic review and meta-analysis study revealed that the most clinically important outcome, viral co- infection did not increase severity and the molecular biology techniques have shown that viruses could be present for an extended duration, often without causing considerable symptoms. As a result, some patients identified as having co-infection may be more accurately described as having co-detection (24). It should be mentioned that since viral genome fragments can persist for up to 5–6 weeks after the onset of infection symptoms, it is possible for multiple viruses to be detected simultaneously. These fragments may not play any significant role in the disease's severity or the general clinical outcomes (17).

A primary limitation of our study is the small number of identified co-infection cases (n=4). This limited sample size reduces the statistical power to detect significant differences in disease severity between co-infected and single-infected patients. Therefore, our observation that co-infection was not associated with increased severity should be interpreted with caution and warrants confirmation in larger, multi-center studies. It is also possible that the low prevalence of co-infection was influenced by the specific study period, during which non-pharmaceutical interventions for COVID-19 may have altered the typical transmission patterns of other respiratory viruses.

Beyond the limited number of co-infection cases, our study has other notable limitations. Our research was confined to hospitalized patients and did not include cases from outpatient clinics, which introduces a selection bias that may limit the generalizability of our findings to milder cases.

5. Conclusion

Our study did not incorporate radiological imaging findings. The primary design was virological and epidemiological, and as such, a systematic review of chest X-rays or CT scans was not performed. This absence of imaging data precludes any analysis of the correlation between viral etiology and specific pulmonary manifestations. Furthermore, while the overall sample size was substantial for a single-center study, the scarcity of co-infection cases hindered a robust statistical analysis of disease severity differences.

Despite these limitations, our findings underscore that influenza and RSV remain significant viral pathogens among hospitalized children suspected of having COVID-19. Consequently, the use of multiplex PCR tests for respiratory viruses is crucial for prompt diagnosis and treatment, as it can prevent ongoing transmission to known risk groups.

6. Declarations

6.1 Acknowledgments

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6.2 Ethical Considerations

All procedures performed in studies involving human participants followed ethical standards, and informed consent was obtained. The Ethics Committee of Babol University of Medical Sciences approved this project (ethics code: IR.MUBABOL.HRI.REC.1400.234).

6.3 Authors' Contributions

Study conceptualization and design performed by Hossein Ghorbani and Farzin Sadeghi. Data analysis and interpretation performed by Farzin Sadeghi and Ali Hasanzadeh. Drafting of the manuscript were performed by Farzin Sadeghi, Ali Hasanzadeh and Mohammad Hossein Alijani

6.4 Conflict of Interest

The authors declare that there is no conflict of interest. All authors mentioned have approved the manuscript. Moreover, the authors have no relevant financial or non-financial interests to disclose.

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6.6 Using Artificial Intelligence Tools (AI Tools)

Artificial intelligence (AI) tools were not used in preparation of this manuscript'

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