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ORIGINAL ARTICLE

Relevance of codetection of respiratory viruses in the severity of acute respiratory infection in hospitalized children

Relevancia de la co-detección de virus respiratorios en la severidad de la infección respiratoria aguda en niños hospitalizados

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What do we know about the subject matter of this study?

The use of molecular biology has significantly contributed as a virological diagnostic tool to identify more and new viral respiratory agents. However, the impact and interpretation of two or more viruses' detection in hospitalized children is still controversial.

What does this study contribute to what is already known?

In hospitalized children due to acute respiratory infection, where is a high presence of viral co-detection (26%) which is associated with an increment in ICU admission. Hospital stay was longer when rhinovirus/enterovirus was identified along with a second respiratory virus.

Abstract

Multiplex polymerase chain reaction (PCR) allows simultaneous detection of respiratory viruses, raising questions about their relevance in the clinical feature. **Objective:** To evaluate the contribution of clinical, epidemiological, and virological factors in the clinical course of children hospitalized due to ARI with viral co-detection. **Patients and Method:** Pediatric patients ≤ 15 years old, hospitalized due to ARI at the UC-CHRISTUS Health Network Clinical Hospital between June and October 2014, and who presented a positive respiratory molecular panel test, were included. Respiratory samples (nasopharyngeal swab, tracheal aspiration, or bronchoalveolar lavage) with positive panel tests by Seeplex® RV15 OneStep ACE Detection Seegene® technique, were analyzed with a second technique (xTAG-RVP-FASTv2 Luminex®, USA), which allows simultaneous and semi-quantitative detection

Keywords:
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of 17 respiratory viruses. Clinical and epidemiological records were collected. **Results:** One virus was identified in 42/57 children (74%) and two or more in 15/57 (26%). Intensive care unit (ICU) hospitalization was significantly more frequent in patients with viral co-detection (OR = 5,5; IC 95%: 1,5-19,6). The most frequently detected viruses were rhinovirus/enterovirus (HRV/EV) (29%) and respiratory syncytial virus (RSV) (25%), and the most common co-detection was HRV/EV-RSV (33%). In x-rays, patients with HRV/EV infection presented interstitial images more frequently, while RSV was associated with condensations (p = 0.002). For HRV/EV, median fluorescence intensity (MFI, semi-quantification) were 1788 and 2456 in co-detection and single agent, respectively (p = 0.022). Children with HRV/EV co-detection had a longer hospital stay compared to isolated identification (5 versus 3 days, p = 0,028). **Conclusion:** In children hospitalized due to ARI, viral co-detection is frequent and associated with more ICU hospitalizations. Our study highlights the presence of HRV/EV in viral co-detection and longer length of stay. More studies are needed to define the relevance of viral co-detection in hospitalized pediatric patients.

Introduction

Acute respiratory infections (ARIs) are a major public health problem due to their morbidity and mortality[1]. This is defined as any acute respiratory infection, whether upper or lower, of less than 15 days from the onset of symptoms[2]. Most of them are caused by a viral etiology, even in children with lower respiratory infections, and are a frequent cause of hospitalization. The clinical presentation and severity of these infections can be very variable, from mild to severe pneumonia, which would be determined by host factors and the viruses involved[5].

For decades, the virological diagnosis of ARIs was made through immunofluorescence or culture techniques, which were progressively replaced by molecular biology techniques[6]. Among these, the polymerase chain reaction (PCR) is a fast and highly sensitive technique, which has allowed increasing the etiological diagnosis, reaching up to 67-92% positivity in children with ARIs[3,4,7-10]. It has also contributed to identify new respiratory viruses such as human metapneumovirus (hMPV) and human bocavirus (HBoV), and has been reported that this technique has an epidemiological impact and eventual on reduction antibiotics prescription[13].

Recently, multiplex PCR assays have been developed that have provided valuable information on the seasonality and clinical spectrum of viral ARIs[9], such as human rhinovirus (HRV), and their association with severe respiratory symptoms. In addition, this technique has allowed a more often detection of more than one virus simultaneously. The most frequently identified viruses in co-detection are HRV, coronavirus (CoV), and HBoV[8,13]. The frequency of viral co-detection by multiplex PCR fluctuates between 6-35% among outpatients and hospitalized ones, with a higher percentage in young children[8,13]. The presence of more than one virus, and the role played by each one in the acute phase of the disease and the temporality when which the viruses has infected the patient, is a challenge. Some studies have shown that co-detection is associated with a greater presence of fever, longer hospital stays, worsening to pneumonia, leukocytosis, and antibiotic use[3,15,16]. However, other authors have not observed differences in symptoms or clinical progression[17-20].

Quantitative viral load of viral agent detected could help to interpreted the the clinical relevance of the different viruses identified in pediatric patients with ARIs. In the case of HRV and CoV, there have been observed significantly higher viral loads in the presence of symptoms compared with viral loads detected in asymptomatic subjects[21], however, a correlation between severity and viral load has not yet been established[15]. A recent systematic review failed to determine the relevance of viral co-detections in the severity of ARIs in children and suggests further studies to clarify this point[20].

Our objective was to determine the pathogenic role of the different viruses in multiplex PCR co-detection, according to clinical, epidemiological, and virological characteristics of children hospitalized with ARI.

Patients and Method

Cross-sectional design study, conducted at the Clinical Hospital of the UC-Christus Health Network, a tertiary level care center in Santiago, Chile. We included all pediatric patients aged ≤ 15 years, hospitalized due to ARI in basic health care services and pediatric ICU, between June and October 2014 (winter-spring), and who presented a positive respiratory molecular panel. ARIs were defined as respiratory tract infections of less than 15 days of evolution, with symptoms such

as cough, rhinorrhea, nasal obstruction, odynophagia, dysphonia, or respiratory distress, with or without fever[2]. Patients hospitalized due to lower ARIs with laryngitis (croup), tracheitis, pneumonia, obstructive bronchitis, bronchiolitis, or other similar pathologies included in ICD-10 were included[22]. Patients with influenza-like illness, whooping cough, cyanosis, apnea, and BRUE (brief resolved unexplained event) were also considered[22]. Patients with respiratory symptoms that started after 48 hours of admission were excluded. Any underlying pathology of the patients was not considered an exclusion criterion.

A classic end-point multiplex RT-PCR (Seeplex® RV15 OneStep ACE Detection, Seegene® Korea) was used for diagnostic, performed at the Laboratorio de Infectologia y virologia molecular. This diagnostic assay consists on the specific multiplex amplification of 15 different respiratory viruses (respiratory syncytial virus (RSV) A and B, adenovirus (AdV), hMPV, influenza type A (FluA) and B (FluB), human parainfluenza viruses (hPIVs) subtypes 1, 2, 3 and 4, HRV, enterovirus (EV), HBoV, and CoV subtypes 229E/NL63 and OC43), using the 2720 Thermal Cycler (Applied BioSystems®, USA), with subsequent DNA visualization in agarose gel, providing a qualitative result.

Co-detection was defined as the identification of 2 or more viruses simultaneously from the same respiratory specimen. Bacterial superinfection was considered when the treating physician recorded it in the clinical record. All radiological reports were performed by a radiologist of the UC-Christus Health Network.

Respiratory samples (nasopharyngeal swab, tracheal aspirate, or bronchoalveolar lavage) with positive panels by Seeplex® RV15 OneStep ACE Detection Seegene® technique were stored at -80ºC and analyzed with a second technique (xTAG-RVP-FASTv2 Luminex®, USA) up to 2 months from sample collection. This second test allows the simultaneous and semi-quantitative detection of 17 respiratory viruses: RSV, AdV, hMPV, FluA subtypes H1, H3, and H1N1 2009, FluB, hPIV subtypes 1, 2, 3, and 4, HRV/EV (not differentiated when identified with common partitions), HBoV, and CoV subtypes OC43, 229E, NL63, and HKU1. The ProFlex™ thermal cycler (Applied Biosystem®, USA) was used for the amplification and hybridization. To visualize the results, the MAGPIX® system (Luminex®, USA) was used which with a signal of median fluorescence intensity (MFI), generated for each target, provides a semi-quantitative estimate of the viral load in the analyzed sample. The MFI values of the viruses were compared when there was a single pathogen or in co-detection.

Statistical analysis was performed with the software Prism v.5 (GraphPad, 2012, USA). The Fisher exact test was used for categorical variables and, for the continuous ones, the Mann Whitney U (unpaired variables) and Wilcoxon (paired variables) nonparametric tests. The relationship between dichotomous variables was evaluated by calculating the Odd Ratio with a 95% confidence interval (OR, 95% CI). A significant p-value < 0.05 was considered significant.

This study was approved by the Ethics committee board from Pontificia Universidad Católica[14-312]. Parents or legal guardians were asked to sign an informed consent form, in addition to the assent of children between 7 and 18 years of age in person or by telephone. The clinical and epidemiological history of the patients was recorded in a special form, through a face-to-face and telephone questionnaire and clinical record review. Subsequently, data were registered in a specially designed database.

Results

During the study period, 73 children hospitalized due to ARIs with positive samples for molecular respiratory viral panel and available frozen samples were identified. 68 subjects were recruited and agreed to participate in the study; two patients were excluded due to incomplete clinical data. When performing PCR by a second semi-quantitative technique on the stored samples, we obtained a positive result for any virus in 57 patients (figure 1). Table 1 describes the 57 children included in the study analysis and their clinical characteristics.

In the 57 samples from these patients, 77 viruses were identified by the second PCR technique. Figure 2a shows the distribution of viral etiologies found, highlighting the presence of HRV/EV (29%), RSV (25%), and hMPV (21%). In 42 samples (74%), only one virus was identified and in 15 samples (26%) more than one agent was detected (12 with 2 viruses and 3 with 3 or more viruses). In the latter group, 11 patients presented co-detection with HRV/EV, 8 with RSV, and 4 with hMPV. The most frequent co-detection was RSV and HRV/EV in 33% (figure 2b).

Table 1 shows the clinical and epidemiological characteristics of patients with a virus or with co-detection. There were no significant differences in any of the characteristics between the two groups, except for ICU hospitalization, which was significantly more frequent in patients with an OR = 5.5 viral co-detection (95% CI: 1.5-19.6).

Patients infected with HRV/EV as a single agent had shorter hospital stays (median 3 days, range 2-17 days) than those with HRV/EV co-detection (median 5 days, range 2-14 days, p = 0.028). Of the 57 patients, 28 (49%) had bacterial superinfection. Most of them presented acute otitis media (n = 15) and pneumonia

(n = 12), followed by *Mycoplasma pneumoniae* superinfection (n = 3), pleuropneumonia (n = 1), and superinfected atelectasis (n = 1). In patients with bacterial superinfection, there was no predominance of any particular type of virus, nor was there any difference in the frequency of bacterial superinfection between the groups with and without viral co-detection (table 1).

Regarding epidemiological history, there was no difference between the groups regarding the number of patients at home, previous visits to crowded places, number of inhabitants in the home, or attendance to a daycare center or school.

When comparing the clinical characteristics of the most frequently identified agents, HRV/EV and RSV, there were no differences regarding age, sex, use of oxygen, bacterial superinfection, days of hospitalization, or admission to the ICU. However, when evaluating radiological images, the presence of interstitial infiltrates was more frequently observed in patients infected with HRV/EV as a single agent (6/11), in contrast to patients infected with RSV, where the most frequent findings were condensations and/or atelectasis associated with an interstitial pattern (11/11) (p = 0.002). In line with these findings, the same was observed regarding discharge diagnoses, where pneumonia was more frequent in patients with RSV (10/11) than those infected by HRV/EV (2/11) as single agents (p = 0.002). Significant differences observed in radiological findings and discharge diagnoses were not demonstrated when comparing HRV/EV and RSV in co-detection.

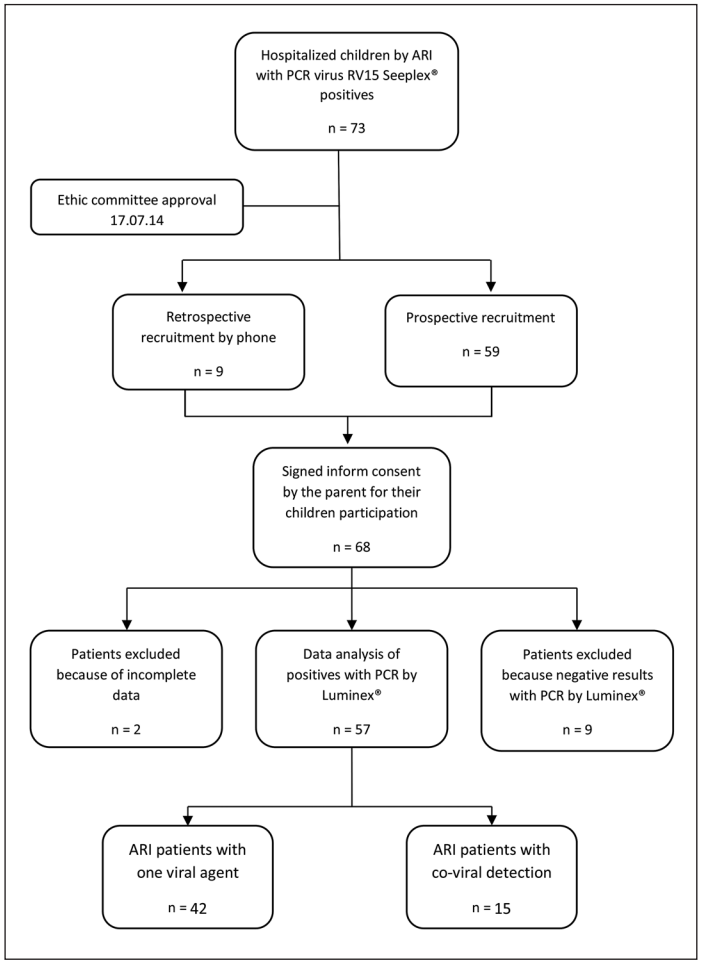

Figure 1. Patients Enrollment diagram. ARI: Acute respiratory infection, PCR: Polymerase chain reaction.

Table 2 shows the characteristics of the subgroup of patients admitted to the ICU (n = 18), where there was a significant HRV/EV predominance. There were no differences between patients with single or co-detection virus infection regarding the reason for admission to the ICU, and high oxygen requirement was the most frequent in both situations. Regarding radiological findings, interstitial lung involvement predominated in both groups. There were no differences in the number of days of hospitalization in the ICU or the final diagnosis. Among these patients, there was one case of septic shock secondary to pulmonary infection with hemodynamic compromise in each group, who responded adequately to fluid support without requiring vasoactive drugs. The number of patients who consulted before admission (considering the last 4 months preceding hospitalization) was significantly higher in the co-detection group (table 2). Of the 11 patients with previous consultations, these were mostly outpatient visits (10/11) and due to respiratory causes (9/11).

There was a tendency towards higher MFI values of viruses when were in isolation compared with co-detection, however, this difference was statistically significant only for HRV/EV (MFI: 2456 and 1788, respectively, p = 0.022, figure 3). Specifically in this virus, no relationship was found between MFI and any of the clinical parameters evaluated (age, fever, condensation on radiography, use of mechanical ventilation, transfer to ICU, duration of hospitalization; results not shown).

Discussion

This study aimed to evaluate the contribution of clinical, epidemiological, and virological factors in the clinical presentation of children hospitalized due to ARIs with viral co-detection. In 26% of the children hospitalized with ARIs, viral co-detection was identified by multiplex PCR; HRV/EV was the most frequently found. Regarding the clinical relevance of viral co-detection in our population, it was associated with higher admission to the ICU and longer hospitalization stay in case of co-detection with HRV/EV.

The frequency of viral co-detection was similar to that described in the literature[8,13]. The clinical manifestations and diagnoses did not differ among children

Table 1. Clinical-epidemiological characteristic of acute respiratory infection (ARI) in hospitalized children with or without co-viral detection

	Unique Virus n = 42/57 (74%)	co-detection n = 15/57 (26%)	p-Value
Sex female, n (%)	18 (42)	7 (47)	1.000
Age, median years (range)	1.41 (0.08-13.8)	0.8 (0.12-3.6)	0.183
Median symptoms onset before hospitalization in days (range)	4 (1-10)	4 (2-10)	0.971
Show Fever, n (%)	32 (76)	11 (73)	1.000
Underlying pathology, n (%)	26 (61.9)	12 (80)	0.339
Influenza Vaccine 2014, n (%)*	16 (47)	9 (69)	0.207
Conjugate Pneumococci Vaccine, n (%)**	30 (99)	14 (100)	1.000
Hospitalization at ICU, n (%)	9 (21)	9 (60)	0.009
Oxygen requirement, n (%)	36 (86)	15 (100)	0.325
Radiology, n (%)***			
Interstitial	38 (90.5)	14 (100)	0.562
Condensation	22 (52.4)	7 (50)	1.000
Atelectasis	16 (38)	6 (43)	0.762
Hospitalization Length , Median in Days (range)	4 (1-17)	5 (2-14)	0.074
Final Diagnostic, n (%)			
Viral Pneumonia	21 (50)	7 (47)	1.000
Bronchiolitis	9 (21)	4 (27)	0.727
BOS/ Asthma Crisis	17 (40)	7 (47)	0.765
Whooping Cough Syndrome/apnea	3 (7)	0	0.559
Laryngitis	2 (5)	1 (7)	1.000
Bacterial Reinfection	20 (48)	8 (53)	0.769

*Patients over 6 month-old are included (n = 34 y n = 13). **Patients over 2 month-old are included (n = 31 y n = 14) and children born after November 2010 (Vaccine incorporate to National immunization program). ***Radiologic report is available for 14 patients in the co-detection group of patients ARI: Acute respiratory infection, BOS: Bronchial obstructive syndrome.

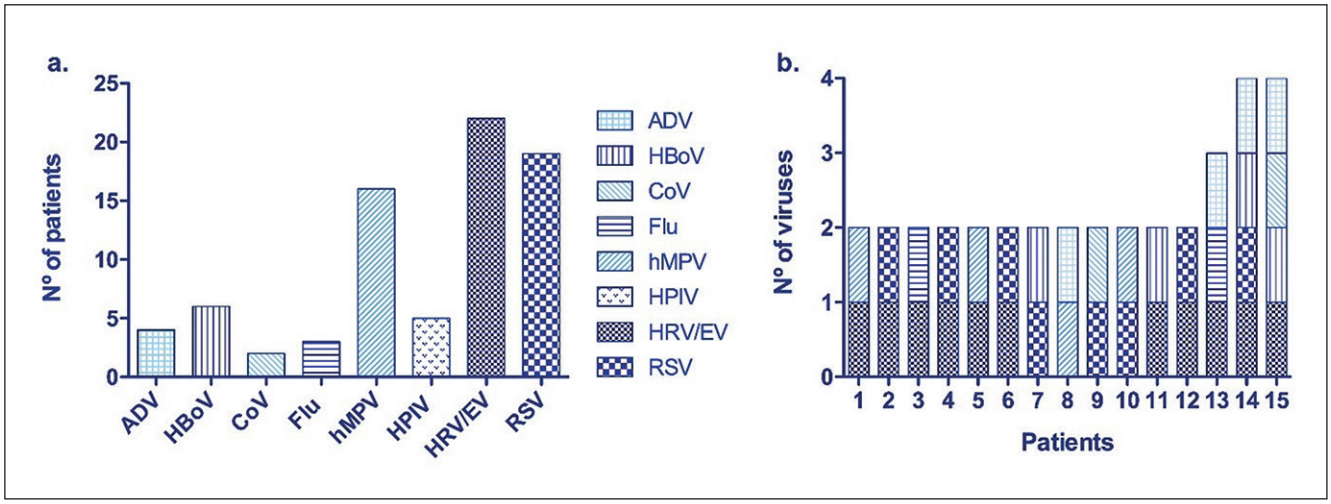

Figure 2. Identified viruses in respiratory secretion from hospitalized children with acute respiratory infection, using Seeplex® RV15 OneStep ACE Detection de Seegene® and xTAG-RVP-FASTv2 Luminex®. a: Identify viruses in 57 ARI-patients with mono or co-detection of virus agents (ADV: adenovirus, HBoV: Human-bocavirus, CoV: coronavirus, Flu: influenza A, hMPV: human metapneumovirus, HPIV: human parainfluenza viruses 3 y 4, HRV/EV: rhinovirus/enterovirus, RSV: respiratory syncytial virus). b: Identified virus distribution in 15 patients with co-detection.

presenting infection by only one virus, compared with those with co-detection. Regarding age, there was no significant difference, however, there was a tendency towards younger age in the group with co-detection, which coincides with the literature, and a higher identification frequency of more than 1 virus in children younger than 24 to 36 months[23,24].

Several publications have studied the etiology of pneumonia in hospitalized children, where in most cases at least one virus was identified (45%-66%). HRV was more frequently identified, which was detected in up to 45% of cases with viral etiology and 25% of children with severe ARIs[25,26]. Likewise, our study highlighted the presence of HRV/EV as the most frequent etiologic agent, especially in children with co-detection hospitalized in the ICU.

Recently, Asner et al. 2015 suggested that the presence of HRV/EV could be a predictor of severity[27], however, in multivariate analysis, this finding did not achieve statistical significance, unlike the baseline pathology. This differs from our study, where no differences were found in the baseline pathologies of ICU patients compared with patients in basic care services (data not shown). This discrepancy could be due to the analysis of a small sample with a low number of ICU patients, selected from a single hospital center. The most frequently found co-detection was RSV and HRV/EV. The identification of these two viruses together is one of the most frequently described in the literature[3,26,28,29]. Our work was performed at the end of winter 2014 when a high circulation of HRV/EV and hMPV was observed, which could explain the frequen-

Table 2. Clinical characteristic in ICU hospitalized Children by acute respiratory infection with or without viral co-detection

	Unique Virus n = 9	co-infection n = 9	p-value
Age, median years (range)	1.83 (0.08-13.8)	0.8 (0.46-3.6)	0.730
Virus distribution, n:	3	8	0.049
HRV/EV			
RSV	3	4	1.000
Previous Consults, n	2	9	0.002
Influenza vaccine 2014, n*	3	6	0.580
ICU cause of admission, n			
Apnea/cyanosis	1	1	1.000
High Oxygen requirements	5	5	1.000
Pulmonary-pathology Preexistence	1	1	1.000
Pulmonary septic shock	1	1	1.000
Other****	1	1	1.000
Radiology, n**			
Interstitial	7	8	1.000
Condensation	4	5	1.000
Atelectasis	4	5	1.000
Oxygen requirements (High FiO2 %)	50	32	0.100
Mechanical ventilation use, n ***	3	2	1.000
Final Diagnosis, n:			
Viral Pneumonia	5	4	1.000
Bronchiolitis	1	1	1.000
BOS/ Asthma Crisis	3	4	1.000
Whooping Cough Syndrome/apnea	2	0	0.471
Laryngitis	0	1	1.000
Bacterial Reinfection	3	5	0.637
Days in ICU, median (range)	3 (1-14)	2 (1-5)	0.300

*Patients over 6 month-old are included (n= 6 y n= 8). **Radiologic report is available for 8 in the co-detection group of patients. ***Ventilator support system Bi-pap use included. ****Other pathology: laryngitis and Whooping Syndrome. ICU: Intensive care unit, ARI: Acute respiratory infection, HRV/EV: rhinovirus/enterovirus, RSV: Syncytial respiratory virus, FiO2: inspired fraction of oxygen, BOS: Bronchial obstructive syndrome.

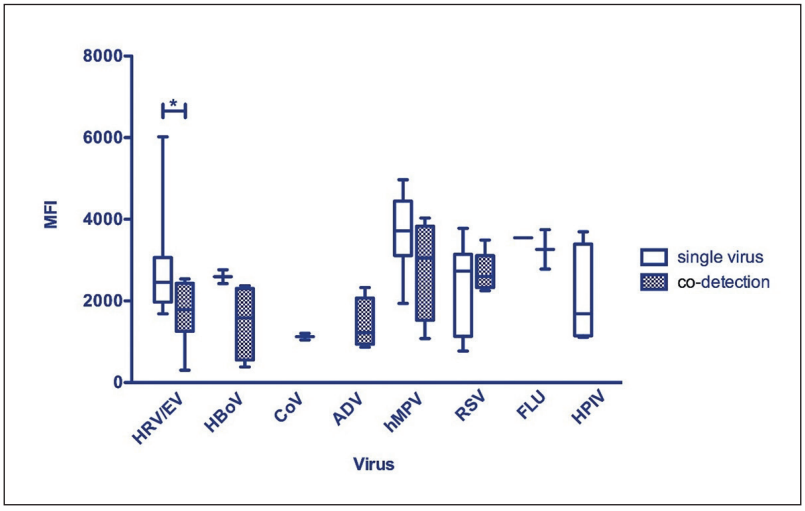

Figure 3. Median fluorescence intensity (MFI) values of the respiratory viruses by infection of single or co-detection of viral agent. Respiratory viruses MFI as a single agent (empty) or in co-detection (hatched) in respiratory samples from pediatric hospitalized patients due to acute respiratory infections (ARI), specifically by each virus. Mean value has drawn by horizontal lines, and minimum-maximum interval by bars. MFI values were compared when there was a single virus or in co-detection using Mann-Whitney test (*p-value <0.05 was considered significant). (ADV: adenovirus, HBoV: Human-bocavirus, CoV: coronavirus, Flu: influenza A, hMPV: human metapneumovirus, HPIV: human para-influenza viruses 3 y 4, HRV/EV: rhinovirus/enterovirus, RSV: respiratory syncytial virus).

cy of the observed distribution. In addition, a differentiated analysis of HRV and EV was not performed in those in which HRV/EV was detected, which is a limiting factor in the interpretation of the high frequency of this etiology.

Regarding the clinical relevance of co-detection, a higher admission to the ICU was observed in this group of patients. This finding is similar to that reported by Richard et al 2008, where the presence of 2 or more viruses in younger infants increased the probability of admission to this unit by 2.7 times[30]. A meta-analysis highlighted the identification of RSV or hMPV in co-detection and the severity in the clinical presentation, while other authors state that this synergism still seems controversial[19,31]. One hypothesis that could explain our results is that the viruses together accounted for greater clinical severity, however, no difference was found in the reasons for admission to the ICU, maximum oxygen requirements, or use of mechanical ventilation compared with patients infected with a single virus.

On the other hand, it is possible to hypothesize that this co-detection is due to a temporal coincidence. Viral co-detection may represent previous inflammation of the airways by an agent, which determines that the following ARI evolves more severely. In our study, a significant difference was observed when there was history of a higher number of consultations before hospital admission. This could explain the co-detection of a new infection in a child with prolonged viral shedding from a previous respiratory infection. Indeed, excretion of viral genetic material has been detected in children up to 5-6 weeks after the onset of the clinical picture, as occurs with HRV and HBoV[25, 32].

In addition, a viral agent has been detected in 28% of asymptomatic children, a percentage that varies according to age, reaching 44% and 19% in children under 1 year of age and adolescents, respectively[21]. HRV and CoV were the most identified agents in these asymptomatic subjects[21,33]. Therefore, in patients with ARIs and a viral respiratory panel with co-detection, it is difficult to differentiate from the clinical presentation alone whether it is an actual co-infection, serial infections, or an asymptomatic excretory patient.

Furthermore, Meskill et al. 2020 did not demonstrate greater severity in the case of viral co-detection in hospitalized children, as opposed to viral-bacterial co-infection[34]. Our report identified a high frequency of bacterial superinfection, similar or higher than that described in the literature[24,27,32]. This could be determined by the greater severity of our patients, hospitalized in a tertiary level care center.

Similar frequencies of bacterial superinfection were observed in the group with and without co-detection, indicating that documenting a bacterial infection would not act as a confounding factor in our results. These findings suggest that, when faced with a suspicion of bacterial superinfection, viral co-detection does not exclude studying its presence.

In our analysis, a longer hospital stay was also observed only in HRV/EV in co-detection compared with a single infection by this virus. Only a few studies have supported this association; indeed, one meta-analysis ruled it out with limited evidence[16,17]. There are other associations in the literature that support a longer hospital stay in co-detection with traditional viruses such as RSV and influenza[35]. Controversially, the systematic review by Scotta et al.2016 did not demonstrate an increased risk associated with length of hospital stay[20].

Finally, regarding the semi-quantification of the viral load, a lower MFI was observed for HRV/EV in co-detection than when identifying it as a single agent.

This could be explained by a lower or null pathogenic value, where the 2nd virus predominates with greater relevance. However, this could also be attributed to the competition that occurs in the initial stage of the PCR reaction (enzymes, nucleotides, etc.). It is important to note that it is not possible to fully compare the MFI values between the different viruses since each partition has a different performance for each of them. Our results did not show an association between MFI and clinical features of the disease.

In recent years, different studies have highlighted the clinical usefulness of viral load in prognosis, as in the case of RSV, where high viral loads have been associated with greater severity, prolonged hospital stay, and longer duration of symptoms in bronchiolitis[36-38]. It has also been used to evaluate response to treatment, as in the case of influenza, where viral load proved to be a reliable tool to evaluate response to oseltamivir therapy in immunocompromised patients[39]. Therefore, further studies with new techniques for the quantification and assessment of viral load and excretion are required to understand the role and clinical relevance of the presence of more than one virus in ARI[40].

Viral co-detection has a high frequency in pediatrics. This is probably due to the immunological characteristics of children and the high transmission rate of respiratory viruses in this age group given the close social contact between them. However, the clinical, epidemiological, and virological factors studied do not yet allow us to determine a change in the clinical behavior of children hospitalized due to ARI with viral co-detection.

Conclusions

Viral co-detections are frequent in hospitalized children and their identification has been optimized by the increasing use of molecular biology techniques. The diagnosis of a co-detection could be associated with unfavorable clinical evolution, increased admission to the ICU, and longer hospital stay in the presence of HRV/EV. The virological analysis performed in our study does not allow us to infer the clinical relevance of each of the viruses identified in co-detection and requires further investigation.

Ethical Responsibilities

Human Beings and animals protection: Disclosure the authors state that the procedures were followed according to the Declaration of Helsinki and the World Medical Association regarding human experimentation developed for the medical community.

Data confidentiality: The authors state that they have followed the protocols of their Center and Local regulations on the publication of patient data.

Rights to privacy and informed consent: The authors have obtained the informed consent of the patients and/or subjects referred to in the article. This document is in the possession of the correspondence author.

Conflicts of Interest

Authors declare no conflict of interest regarding the present study.

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