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REVIEW ARTICLE

Diversity of Non-Influenza Respiratory Viruses Associated with Influenza-Like Illness during 2009 pre and pandemic periods in São Paulo, Brazil, a Historical Overview

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Abstract

Background and objective: During the 2009 influenza A (H1N1) pandemic, 60% of the respiratory specimens collected from patients under surveillance for influenza-like illness (ILI) in São Paulo, Brazil, tested negative for influenza A and B by Real-Time RT-PCR (RT-qPCR) assays. In this retrospective study, we identified the diversity of other respiratory viruses associated with ILI during the pandemic period and added the benefit of RT-qPCR for their detection.

Methods: Five-hundred and forty-two influenza RT-qPCR negative respiratory specimens from ILI patients collected from Jan - Dec 2009, that also tested negative by a commercial Indirect Immuno Fluorescence (IIF) assay for the Respiratory Syncytial Virus (RSV), Parainfluenza (PIV) PIV1, PIV2, PIV 3 and Adenovirus (AdV) were retested by a panel of RT-qPCR assays for the other respiratory viruses, including RSV, Human Metapneumovirus (HMPV), PIV 1-4, AdV, Human Coronaviruses (HCoV) OC43, 229E, NL63 and HKU1, Rhinovirus (RV), Enterovirus (EV) and Human Bocavirus (HBoV).

Results: Of the 542 negative specimens, 428 (78%) were positive by RT-qPCR for another respiratory virus: In descending order: RV (149, 27%); RSV (41, 8%); AdV (32, 6%); HMPV (29, 5%); PIV3 (19, 4%); PIV1 (3, 1%); HCoV-OC43 (9, 2%); HBoV (9, 2%); PIV4 (6, 1%); HCoV-HKU1 (6, 1%); EV (5, 1%); HCoV-229E 4 (4, 1%); HCoV-NL63 (3, 1%). Co-detections of two viruses (98, 18%) and three viruses (12, 2%) were common.

Conclusion: This retrospective study revealed a high prevalence of respiratory viruses other than influenza associated with ILI during the pandemic period and highlights the superiority of RT-qPCR over IIF for ILI surveillance.

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Introduction

Influenza-Like Illness (ILI), defined by the World Health Organization (WHO) as an acute respiratory infection with fever $\geq 38^{\circ}\text{C}$ and symptoms onset within 10 days [1], is a leading cause of human morbidity and mortality worldwide [2]. As the name implies, influenza viruses are a predominant cause of ILI and consequently a major contributor to the

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human disease burden. However, ILI is a syndrome that is not specific to influenza virus and can be caused by many other respiratory pathogens as revealed by the increasing use of molecular diagnostics [3]. In addition to the plethora of historically well-known respiratory viruses, molecular diagnostics has also contributed to the discovery of an increasing number of “new” respiratory viruses responsible for ILI in the last two decades, including Human Metapneumovirus (HMPV), 2001 [4]; Human Coronaviruses NL63 (HCoV-NL63), 2004 [5]; Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), 2003 [6,7]; (HCoV-HKU1), 2005 [8]; Human Bocavirus (HBoV), 2005 [9]; two new polyomaviruses WU (2007) [10] and KI (2007) [11]; new Human Rhinovirus (HRV - QPM), 2007 [12]; Middle East Respiratory Syndrome Coronavirus (MERS-CoV), 2012 [13].

From January through December 2009, 52,929 respiratory specimens were collected from patients presenting with ILI, until five days after the clinical symptoms onset, were sent to the Respiratory Virus Laboratory (RVL)/Institute Adolfo Lutz (IAL), a National Influenza Centre/World Health Organization (NIC/WHO), to investigate an outbreak of a new pandemic strain of influenza virus, A(H1N1) [14]. Sixty percent of these specimens tested negative for influenza A(H1N1), A(H1N1)pdm09, A(H3N2) and B viruses by real-time RT-qPCR assays (Figure 1). The aim of this retrospective study was to test a subset of these influenza-negative specimens using RT-qPCR assays to investigate the diversity of other respiratory viruses associated with ILI before and during the 2009 influenza pandemic.

Materials and Methods

Pre-study surveillance

IAL is an accredited NIC/WHO established in São Paulo, Brazil in 1960. In 2000, the Brazilian National Influenza Surveillance Network (NISN), sponsored by the Brazilian Ministry of Health, initiated surveillance for ILI in Brazil, and in 2002, IAL joined the NISN. Routine ILI surveillance consisted of receiving systematically collected Nasopharyngeal (NP) swab specimens from 5 children aged < 5 years old, per week, presenting with ILI symptoms at selected sentinel units: a children’s hospital, Hospital Municipal Infantil Menino Jesus; and two general hospitals, Municipal Ver José Storopolli and Geral de Guarulhos. Following the first confirmed case of

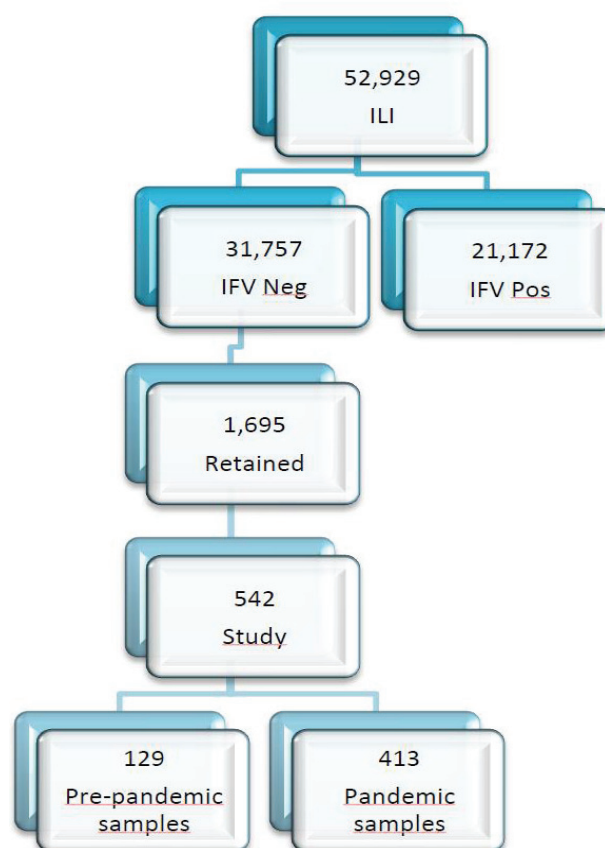


Figure 1 Diagram showing the negative samples selection schedule towards to investigate other respiratory viruses’ non influenza.

influenza A (H1N1)pdm09 in São Paulo on May 07, 2009, ILI samples collection substantially increased from an expanded network of sentinel units at public and private hospitals located in São Paulo state and other Midwestern states: Mato Grosso (MT); Mato Grosso do Sul (MS), Goiânia (GO); Distrito Federal (DF); Northeast: Piauí (PI); and North: Acre (AC), Rondônia (RO); Tocantins (TO).

From January through December 2009, 52,929 respiratory specimens were collected at sentinel clinics from patients presenting with ILI and sent to the RVL//IAL/NIC/WHO for rapid processing and screening for influenza and other respiratory viruses Figure 1. All specimens were collected after the informed consent of parents or guardians following the Brazilian Ministry of Health protocol and with the approval of the IAL institutional review board.

After the first confirmed case of influenza pandemic strain A(H1N1)pdm09, in São Paulo (April 2009), from a respiratory secretion collected from

an individual with a travel history to a risk area, the number of respiratory secretions collected from patients, presenting with Influenza-Like Illness (ILI) by the Children and General Sentinel Hospitals and public and private Hospitals, increased substantially. These clinical specimens were sent to the RVL/IAL/NIC/WHO for rapid processing and screening by Indirect Immunofluorescence (IIF) assays for other respiratory viruses (Figure 1). The clinical specimens corresponding to both pre- (129) and pandemic (413) periods were also tested by real-time (RT-qPCR) assays developed by the Centers for Disease Control and Prevention (CDC), Atlanta (USA), for the new influenza A(H1N1)pdm09 strain [15], seasonal influenza strains A(H1N1, H3N2)) and influenza B viruses were also tested by real-time RT - PCR (RT-qPCR). All samples were maintained at -80°C at the RVL/IAL/NIC/WHO. Approximately 60% of these specimens were negative for influenza viruses, raising the question of what other respiratory viruses might be responsible for ILI during this period. To answer this question, we selected 542 specimens, among the 1695 negative samples, by epidemiological week from January – December 2009, to test by CDC real-time RT-PCR (RT-qPCR) assays for non-influenza respiratory viruses. Results were negative using differential diagnosis (Influenza A, Influenza B, Respiratory Syncytial Virus (RSV), Parainfluenza Virus (PIVs) PIV-1, PIV-2, PIV3 and Adenovirus (AdV) by IIF, using Respiratory Panel 1 Viral Screening and Identification kit (Light Diagnostics, Chemicon, Temecula, CA) catalog number 3105 and also negative for influenza viruses by real-time RT - PCR (RT-qPCR).

Pre-study laboratory procedures

Routine viral surveillance was performed using commercial indirect immunofluorescence assays IIF assays for influenza viruses A and B, Respiratory Syncytial Virus (RSV), Parainfluenza (PIV1, PIV2, PIV3) and Adenovirus (AdV) available with the respiratory panel 1 viral screening and identification kit (Light Diagnostics, Chemicon, Temecula, CA) catalog number 3105. Assays were performed according to the manufacturer's instructions.

Beginning on (29 May 2009), (RT-qPCR) assay for influenza A(H1N1), A(H1N1) pdm09, A(H3N2), and influenza virus B following procedures developed by the U.S. Centers for Disease Control and Prevention

(CDC) were introduced [15].

Study laboratory procedures

Of the 52,929 surveillance specimens obtained 31,757 (60%) were negative for influenza A and B by the IIF panel and 1,695 of these were negative for influenza A and B by RT-qPCR assay and all other respiratory viruses covered by IIF panel Figure 1. These specimens were retained at -80C at the RVL/IAL/NIC/WHO.

Phases: In-house single plex real-time RT - PCR (RT-qPCR) assays for RSV, HMPV, PIV1 – PIV4, AdV, RV, enterovirus (EV), HBoV, and HCoV 229E, OC43, NL63, and HKU1 following procedures developed by CDC. The primers and probes reflect the respiratory virus sequences obtained from the databases at the time. The CDC respiratory virus group likely had them updated. Real-Time RT-PCR (RT-qPCR) was conducted by using AgPath _ ID TM (Applied Biosystems) A positive test result was considered a well-defined fluorescence curve crossing the threshold within 40 cycles. All runs included positive and negative virus template controls to monitor assay performance. The Ct values of positive samples are shown in table 1.

Results

Of the 542 patients selected from Jan - Dec 2009 for testing for other respiratory viruses, 307 (56.7%) were males and their mean age was 3.2 years (range 0.0 to 77 years). For this analysis, patients were grouped by age as follows: 20 (3.7%) were newborns; 354 (65.3%) were infants 28 days to 1 year old; 106 (19.6%) were preschool children 2 to 6 years old; 27 (5%) were school-age children; 10 (1.8%) were school-age adolescents; and 25 (4.6%) were adults ≥ 19 years old.

Overall, 425 of the 542 (78.4%) patients were real-time RT-PCR (RT-qPCR) positive for one or more other respiratory viruses. These included single detections of RV (149, 27.5%); RSV (41, 7.6%); AdV (32, 9.0%); HMPV (29, 5.4%); PIV (28, 5.2%); HCoV (22, 4.1%); HBoV (9, 1.7%); and EV (5, 0.9%). Patients with co-detections (110, 20.3%) of two (98, 18%) and three viruses (12, 2%) were also identified in figure 2. RV was the most common virus detected overall (27.5% of all detections) followed by RSV (7.6 % of all detections).

Of the 542 specimens negative for influenza by real-time RT-PCR (RT-qPCR) and a panel of other respiratory viruses by IIF, 219 were collected in the pre-pandemic period from January - April 2009. Of these, 114 (52.1%) were positive by RT-qPCR for respiratory viruses other than influenza. These included single detections of RV (42, 36.8%); RSV (18, 15.8%); AdV (8, 7.0%); HCoV-NL63 (3, 2.6%); EV (2, 1.75%); HMPV (1, 0.87%); PIV3 (1, 0.87%); HCoV-OC43 (1, 0.87%); HBoV (1, 0.87%); and PIV4 (1, 0.87%). HCoV-HKU1 and -229E were not identified during the pre-pandemic period. We also observed co-detections with two viruses (17, 14.91%) and three viruses (1, 0.87%). It is noteworthy that non-influenza respiratory viruses had been circulating before the emergence of the influenza pandemic strain. The Ct values of positive samples are shown in table 1.

RVs were the most common viruses detected in both pre-and-pandemic periods. Molecular methodologies such as real-time RT - PCR (RT-qPCR) recovered the following non-influenza viruses from January to April 2009: HMPV, PIV4, HBoV, EV, and coronaviruses OC43, NL63 was detected only in week 5, corresponding to the summer season. Regarding coronavirus 229E, its circulation was identified from weeks 20 to 22 besides weeks 25 and 27 (autumn-winter) whereas HKU1 was detected from

week 32, following weeks 36 to 41 and 44 (spring); besides those identified by IIF differential diagnosis. Due to the high specificity of the real-time RT-PCR (RT-qPCR), RSV, AdV, PIV1, PIV2, and PIV3 viruses were also recovered from the IIF-negative samples selected for this study. The trends of seasonality of influenza viruses and other respiratory viruses, using IIF, during the pre- and pandemic periods, in 2009 are summarized in figure 4, showing the circulation of RSV, AdV, PIV1, PV3 during the pandemic period. On the other hand, PIV2 circulation was detected only in the 2009 pre-pandemic period.

A retrospective study in 2009 using real-time RT-PCR (RT-qPCR), revealed the concomitant circulation of RVs, ADV, RSV, HMPV, PIV1, PIV3, PIV4, coronaviruses 229E, OC43, and HKU1, during the pandemic period. Coronavirus NL63 circulation was observed during the pre-pandemic period as shown in figure 3. With the exception of RV which predominated in all four seasons, the following trends in the RSV and HMPV seasonality were observed: RSV peaking during autumn - winter, and HMPV peaking during spring; AdV was detected all year round as shown by IIF, although a retrospective study using real time RT - PCR (RT-qPCR) revealed an increasing activity during the winter and spring. PIV3 activity was predominant during the pandemic period

Table 1: In house assay Ct values, mean, range. In house assay results classified as strong (Ct < 30) moderate (Ct ≥ 30 to ≤ 37) or weak (Ct > 37 to < 40) positive.

Target		(n)	Mean Ct	SD	Ct Range	
					Min	Max
RV	Samples	195	29.29	4.49	15.94	37.71
	Control	63	28.93	1.58	26.55	34.36
AdV	Samples	74	31.67	5.93	13.75	39.48
	Control	63	29.10	1.18	24.36	32.23
RSV	Samples	63	27.84	6.21	17.44	38.72
	Control	63	30.57	1.16	26.68	32.84
HMPV	Samples	56	27.80	4.85	20.30	38.97
	Control	63	30.06	0.87	26.73	32.42
PIV3	Samples	36	26.12	6.13	15.10	39.96
	Control	63	30.81	1.09	27.60	33.16
PIV4	Samples	6	35.92	3.22	31.76	39.96
	Control	22	33.20	1.39	29.30	33.99
PIV1	Samples	5	30.39	8.69	16.53	36.76
	Control	63	29.93	0.95	26.23	32.03

PIV2	Samples	0	0.00	0.00	0.00	0.00
	Control	63	30.70	0.88	26.95	32.89
HCoV-HKU1	Samples	10	25.82	5.02	20.66	37.71
	Control	22	32.43	1.79	26.77	36.88
HCoV-229E	Samples	7	30.41	3.60	25.98	35.86
	Control	22	35.05	2.38	27.21	37.94
HCoV-OC43	Samples	7	25.16	4.64	17.51	30.88
	Control	22	33.31	1.64	28.21	36.90
HCoV-NL63	Samples	4	29.69	4.35	25.67	34.88
	Control	22	34.65	1.55	29.92	37.95
HBoV	Samples	14	29.58	10.74	11.79	40.00
	Control					
Resp/ E	Samples	9	28.46	2.78	24.82	32.32
	Control					
RNaseP	Samples	512	25.29	3.32	19.66	37.65
	Control	22	30.89	1.24	28.78	34.17

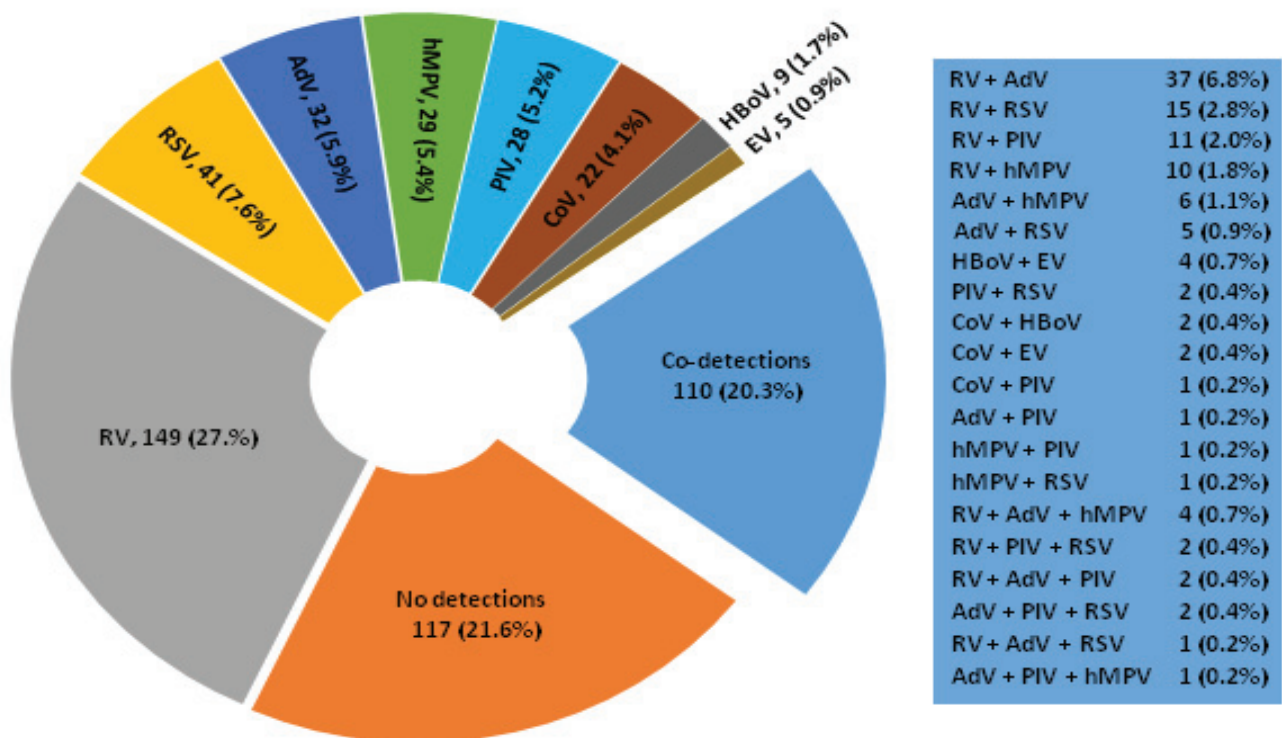


Figure 2 Prevalence of detection of single and mixed viral infection in 542 patients with ILI by using RT – qPCR.

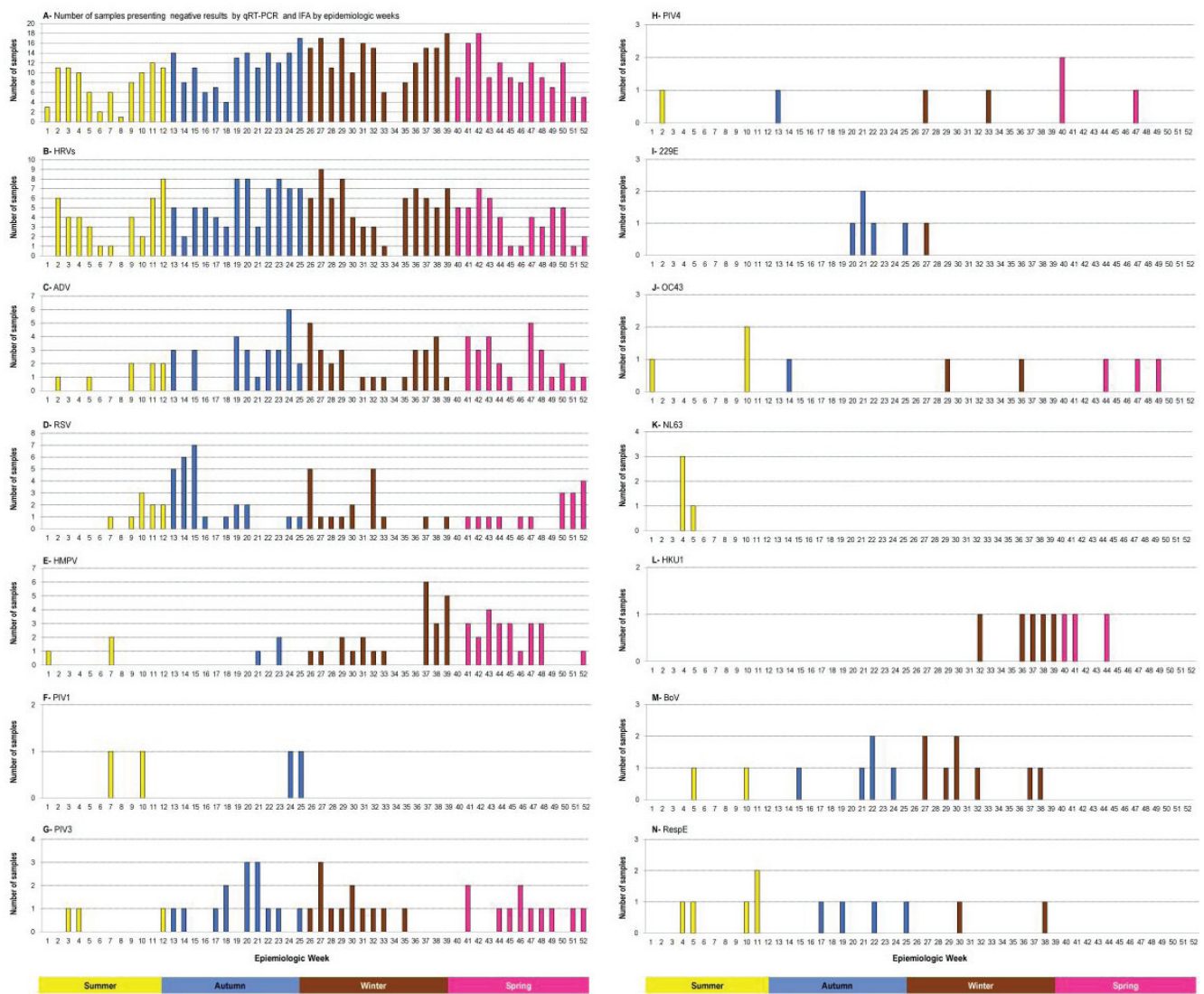


Figure 3 Respiratory viruses' seasonal profile was determined by RT-qPCR during the influenza A (H1N1) pdm09 emergence in São Paulo, SP, Brazil. (A) The lack of respiratory samples on epidemiologic week 34 is demonstrated.

in autumn–winter. The remarkable prevalence of HMPV and the low activity of PIV3 and RSV during spring provide insights concerning the seasonality of these viruses during the pandemic period (Figure 3). Apart from the activity of coronaviruses 229E and HKU1 during winter and spring, respectively, a low activity of NL3 was observed during summer, while a low activity of OC43 was noted in all four seasons (Figure 3). Although HBoV also presented low activity in autumn and early spring, its detection was concentrated during the winter period; similarly, a low detection of Resp/E was observed from autumn to early spring. The high activity of RVs in all four seasons is clearly demonstrated; these data clarify the

high co-detection between RVs and other respiratory viruses as shown in figure 2.

Discussion

The results of this retrospective study revealed the high prevalence of non-influenza respiratory viruses identified in patients presenting with influenza-like illness during the influenza A (H1N1) pdm09 pandemic period. Of these, RVs were the most prevalent followed by RSV, AdV, HMPV, PIV3, PIV1, HBoV, OC43, PIV4, HKU1, Resp/E, 229E, NL63. Historically, RVs were not considered a pathogen responsible for severe respiratory diseases, although this scenario has changed after the molecular diagnosis [20–23]. According to literature data, 20 to 25% of suspected

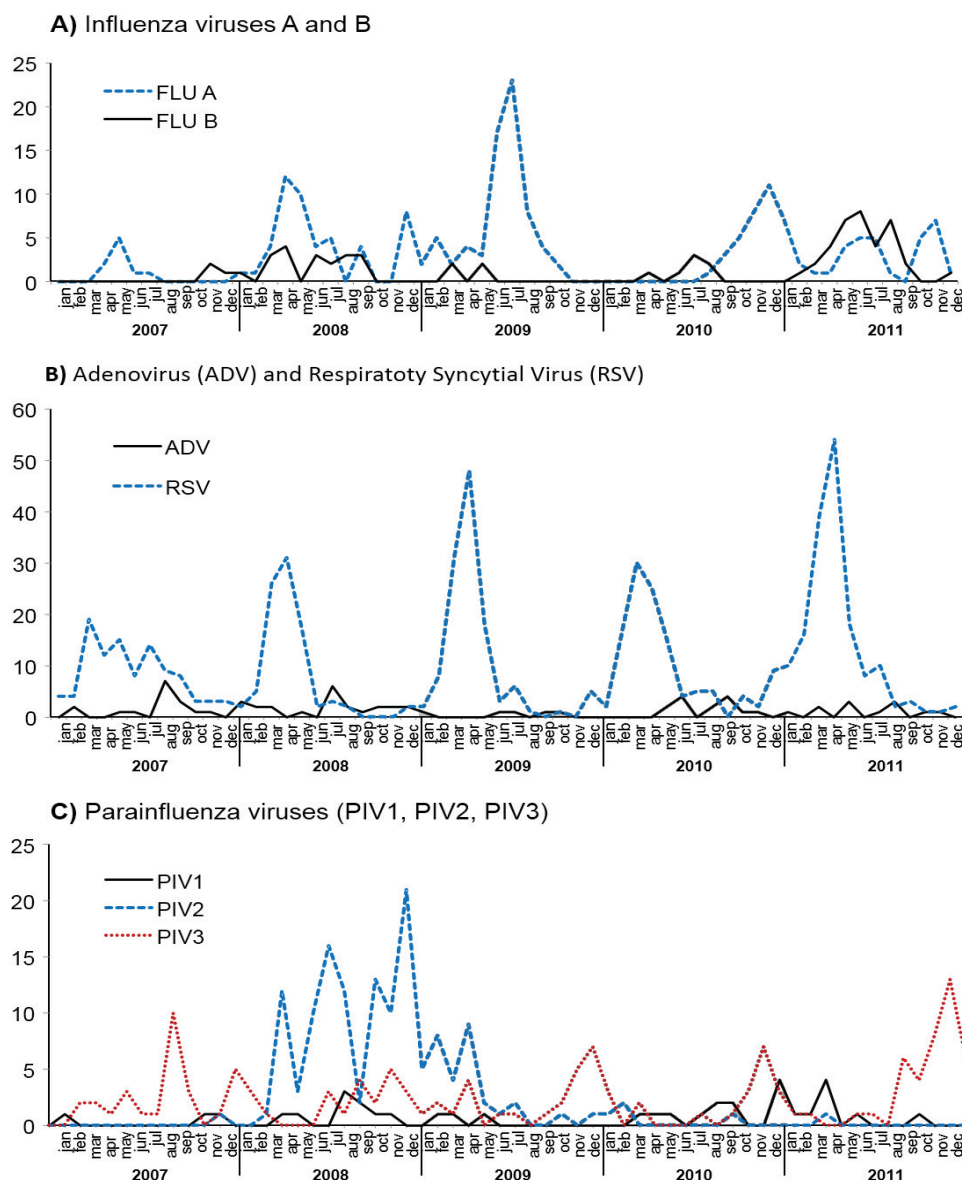


Figure 4 Prevalence of respiratory viruses, determined by IIF, from 2007-2011 influenza seasons.

cases of influenza virus infection were diagnosed as RVs [24,25]. In fact, high rates of HRV responsible for ILI outcomes, during the pandemic period, have already been described [30–36]. Tregoning JS and Schwaew J [31] have reported viral co-infections around 20%. In this retrospective study, from 542 respiratory specimens under investigation, a total of 425 tested positive as follows: 315 (58%) of mono-infection, 98 (18%) due to double infection, and 12 (2%) as a result of triple infection as shown in figure 2.

A limitation regarding mixed infections with PIV4, HCoV (OC43, 229E, HKU1, NL63), HBoV, and EV also has been identified due to our laboratory routine

procedures. Firstly, 542 selected samples presenting negative results for Flu by both methodologies IIF and real time RT – PCR (RT-qPCR) were investigated using the CDC real-time RT – PCR (RT-qPCR) protocol containing the following respiratory pathogens primers/probes: RSV, PIV(1, PIV2, PIV3), Adv, HMPV, and HRV. This protocol provided 170 negative results. In this context, only the negative samples were submitted to the protocol represented by primer/probes to identify the other respiratory pathogens included in the CDC real-time RT – PCR (RT-qPCR) as following PIV4, HCoV (OC43, 229E, HKU1, NL63) and EV. This work protocol has been limited to generating information regarding co-detection with PIV4, HCoV,

HBoV, and Resp/E among the most prevalent viruses causing ILI clinical symptoms RSV, PIV1, PIV2, PIV3, AdV, HMPV, and HRV). A limitation regarding the detection of Resp/E and HBoV has also been observed due to the lack of Resp/E and HBoV positive control as shown in table 1.

The samples selected for investigation of non-influenza respiratory viruses were not tested for respiratory bacterial pathogens, a study limitation.

The improvement of molecular methodologies by real-time RT-PCR (RT-qPCR) multiplex platforms has contributed to identifying a large panel of respiratory viruses, other than influenza, and to clarifying the etiology of acute respiratory infection. The study clearly demonstrated that RV circulating, during 2009, did not follow a seasonal pattern which means its detection has occurred during all four seasons. In this context independently of the seasonality profile of AdV, RSV, PIVs, and HMPV virus's co-detection with RSV has been observed as shown in figure 3. Otherwise, the co-detection between AdV and RSV was favorable due to the RSV seasonal profile during the autumn and between AdV and HMPV due to the HMPV seasonal profile at the end of winter and during the spring. It is interesting to observe that despite the low circulation of the HCoV, HBoV, and Resp/E co-detection has been observed among them during 2009.

This retrospective study demonstrates that with the exception of RVs and AdVs, the other respiratory

viruses circulating in São Paulo, followed a seasonal pattern during the pandemic period as shown in figure 3. A limitation of this retrospective study concerns the lack of archived respiratory samples corresponding to the epidemiological week 34.

The present study shows the prevalence of viral co-detection in children under two years of age, however despite the diversity of circulation of other non-influenza respiratory viruses' mono-infection dominates each age group as shown in figure 5.

The diversity of respiratory non-influenza viruses involved in ILI cases, during the pandemic period, certainly was revealed due to the increase in medical consultations, resulting from the panic caused by the new influenza strain.

A retrospective analysis from 2007 - April 2011, based on laboratory-confirmed cases by IIF, demonstrates that RSV was the most prevalent non-influenza virus circulating in São Paulo, with the exception of 2008 year when a remarkable PIV2 prevalence was observed. Respiratory virus differential diagnosis by using real-time RT-PCR (RT-qPCR) revealed the high prevalence of RVs circulating in 2009 in patients suspected of infection by the influenza pandemic strain. In contrast, by using the routine IIF respiratory panel RSV was the most prevalent non-influenza respiratory virus in the same period as shown in figure 4.

Considering the impact of infection by respiratory viruses in all age groups, especially among children <

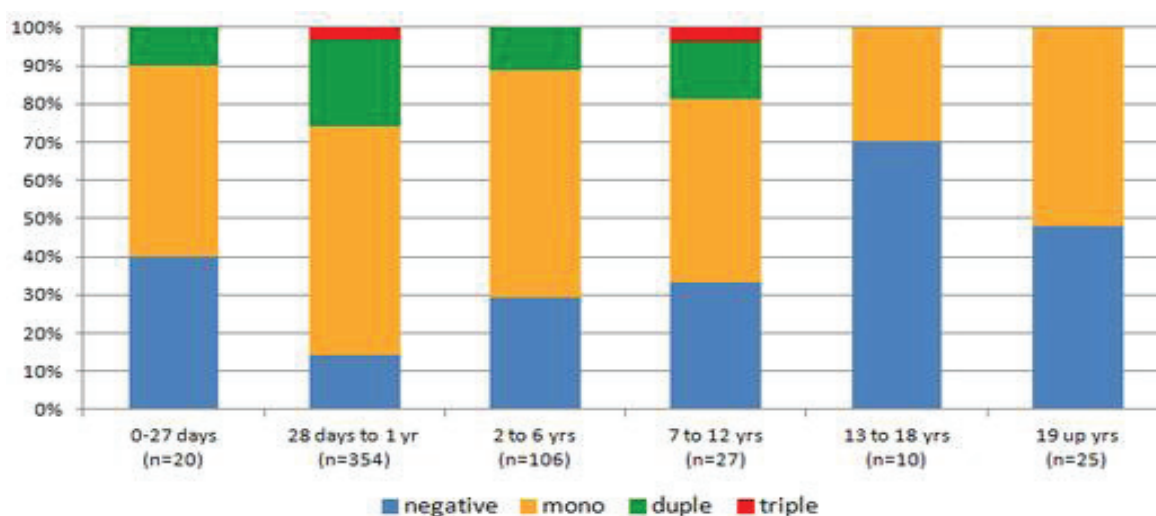


Figure 5 Virus co-detection, by age groups, identified during the emergence of the influenza A (H1N1) pdm09.

5 years of age [32–34] patients with chronic diseases and adults ≤ 60 years of age, a rapid diagnosis is important to improve patient clinical management.

This retrospective study highlights how powerful real-time RT – PCR (RT-qPCR) respiratory differential diagnoses are to clarify ILI and SARI viral etiologies, during pre-and pandemic periods by recovering viruses among samples that previously tested negative by IIF and showing the real scenario of virus circulation, during a pandemic period in the city of São Paulo, as requested by the local scientific community. Our findings revealed to the Brazilian Public Health Authorities the high incidence of RVs in suspected cases of infection by the influenza pandemic strain A (H1N1) pdm09. The improvement of respiratory virus diagnosis using real-time RT – PCR (RT-qPCR) CDC protocol for other non-influenza respiratory viruses will cooperate with studies to determine the burden of the whole range of respiratory viruses involved in ILI cases, as already proposed in the global context [35]. Also, the Institute Adolfo Lutz has improved its routine respiratory virus surveillance differential diagnosis by replacing IIF with the RT – qPCR to identify RSV, AdV, PIV1, PIV2, PIV3, HMPV, and RVs based on the results from the present study. In addition, the World Health Organization (WHO) encourages epidemiological surveillance for non-influenza respiratory viruses, to identify the most prevalent viruses aiming for vaccine production. Briefly, this research will contribute to the information obtained – during the first influenza pandemic of the 21st century – from a tropical country in Latin America, the South Hemisphere, in the global scenario context.

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Competing Interests

The authors have no conflicts of interest to declare.

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