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Original Article

Epidemiological and clinical features of human coronavirus infections among different subsets of patients

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Background Epidemiological and clinical data of human coronaviruses (HCoVs) infections are restricted to span 1–3 years at most. We conducted a comprehensive 9-year study on HCoVs by analyzing 1137 respiratory samples from four subsets of patients (asymptomatic, general community, with comorbidities, and hospitalized) in São Paulo, Brazil.

Methods A pan-coronavirus RT-PCR screening assay was performed, followed by species-specific real-time RT-PCR monoplex assays.

Results Human coronaviruses were detected in 88 of 1137 (7.7%) of the samples. The most frequently detected HCoV species were NL63 (50.0%) and OC43 (27.3%). Patients with comorbidities presented the highest risk of acquiring coronavirus infection (odds ratio = 4.17; 95% confidence interval = 1.9–9.3), and children with heart diseases revealed a significant HCoV infection presence. Dyspnea was more associated with HCoV-229E infections (66.6%), and cyanosis was reported only in HCoV-OC43 infections. There were interseasonal differences in the detection frequencies, with HCoV-229E being predominant in the year 2004 (61.5%) and HCoV-NL63 (70.8%) in 2008.

Conclusions Our data provide a novel insight into the epidemiology and clinical knowledge of HCoVs among different subsets of patients, revealing that these viruses may cause more than mild respiratory tract disease.

Keywords Clinical features, epidemiology, human coronaviruses, respiratory infection.

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Introduction

The genus *Coronavirus* belongs to the family *Coronaviridae* in the order *Nidovirales*. Coronaviruses (CoVs) infect a variety of livestock, poultry, and companion animals, in which they can cause serious and often fatal respiratory, enteric, cardiovascular, and neurological diseases.[1] The first reports on human coronaviruses (HCoVs) appeared in the mid-1960s. The human viruses were isolated from persons with the common cold, and two species were detected: HCoV-229E and HCoV-OC43.[2]

Almost 40 years later, an emergent HCoV was identified as the causative agent of severe acute respiratory syndrome (SARS).[3] While SARS-CoV was eradicated from the human population after a highly effective global public health response,[4] its emergence necessitated a rethinking of the role that CoVs play as a potential cause of more than just the common cold. Thereafter, there was an increase in research on HCoVs worldwide, which has led to the discovery of two other HCoVs, HCoV-NL63 in the Netherlands and HCoV-HKU1 in China.[5,6] Reports have documented that HCoVs are involved in a wide spectrum of clinical presentation in upper and lower respiratory tract illnesses,[7–15] as well as being a contributor to exacerbations of chronic obstructive pulmonary disease[16] and among patients presenting cystic fibrosis.[17] The involvement of other non-respiratory organ systems is still controversial but can occur, for example the enteric and nervous systems.[18,19] Additionally, the role of HCoV infections among susceptible groups, outbreaks, and fatal cases has been documented.[20–25] However, the majority of reports describe HCoV infections only among children. Moreover, longitudinal studies of CoV epidemiology in the literature are lacking and are restricted to descriptions spanning 1–3 years at most.[26–28] In this study, we conducted a comprehensive 9-year study of the epidemiological and

clinical features of all four circulating HCoVs (OC43, 229E, HKU1, and NL63) among four different subsets (asymptomatic, general community, with comorbidities, and hospitalized) of adult and child patients.

Material and methods

Study design and population

During the 9-year study, respiratory samples were collected from patients presenting acute respiratory illnesses and those who were asymptomatic. The study period began in June 2001 and concluded in September 2010. We collected 1137 samples and stored them at −80°C. The clinical specimens from the studied patients were collected from specific units by trained professionals (nurses or doctors). The specimens comprised 464 nasopharyngeal swabs, 208 nasal aspirations, and 465 nasal lavages. The subjects were 50 asymptomatic adults and 1087 patients presenting acute respiratory infections (ARIs): 465 from the general community (adults, children, and healthcare workers), 410 with comorbidities (children with heart diseases, patients with stem cell and renal transplants), and 212 hospitalized patients (adults and children) originally suspected of H1N1 2009 influenza infection. Of the 1137 studied patients, 591 (51.9%) were adults, while the rest were children. The patients were treated at the primary care health service section of São Paulo Federal University/São Paulo University Hospital in ambulatory or specific units and the emergency room. Each of these specific health services had a variable flow of sampling to respiratory viruses diagnoses, which resulted in collections of different patient groups in this study. Hence, the subjects groups were unevenly distributed throughout the study. The distribution of the studied years and clinical specimens by studied patients is shown in Table 1.

Inclusion criteria

The inclusion criteria were as follows: clinical diagnosis of influenza-like illness (ILI) defined by the presence of fever plus at least one respiratory symptom (cough and/or sore throat) and one constitutional symptom (headache, myalgia, sweat, or chills); clinical diagnosis of ARI, defined by the presence of respiratory symptoms that did not fulfill the definition of ILI; hospitalized patients suspected of 2009 H1N1 influenza A infection presenting fever >38°C, cough, and dyspnea. Lower respiratory tract illness was defined as having suggestive clinical findings and one of the following: chest radiograph revealing possible or definite infiltrates, mechanical ventilation requirement, or supplemental oxygen requirement.

Demographic and clinical data

Epidemiological and clinical data from outpatients were obtained using a standardized questionnaire, while data from hospitalized patients were extracted from medical charts using a standardized form. Age, gender, occupation, presence of underlying conditions or diseases, and clinical findings at first attendance (for outpatients) or during the first 24 hours (for hospitalized patients) were recorded for analysis. The number of days between admission and a positive CoV sampling, as well as the total length of respiratory illness, was evaluated for evidence of nosocomial acquisition. Institutional review board approval was received before we reviewed any records. The Ethics Committee of São Paulo Federal University (CEP 1968/09) approved this study, and written

Table 1. Distribution of studied patients by year and clinical specimen

		Years										
Populations	**Tested, no.**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**Specimen**
Adults*	50	•	•	•								NL
Healthcare workers*	86	•	•	•								NL
Children*	329								•	•	•	NPS
Children with heart diseases†	81					•	•	•				NA
Renal transplant patients†	148		•	•	•							NL
ST transplantation program†	181								•	•		NL
Hospitalized	212									•	•	NPS
Asymptomatic subjects	50									•		NPS
Total samples	1137	45	76	67	96	42	3	20	231	370	187	

•, Sampling.
NL, nasal lavage; NPS, nasopharyngeal swab; NA, nasal aspiration; ST, stem cell.
*Patients from the community.
†Patients with comorbidities.

consent was obtained from all patients or those responsible for the individual patient.

Pan-CoV RT-PCR one-step assay

Viral genomic RNA was extracted from the collected specimens with a QIAamp Viral RNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A previously described pan-CoV RT-PCR assay[29] with minor modifications (from 50 amplification cycles to 40 and melting temperature from 48 to 50°C) was performed as a screening step to detect human and animal CoVs in the samples. The amplification of a 251-bp fragment of the polymerase gene was carried out using the following primer set: Cor-FW (5′-ACWCARHTVAAYYTNAARTAYGC-3′) and Cor-RV (5′-TCRCAYTTDGGRTARTCCCA-3′). These one-step RT-PCR assays (OneStep RT-PCR kit; Qiagen) were undertaken in a 50-μl reaction volume containing 10 μl RNA extract, 10 μl 5 × Qiagen OneStep RT-PCR Buffer, 2 μL dNTP mix (final concentration of 400 μM of each dNTP), 1.8 μl Qiagen OneStep RT-PCR Enzyme Mix (a combination of Omniscript and Sensiscript reverse transcriptase and HotStarTaq DNA polymerase), 4 μM of each primer, and RNase-free water. Positive and negative controls were included in each PCR experiment. The reaction was carried out with an initial reverse transcription step at 50°C for 30 minutes, followed by PCR activation at 95°C for 15 minutes, 40 cycles of amplification (30 seconds at 94°C; 30 seconds at 50°C; 1 minutes at 72°C), and a final extension step at 72°C for 10 minutes in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were verified on an agarose gel, stained with ethidium bromide, and visualized under UV light. The sensitivity of the assay was assessed by testing 10-fold dilutions of HCoV-NL63 RNA standard curve, which showed sensitivity of 5×10^5 RNA copies/μl. The specificity was assessed by testing controls of other respiratory viruses (rhinovirus, bocavirus, human parainfluenza 1, 2, and 3, influenza A and B, metapneumovirus, adenovirus, and respiratory syncytial virus) through the methods for HCoVs detection. There was no amplification of the non-HCoV controls through the method used for CoVs detection.

Real-time RT-PCR for HCoV detection

Positive samples on the pan-CoV RT-PCR assay were tested to identify the HCoV species OC43, 229E, NL63, and HKU1 using a previously described real-time RT-PCR panel consisting of four different HCoV monoplex assays run under identical amplification conditions. Reaction mixtures were prepared using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems). Reactions (25 μl) contained 2 × RT-PCR buffer, 25 × RT-PCR enzyme mix, primers, probe, nuclease-free water, and nucleic acid. Amplification was performed on a 7500 Standard Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 48°C for 10 minutes (1 cycle); 95°C for 5 minutes (1 cycle); and 95°C for 15 seconds followed by 55°C for 1 minutes (45 cycles). Each specimen was also tested for the human ribonuclease P gene to measure nucleic acid integrity.[30] The sensitivity of the assay was assessed by testing 10-fold dilutions of each HCoVs RNA standard curve, which showed sensitivity of 5×10^2 copies/μl for HCoV-OC43, 5×10^4 copies/μl for HCoV-229E, 5×10^3 copies/μl for HCoV-NL63, and 5×10^3 copies/μl for HCoV-HKU1. The specificity was assessed as described in the Pan-CoV assay.

Statistical analysis

Statistical analysis consisted of Pearson's χ^2 test for the comparison of categorical values, with a significance level of $P < 0.05$. Non-conditional logistic regression was used to identify associations between CoV positivity and the studied populations. All reported values were two-tailed. Results are presented as odds ratios (OR) and the respective 95% confidence intervals (CI), and P values. All data were analyzed using the Statistical Analysis Software, version 9.0 (SAS Institute Inc., Cary, NC, USA).

Results

Coronavirus detection

The pan-CoV assay detected 128/1137 (11.2%) CoV-positive samples, and the real-time RT-PCR panel identified 88 HCoV of the 128 (68.7%) screened samples. The most frequently detected HCoV species were NL63 (50.0%) and OC43 (27.3%). The 229E and HKU1 species were detected in 17.0 and 5.7% of the samples, respectively. The CoV infection among the studied settings was evaluated by logistic regression analysis. When compared with the hospitalized, significant difference ($P < 0.05$) was observed among all subsets, except in children from the community ($P = 0.3076$) and asymptomatics ($P = 0.9879$; Table 2). Patients with comorbidities presented the highest risk of acquiring CoV infection (OR = 4.17; 95% CI 1.9–9.3; $P = <0.05$) when compared with the other subsets. Children with heart diseases and renal transplant patients had the next highest rates of CoV infections after the comorbidities group, tallying 17.3 and 12.2%, respectively. An important rate of detection was also found among healthcare workers (10.5%) and patients in stem cell transplantation programs (10.5%). No HCoV infection was found among asymptomatic patients. Figure 1 illustrates the distribution of HCoV species among the positive cases based on the studied patient subsets. HCoV-NL63 was the species most frequently associated with children, both from the community (100%) and presenting heart diseases (50%), while there was a high rate of HCoV-229E detection among renal transplant patients (44%). Patients in stem cell transplantation programs were more

Table 2. Detection of HCoVs over 9 years from 1137 samples

Populations	Tested no.	Detection rate*	*P* value
Adults†	50	4 (8%)	<.0001
Healthcare workers†	86	9 (10.5%)	0.0183
Children†	329	17 (5.2%)	0.3076
Children with heart diseases‡	81	14 (17.3%)	0.0002
Renal transplant patients‡	148	18 (12.2%)	0.0023
ST transplantation program‡	181	19 (10.5%)	0.006
Hospitalized	212	7 (3.3%)	ref
Asymptomatic subjects	50	0 (0%)	0.9879
Total	1137	88 (7.7%)	–

ST, stem cell; HCoVs, human coronaviruse.
*Real-time RT-PCR for human coronavirus detection.
†Patients from the community.
‡Patients with comorbidities.

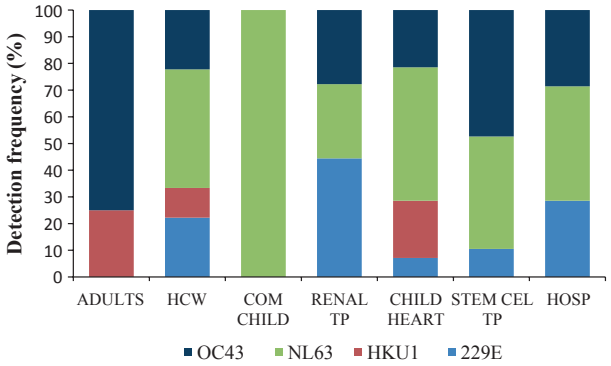

Figure 1. Proportions of samples positive for each human coronavirus from subsets of studied patients. HCW, healthcare workers; Com child, children from the community; Renal TP, renal transplant patients; Child heart, children with heart diseases; Stem cel TP, patients in stem cell transplantation programs; Hosp, hospitalized.

frequently infected with HCoV species OC43 (47%) and NL63 (42%) when compared with the other HCoV species. HCoV-NL63 was not detected among adults from the community, and HCoV-HKU1 was detected only among adults, healthcare workers, and children with heart diseases.

Circulation trend

Coronaviruses were most frequently detected during winter and autumn (75%) (Figure 2). Peak detection frequency was observed in the years 2001, 2004–2005, and 2008, with the highest rate of detection recorded in 2005 (8/42; 19%). There were additional longer-term differences in detection frequencies between seasons, with HCoV-229E being predominant in 2004 (61.5%) and HCoV-NL63 (70.8%) in 2008.

HCoV-OC43 was detected in all studied years (except in 2006, when no CoV cases were detected) from March to November (autumn to spring), with detection frequencies peaking in August (winter). Interseason HCoV-OC43 detection frequencies were more consistent than that for the other CoVs.

HCoV-HKU1 was detected only in July (winter) of 2001, 2005, and 2008. A decrease in HCoV-OC43 detection was observed during the period of HCoV-HKU1 detection.

HCoV-229E displayed a marked autumn seasonality (between March and April), with the highest rate of detection recorded in May (autumn) in 2002, 2004–2005, and 2008–2010.

HCoV-NL63 was the most frequently detected species in the study, circulating in all seasons. Although the NL63 species presented a high frequency of detection in July (winter), it was a unique HCoV species with cases also being detected in the summer.

Patient parameters

The median age of HCoV-infected patients was 28 years (range, 1 month to 68 years). The frequency was higher in patients aged 50–59 years (15.9%). Figure 3 depicts the distribution of CoV-infected cases by age. Predominantly, infected patients were female (60×28, $P < 0.001$). Sixteen percent (16/88) of the infected patients reported underlying conditions such as human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV)-type infection, diabetes mellitus, liver transplant, megacolon syndrome, hypertension, Wiskott–Aldrich syndrome, and congenital kidney and lung diseases. In addition, four patients were born prematurely and seven were smokers.

Clinical associations of CoV infections

Clinical data were available for all 88 HCoV-infected patients (Table 3). Coryza, cough, and fever were the most common symptoms at presentation, being reported in 86%, 77%, and 51% of positive cases, respectively. There were differences in clinical expression among the studied populations. Fever was found less frequently among renal transplant patients and patients in stem cell transplantation programs when compared with the other patient subsets. Cyanosis was observed among children with heart disease, and wheezing was reported among hospitalized patients, children from the community, and children with heart diseases. Dyspnea, another lower respiratory tract infection symptom other than wheezing, was documented among patients in stem cell transplantation programs, children with heart diseases, children from the community, and hospitalized patients. Healthcare workers presented more cases of sore throat and myalgia than did adults from the community group, patients under transplantation program, and hospitalized patients. Differences in clinical presentation were observed among

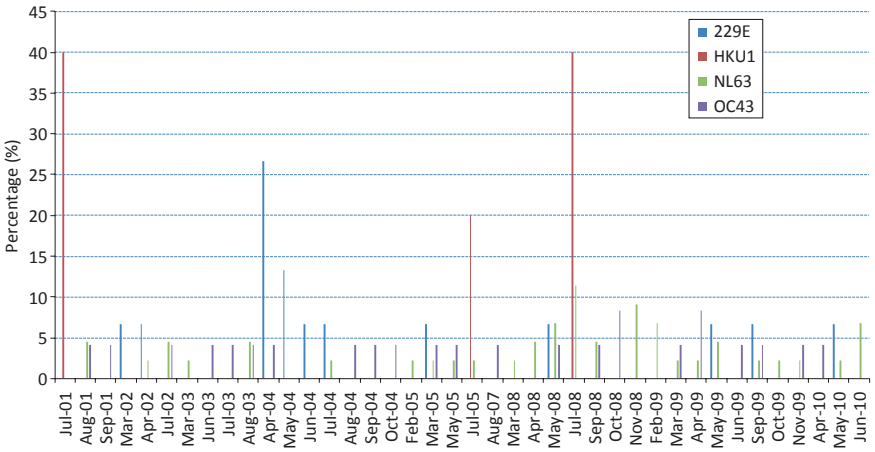

Figure 2. Human coronavirus detection frequencies by month over 9 years.

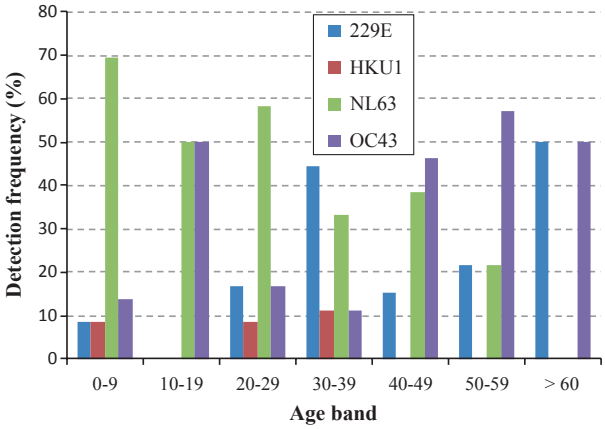

Figure 3. Percentages of detection frequencies of the four human coronaviruses by age.

each HCoV species infection (Figure 4). Patients infected with HCoV-HKU1 presented more fever, coryza, and myalgia when compared with the other HCoV-infected cases ($P < 0.05$). Dyspnea was more frequently associated with patients infected with HCoV-229E (66.6%). Cyanosis was reported only among patients infected with HCoV-OC43, and diarrhea was reported only in HCoV-NL63 cases.

The median time for hospitalized CoV-infected patients to be discharged was 12 days (range, 5–30 days); of the seven hospitalized CoV-infected patients, three were infected with HCoV-NL63, two with HCoV-229E, and the other two with HCoV-OC43. Patients infected with HCoV-OC43 stayed longer in hospital (average, 19 days) than did patients infected with HCoV-NL63 or HCoV-229E. Four hospitalized patients of seven (57.1%) were admitted to the intensive care unit. Lower respiratory tract infections were found in 6/7 (85.7%) hospitalized CoV-infected patients. The remaining patient was infected by HCoV-OC43 and presented upper respiratory tract infection. This patient was a nosocomial case documented in a child who had been admitted for an intestinal surgery due to megacolon. Six of the patients had a good outcome, but 1 diabetic adult died of HCoV-NL63-associated pneumonia 9 days after admission.

Coinfections

Investigations into other respiratory viruses (rhinovirus, bocavirus, human metapneumovirus, adenovirus, influenza A or B, and respiratory syncytial virus) were available in 87 of 88 CoV-infected cases, in which at least 1 respiratory virus other than CoV was detected in 26 samples (29.8%). Rhinovirus (46.1%) and adenovirus (26.9%) were the most frequently codetected viruses in CoV-positive cases. HCoV-NL63 was associated most frequently with coinfection (57.7%), followed by HCoV-229E (23.1%) and HCoV-OC43 (19.2%). There was no codetection of HCoV-HKU1 with other respiratory viruses. The most common associations were HCoV-NL63 plus adenovirus (46.1%) and HCoV-NL63 plus rhinovirus (38.5%). The methods used for detecting the coinfections were conventional RT-PCR (for rhinovirus, bocavirus, and human metapneumovirus detection) and direct immunofluorescence (adenovirus, influenza A or B, and respiratory syncytial virus detection).

Discussion

Since the emergence of SARS-CoV, numerous studies have been carried out on the clinical impact of different HCoV species.[8,9,13,14,16,26–28,30–32] However, the majority of these studies were only performed in children, with small sample sizes and short sample collection periods. This is the first study reporting on the epidemiological and clinical features of non-SARS HCoV infections among different clinical patient subsets, both adult and child, over a 9-year period.

Human coronaviruses were detected among all studied subjects presenting respiratory tract illnesses, but not among those who were asymptomatic. Recently, studies have demonstrated HCoV detection among asymptomatic

Table 3. Clinical data of CoV-infected patients, 2001–2010

	Populations						
Symptoms	**Adults (%)**	**HCW (%)**	**Com child (%)**	**Child heart (%)**	**Renal TP (%)**	**Stem cel TP (%)**	**Hospitaliz (%)**
Fever	100.0	55.5	64.7	50.0	16.6	42.1	100.0
Cough	100.0	44.4	88.2	85.7	66.6	73.7	100.0
Wheezing	–	–	31.2	41.6	–	0	28.6
Cyanosis	–	–	0.0	7.1	–	0	0
Dyspnea	–	–	23.5	35.7	–	26.3	71.4
Coryza	100.0	100.0	100.0	85.7	88.8	78.9	42.8
Sore throat	50.0	55.5	–	–	33.3	21.0	16.6
Headache	100.0	77.7	–	–	50.0	15.8	28.6
Diarrhea	0.0	0.0	0.0	0.0	0	0.0	16.6
Myalgia	75.0	77.7	–	–	27.7	21.0	14.3

–, Not evaluated.
HCW, healthcare workers; Com child, children from the community; Child heart, children with heart diseases; Renal TP, renal transplant patients; Stem cel TP, patients in stem cell transplantation programs; Hospitaliz, hospitalized; CoV, coronavirus.

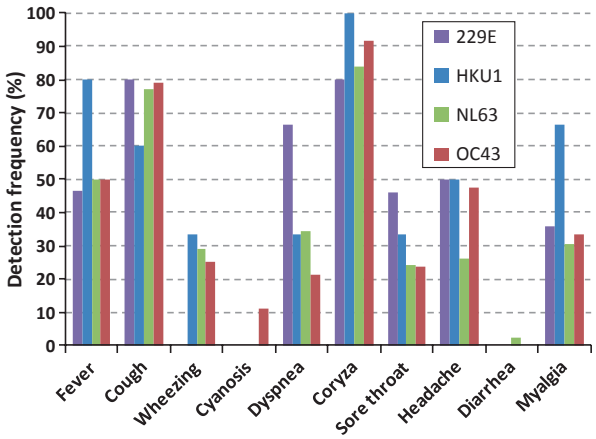

Figure 4. Distribution of symptoms by each human coronaviruse-infected case.

patients, with a positive rate of approximately 4–7%.[33,34] In contrast to our study, the evaluated asymptomatic patients from those reports were children, and we hypothesize that asymptomatic respiratory infection may be more frequent among children than in adults.

An important CoV detection rate was found among the subset of patients with comorbidities, being most frequent in children with heart diseases and in renal transplant patients. Additionally, underlying conditions other than cardiac disease/transplantation were observed in 18% of CoV-infected patients. These data point to the relevance of CoVs causing respiratory tract illness in patients with comorbidities. Some reports have demonstrated a correlation between CoV-induced respiratory tract illness and populations with comorbidities.[22,35–37]

In spite of there being a lower HCoV detection rate in the hospitalized subset, the majority of positive cases presented with lower respiratory tract infections, and one fatal case was documented. This result supports the premise that HCoV infection may cause more than mild respiratory tract disease.

A significant frequency of HCoVs among healthcare workers was also documented. Previous studies have demonstrated the important burden of healthcare workers associated with respiratory tract infections, as well as their being strongly implicated in HCoV outbreaks.[20,25,38–40]

Although HCoV is considered one of the most common respiratory viruses associated with respiratory tract infections in children, the present study revealed a higher rate of detection among adults in the 50- to 59-year range. As our study cohort contained a significant number of adult patients with comorbidities, this result can represent a bias.

As different age groups were tested for HCoV infections in different years in our study, this represents a flaw in the study design which prevents us from comparing the frequency of infection in one age group to another, and it is considered as the major limitation of this work. Nevertheless, the results of our study showed interesting findings on HCoVs infection, which means that HCoVs may be the causal agents of respiratory diseases among patients under different clinical conditions, either adults or children.

Our data revealed that NL63 and OC43 were the most commonly detected HCoV species in our study. Recently, Dijkman *et al.*[41] demonstrated that HCoV-OC43 and HCoV-NL63 may elicit immunity that protects from subsequent HCoV-HKU1 and HCoV-229E infection, respectively, which would explain why HCoV-OC43 and HCoV-NL63 are the most frequently infecting HCoVs. Other studies have demonstrated a higher frequency of

HCoV-NL63 among children[12,32,42] and HCoV-OC43 among adults.[43]

Although it is possible to verify the periodicity of HCoVs infection in the presented data of this study, that is, HCoV-HKU1 (Figure 2), our study focuses on HCoV-type specificity frequency in particular patient groups. In this regard, HCoV-229E was mainly associated with renal transplant patients and HCoV-NL63 was involved in a death. HCoV-229E has previously been associated with infections in immunocompromised individuals,[37,44] and some reports have demonstrated the further association of HCoV-NL63 with fatal cases.[22–24] However, there are no consistent indications that infection by one HCoV is more pathogenic than that with another. Thus, our data provide evidence that the detection and diagnosis of HCoV infections should be considered for immunocompromised individuals with respiratory tract symptoms.

Coronaviruses display the marked seasonality typical of other respiratory viruses, with high detection frequencies in the winter months but few or no detections in the summer. The climate of the city of Sao Paulo (Southeast of Brazil) is under constant influence of both the southern coast and the northern part of the state, which presents a subtropical climate. Thus, this city has an uncharacterized climate marked by a wide thermal and meteorological variety. In this regard, it is not possible for us to trace any interaction between climatic and meteorological factors and HCoVs detection. However, due to the long-term HCoV surveillance performed in the present study, it was possible to demonstrate particular trends concerning CoV species seasonality, where different marked peaks of detection were observed for each HCoV species during the 9-year study. Other studies have demonstrated differences between CoV species seasonalities and a winter preference. van der Hoek *et al.*[45] performed a study on HCoV-NL63 revealing that this species infection follows a 2-year interepidemic period with peaks of infection in the winters. Gaunt *et al.*[28] in a study carried out over a period of 3 years in the UK demonstrated a high detection of HCoVs species during winter months.

Our study showed a very low HCoVs detection rate in 2007 and none in 2006. These were the years that presented the lowest number of samples included in the study, and we believe this might have influenced the HCoVs detection during these 2 years.

Different clinical specimens were used in search of HCoVs. Although some studies have shown the sensitivity of different kinds of specimens for respiratory viruses diagnosis through many different detection methods, we did not evaluate data on HCoVs detection from the different specimens collected using the molecular methods applied in our study.

In conclusion, our data provide a novel insight into the epidemiology and clinical knowledge of HCoV among different subsets of patients, supporting the notion that HCoVs play an important role among patients with comorbidities. HCoV infections were found not only in children, but also among adults, mainly in healthcare workers. Therefore, HCoVs are important pathogens involved in unexplained respiratory illness among patients with different clinical patterns.

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Conflict of interest

The authors have no conflicts of interests to declare.

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