Low circulation of Influenza A and coinfection with SARS-CoV-2 among other respiratory viruses during the COVID-19 pandemic in a region of southern Brazil

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Abstract
With the arrival of coronavirus disease 2019 (COVID-19) in Brazil in February 2020, several preventive measures were taken by the population aiming to avoid severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection including the use of masks, social distancing, and frequent hand washing then, these measures may have contributed to preventing infection also by other respiratory viruses. Our goal was to determine the frequencies of Influenza A and B viruses (FLUAV/FLUBV), human mastadenovirus C (HAdV-C), Enterovirus 68 (EV-68), and rhinovirus (RV) besides SARS-CoV-2 among hospitalized patients suspect of COVID-19 with cases of acute respiratory disease syndrome (ARDS) in the period of March to December 2020 and to detect possible coinfections among them. Nucleic acid detection was performed using reverse-transcription quantitative polymerase chain reaction (RT-qPCR) in respiratory samples using naso-oropharyngeal swabs and bronchoalveolar lavage. A total of 418 samples of the 987 analyzed (42.3%) were positive for SARS-CoV-2, 16 (1.62%) samples were positive for FLUAV, no sample was positive for FLUBV or EV-68, 67 (6.78%) samples were positive for HAdV-C, 55 samples were positive for RV 1/2 (26.3%) and 37 for RV 2/2 (13.6%). Coinfections were also detected, including a triple coinfection with SARS-CoV-2, FLUAV, and HAdV-C. In the present work, a very low frequency of FLUV was reported among hospitalized patients with ARDS compared to the past years, probably due to preventive measures taken to avoid COVID-19 and the high influenza vaccination coverage in the region in which this study was performed.

Keywords
ARDS, coinfection, COVID-19, EV-68, HAdV-C, influenza, rhinovirus
1 | INTRODUCTION

By the end of 2019, scientists announced the circulation of a new type of coronavirus in China, which was later officially named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belonging to the Severe acute respiratory syndrome-related coronavirus species together with the first SARS-CoV.1 Coronavirus disease 2019 (COVID-19), the disease caused by it, was linked to a seafood market in the city of Wuhan, Hubei province, and since then, the disease quickly spread around the world causing 87,589,206 confirmed cases by January 9th and 1,906,606 deaths.2-4 COVID-19 was rapidly declared a pandemic, in about 3 months since its beginning. Regarding being a burden for public health, the present pandemic is only comparable to the Spanish Flu back in 1918, caused by Influenza A virus (FLUAV) H1N1.5

Respiratory pathogens like viruses, bacteria, and fungi are commonly found infecting the same host simultaneously, in fact, co-infection with several different respiratory viruses is a common finding as well as viral-bacterial co-infection, mainly taking into account that seasonality overlaps in some of them.6-8 Among respiratory viruses, FLUAV and Influenza B virus (FLUBV) species are prevalent, as well as Rhinovirus (RV) of Enterovirus genus and Human mastadenovirus C (HAdV-C).9,11

The advent of COVID-19 forced the adoption of several non-pharmaceutical preventive measures aiming to avoid SARS-CoV-2 infection like wearing masks, social distancing, and frequent hand washing. Furthermore, as an attempt to contain COVID-19 and maintain the economy, the state government created a system of “controlled distancing” which has been updated weekly through a system of color-based risk flags that represent a low risk (yellow), medium risk (orange), high risk (red) and very high risk (black). Depending on the flag’s color, the measures required for commerce and services establishments are more restricted and some activities may be prohibited.13,14 These measures may have contributed to preventing infection by other respiratory viruses besides SARS-CoV-215,16 and their circulation may have been reduced along their seasonal months in 2020.

Therefore, the goal of this study was to evaluate the frequency of other viral agents of respiratory illness, including FLUAV and FLUBV, RV, Enterovirus D68 (EV-D68), and HAdV-C in samples collected from hospitalized patients suspected of acute respiratory distress syndrome (ARDS) by SARS-CoV-2 as well as to detect possible viral co-infections among them.

2 | MATERIALS AND METHODS

2.1 | Samples

Respiratory samples from 40 different municipalities of Rio Grande do Sul, the southernmost state of Brazil (Figure S1), were received for COVID-19 molecular diagnosis in Laboratório de Microbiologia Molecular of Universidade Feevale, Novo Hamburgo, RS, Brazil. Samples in this study were chosen randomly among clinical samples from hospitalized patients presenting typical cases of ARDS and suspected of having COVID-19. For this study, 987 cases were selected along a period comprising months of March to December of 2020. This corresponds to the previous phase of the first peak of COVID-19 cases in the region (which occurred in the end of July), to the beginning of the second wave that started in October 2020. Respiratory samples were naso-oropharyngeal swabs or bronchoalveolar lavage, collected by local healthcare professionals in sterile saline solution following standard guidelines, and transported to the laboratory under refrigeration until 24 h after sampling. This study was approved by the University’s Ethical Review Board (protocol number: 33202820.7.1001.5348), following Brazilian regulations and international ethical standards.

2.2 | Nucleic acid extraction

Samples were stored at 4°C until the nucleic acid extraction was performed before completing 24 h. Extractions were made with a commercial kit MagMAX™ CORE Nucleic Acid Purification Kit (Applied biosystems™) using the automated equipment KingFisher™ Duo Prime (Thermo Fisher Scientific™). Saline was also extracted and used as a negative control of the extraction (CE) in each round to ensure the absence of contamination during this process. Nucleic acids were then stored at −80°C until further analysis.

2.3 | Nucleic acids amplification by reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

FLUAV, FLUBV, types of FLUAV H3, SARS-CoV-2, HAdV-C, RV, and EV-D68 were aimed for detection by nucleic acid amplification. RT-qPCR reactions were made in a 25 µl total volume for FLUVs detection, comprising 12.5 µl of 2× RT-PCR buffer and 1 µl of 25X Enzyme mix of AgPath-ID™ One-Step RT-PCR Reagents (Thermo Fisher Scientific™), 0.5 µl of each primer and probe, 5 µl of nuclease free-water (NFW) and 5 µl of sample. SARS-CoV-2 detection reactions were made in a 20 µl total volume, comprising 10 and 0.8 µl of the same buffer and enzyme mix respectively, as well as 0.8 µl of each primer, 0.4 µl of the probe, 2.2 µl of NFW, and 5 µl of the sample. Amplification cycle was the same for FLUV and SARS-CoV-2 detection, beginning at 50°C for 15 min for reverse transcription, followed by denaturation at 95°C for 10 min and by 40 cycles of 95°C for 15 s and 60°C for 45 s. For HAdV-C detection, qPCR reactions were made in a 20 µl final volume, with 10 µl of Taqman® Fast Advanced Master Mix (Thermo Fisher Scientific™), 2 µl of NFW, 1 µl of each primer and probe, and 5 µl of the sample. Amplification cycle was according to Wolf et al.17

For RVs and EV-68 detection the primer-probe kit of TaqMan® Respiratory Tract Microbiota Profiling Experiments (Applied Biosystems™) was used in a total volume reaction of 15 µl, with 7.5 µl
of 2X RT-PCR Buffer, 0.6 µl of 25X Enzyme mix of AgPath-ID™ One-Step RT-PCR Reagents (Thermo Fisher Scientific™), 0.9 µl of NFW and 1 µl of primer-probe in each independent reaction, the human rhinovirus 1/2, human rhinovirus 2/2 and human enterovirus D68.

In all analyses, a no template control (NTC) was used as a negative control to ensure absence of contamination during the RT-qPCR process and positive controls were also used in all plates to ensure the effectiveness of all reactions. Positive controls used were a FLUAV swine H1N1 pandemic virus isolated in MDCK cells (FLUAV), H3N2 virus isolated in MDCK cells (H3 types), a human FLUBV isolated in MDCK cells (FLUBV), HAdV-5 isolated in A549 cells (HAdV-C), and a poliovirus 2 sample (RV).

Descriptions of primers and probes with their references can be seen in Table 1, except for RVs and EV-68 which were not described in the kit of TaqMan® Respiratory Tract Microbiota Profiling Experiments.

3 | RESULTS

A total of 987 clinical samples from ARDS hospitalized patients were analyzed from March to December of 2020. Most samples were naso-oropharyngeal swabs (91.3%). Among the patients, 51.8% were male and 48.2% female, distributed further into young (<18 years old), young adults (19–35), adults (36–59), and elderly (>60)

and 48.2% female from 41 different municipalities, although 46.2% of samples included in this study were from the same city, Novo Hamburgo. The patient included with the highest age was 99 years old and the lowest was 2 months old, most (59.4%) patients were elderly (>60 years old), 30.6% were adults (36–59 years old), 9.9% were young adults (19–35 years old) and 2.1% were children or teenagers (<18 years old), age and gender distribution are shown in Figure 1.

### TABLE 1: Descriptions of primers and probes used in SARS-CoV-2, FLUV, and HAdV-C detection

| Oligonucleotides   | Sequence 5’→ 3’                           | Concentration, µM | References                   |
|--------------------|-------------------------------------------|-------------------|------------------------------|
| InfA primer F      | GACCRATCTGTCACCTCTGAC                     | 40                | CDC18                        |
| InfA primer R      | AGGGCTTYGGGAAAKCGTGCTA                    | 40                |                              |
| InfA sonda         | TGCAGTCTGCTCAGTGGCAGG                     | 10                |                              |
| InfB primer F      | TCCTCAAYTCACTCTTCGAGG                     | 40                | Selvaraju and Selvarangan19  |
| InfB primer R      | CGTGTTCTTGACAAATTGG                      | 40                |                              |
| InfB sonda         | CCAATTCGAGGGACTGAACTCCGCGGTG             | 10                |                              |
| pdmH1 primer F     | GTGCTTAAACACACGACCCTAG                   | 40                | Rönkkö et al.20              |
| pdmH1 primer R     | CGGATTTCTCTAATCTCTGTRG                   | 40                |                              |
| pdmH1 sonda        | CAGAATATACATCGTCACAGTTGGR                | 10                |                              |
| pdmInfA primer F   | GCACGTCAGCAGTATATATCTATCT               | 40                | CDC18                        |
| pdmInfA primer R   | GTGCTTGGTGTTTCTTCTTGT                   | 40                |                              |
| pdmInfA sonda      | CYACTGCAAGCCCATAACACACAGCACGGA          | 10                |                              |
| H3 primer F        | AAGCTTCTCCAATGCAACAC                    | 40                | Tse et al.21                 |
| H3 primer R        | ATGGCTTATAGTGGCTTGT                    | 40                |                              |
| H3 sonda           | CAGGATATACATGCGCCGGTCCG                 | 10                |                              |
| E_Sarbeco_F1       | ACAGGTAGTGTAATATAGTTAAATGCGT           | 5                 | Corman et al.22              |
| E_Sarbeco_R2       | ATATTTGAGCGATCGACACAA                   | 5                 |                              |
| E_Sarbeco_P1       | ACAGTACCATCCTACTCCGCTCGTT              | 5                 |                              |
| VTB2-HAdVCf        | GAGAGTACTCTTGGCGTGAAT                   | 25                | Wolf et al.17                |
| VTB2-HAdVCr        | GATAGACGCCAGCGTCAA                     | 25                |                              |
| VTB2-HAdVC probe   | CCTACGCGACGCGTACCAGA                    | 15                |                              |
Of the 987 samples included in this study, all of them were analyzed for SARS-CoV-2, FLUAVs, FLUBV and HAdV-C. For RV 1/2, 277 were analyzed, 272 for RV 2/2 and 76 for EV-68. A total of 418 (42.3%) samples were positive for SARS-CoV-2, 16 (1.62%) samples were positive for FLUAV, 15 with pdmInfA primer and one with pdmH1, no sample was positive for FLUBV or EV-68 and 67 (6.78%) samples were positive for HAdV-C. Seventy-three samples were positive for RV 1/2 (26.3%) and 37 for RV 2/2 (13.6%), these results can be seen in Table 2 and Figure 2. Figure 4 shows positive cases for all respiratory viruses tested distributed throughout the year.

In addition, some coinfections were also detected. SARS-CoV-2 was detected in coinfections with all the other three viruses found in this study including a triple coinfection with FLUAV and HAdV-C. Also, RV and HAdV-C were the viruses most found in coinfections with SARS-CoV-2. Viral combinations in coinfections and their frequencies can be more clearly visualized in Figure 3.

### 4 | DISCUSSION

This past year had several unusual aspects due to the arrival of SARS-CoV-2 all around the world, Brazil's first case was in February, and in March several states already had cases, including Rio Grande do Sul state where this study took place. One of the peculiar aspects of COVID-19 pandemic was the preventive measures taken to avoid infection, like face masking by populations that usually did not have this habit, the isolation of people presenting respiratory symptoms, frequent hand washing, and social distancing, therefore these precautions probably had an effect in other viruses besides SARS-CoV-2. In this study, it was possible to verify a low frequency of FLUAV in hospitalized patients with cases of ARDS during the COVID-19 pandemic. Only 1.62% of the samples analyzed were positive for this virus even if FLUAV is an important cause of ARDS during autumn/winter months mainly in elderly people that, in fact, compose most of the patients included in this study. For comparison, in 2019, about 17.8% (5.714) of 32,048 ARDS' samples were attributed to one of FLUVs throughout Brazil. One probable explanation for this is the vaccination status of the population, which is above 90% coverage in the state. HAdV-C also presented a low frequency (6.78%) which was also described in similar studies during 2020 for other respiratory viruses and in the case of RV, it remains present at a considerable rate (~20%) in comparison to the past years, even though being higher than that found by Nowak et al. Notwithstanding, in this study, a smaller number of samples were analyzed for RV.

About the distribution of cases over time, in Figure 4 it is possible to evaluate that after a few weeks under the orange flag of the controlled distancing system of the state government that indicates a medium risk of contagion, the positivity of SARS-CoV-2 rose.

### TABLE 2 Number of positive samples for other respiratory viruses and their percentages relative to SARS-CoV-2 positivity status

| Virus          | SARS-CoV-2 positive samples | SARS-CoV-2 negative samples |
|----------------|----------------------------|----------------------------|
|                | Tested | Detected | Percent | Tested | Detected | Percent |
| Influenza A    | 418    | 6        | 1.43     | 569    | 10       | 1.75     |
| Influenza B    | 418    | 0        | 0.00     | 569    | 0        | 0.00     |
| Adenovirus     | 418    | 18       | 4.30     | 569    | 49       | 8.61     |
| Rhinovirus 1/2 | 67     | 18       | 26.8     | 210    | 55       | 26.2     |
| Rhinovirus 2/2 | 73     | 9        | 12.3     | 199    | 28       | 14.1     |
| Enterovirus 68 | 20     | 0        | 0.00     | 56     | 0        | 0.00     |

**a**Names of the primer-probe kit according to the manufacturer of the commercial kit used.
After the second and longer period with orange flags, around eight weeks, most of the FLUAV positive cases of this study occurred in November accompanied by an increase also in the number of positive SARS-CoV-2 cases, probably because people may have circu-
lated more due to the orange flag that must have conveyed a false sense of safety to the population.

We have detected some viral coinfections including SARS-CoV-2 as presented in Table 2. SARS-CoV-2 + HAdV-C and SARS-CoV-2 + RV coinfections occurred more often probably due to the greater frequency of these viruses in samples tested, and in spite of low FLUAV frequency, a dual and a triple coinfection with this virus were detected. Viral respiratory coinfections are very common and several studies already

FIGURE 3 Coinfections cases between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Influenza A (FLUAV), adenovirus (HAdV) and rhinovirus. Different dual coinfections were found as well as a triple coinfection with SARS-CoV-2, FLUAV and HAdV

FIGURE 4 Distribution of positive cases along the year and current risk flag of the government for certain measures of social distancing. The red one being a high risk of contagion which imposes more restrictive measures on businesses and establishments and the orange, moderate risk, with more flexible measures. (A) Distribution of 418 SARS-CoV-2 and 16 influenza A positive patients. (B) Positive cases of adenovirus and rhinovirus (RV) over the year. *Fewer samples were tested for RV and so distribution may be biased
reported viral coinfections with SARS-CoV-2, including with FLUAV, although it is probably not occurring as often as it might be expected, which may be due to a lack of diagnosis for other viruses in this pandemic period.32-39

Still, on the topic of coinfections, there is a risk of disease exacerbation in these cases, considering that the immunologic system is already disturbed due to the first infection. In studies that reported viral coinfections with COVID-19, it is not yet clear if these cases are associated with more severe disease,38-40,42 except for the study of Yue et al.43 that suggested that SARS-CoV-2 and FLUAV coinfection might be associated with more severe cases, but the authors commented that this virus was circulating more than FLUAV so this finding may be biased. Another exception is the work of Li et al.44 which reported that coinfections with COVID-19 are more associated with patients in the intensive care unit but did not separate viral from bacterial coinfections. In sum, the understanding of possible COVID-19 aggravation by coinfection with other respiratory viruses is still largely unknown, and in this study, we did not aim to evaluate clinical outcomes, so this topic still needs further investigation in future studies.

5 | CONCLUSION

In the present work, FLUV was reported in a very low frequency among hospitalized patients with ARDS compared to the past years, probably due to preventive measures taken to avoid COVID-19 and the high influenza vaccination coverage in 2020. We also demonstrated a relatively normal frequency of RV. Some viral coinfections were detected, including SARS-CoV-2 and FLUAV and a triple coinfection of SARS-CoV-2, FLUAV, and HAdV-C, although it is still not clear if viral coinfection is associated with more severe disease.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHORS CONTRIBUTIONS

Ana Karolina Antunes Eisen: Sample selection, extraction, analysis, result analysis, writing. Juliana Schons Gularte: Extraction, analysis, language review. Meriane Demoliner: Analysis, figure elaboration. Vytoria Malayhka de Abreu Goês Pereira: Sample selection, analysis. Fágner Henrique Heldt: Extraction, figure elaboration. Micheli Filippi: Sample selection, analysis. Paula Rodrigues de Almeida: Textual review. Alana Witt Hansen: Sample selection, result analysis. Juliane Deise Fleck: Textual review. Fernando Rosado Spilki: Study planning, textual review.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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