Archives of Virology (2023) 168:87
https://doi.org/10.1007/s00705-023-05717-6

BRIEF REPORT

A multiplex-NGS approach to identifying respiratory RNA viruses during the COVID-19 pandemic

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Received: 15 December 2022 / Accepted: 6 January 2023 / Published online: 14 February 2023

Abstract

A methodological approach based on reverse transcription (RT)-multiplex PCR followed by next-generation sequencing (NGS) was implemented to identify multiple respiratory RNA viruses simultaneously. A convenience sampling from respiratory surveillance and SARS-CoV-2 diagnosis in 2020 and 2021 in Montevideo, Uruguay, was analyzed. The results revealed the cocirculation of SARS-CoV-2 with human rhinovirus (hRV) A, B and C, human respiratory syncytial virus (hRSV) B, influenza A virus, and metapneumovirus B1. SARS-CoV-2 coinfections with hRV or hRSV B and influenza A virus coinfections with hRV C were identified in adults and/or children. This methodology combines the benefits of multiplex genomic amplification with the sensitivity and information provided by NGS. An advantage is that additional viral targets can be incorporated, making it a helpful tool to investigate the cocirculation and coinfections of respiratory viruses in pandemic and post-pandemic contexts.

Keywords COVID-19 pandemic · SARS-CoV-2 · human respiratory RNA viruses · multiplex PCR-NGS · viral coinfections

Respiratory viral coinfections have often been described, focusing mainly on viruses that are associated with acute bronchiolitis in children, although their clinical consequences are not always well understood. Among the viral coinfections in children, the most studied viruses in a non-pandemic setting are human respiratory syncytial virus (hRSV), human metapneumovirus (hMPV), influenza A virus, and human parainfluenza virus (hPIV) [1, 2]. Although less is known about human rhinoviruses (hRVs), some studies have shown their importance in coinfections with other pathogens [3, 4]. During the H1N1pdm09 influenza pandemic, the cocirculation pattern of traditional seasonal viruses was altered, and coinfections were usually identified in younger patients, most frequently with hRSV [1, 5]. At the beginning of the coronavirus disease 2019 (COVID-19) pandemic, reports about viral coinfections mainly focused on case reports [6, 7]. Since 2021, there have been additional studies concerning SARS-CoV-2 and other respiratory virus coinfections [8, 9]. In addition, changes in the seasonal cycles of respiratory viral diseases and the impact of the pandemic on the circulation of respiratory viruses have also been examined. However, regional data about the

Handling Editor William G Dundon

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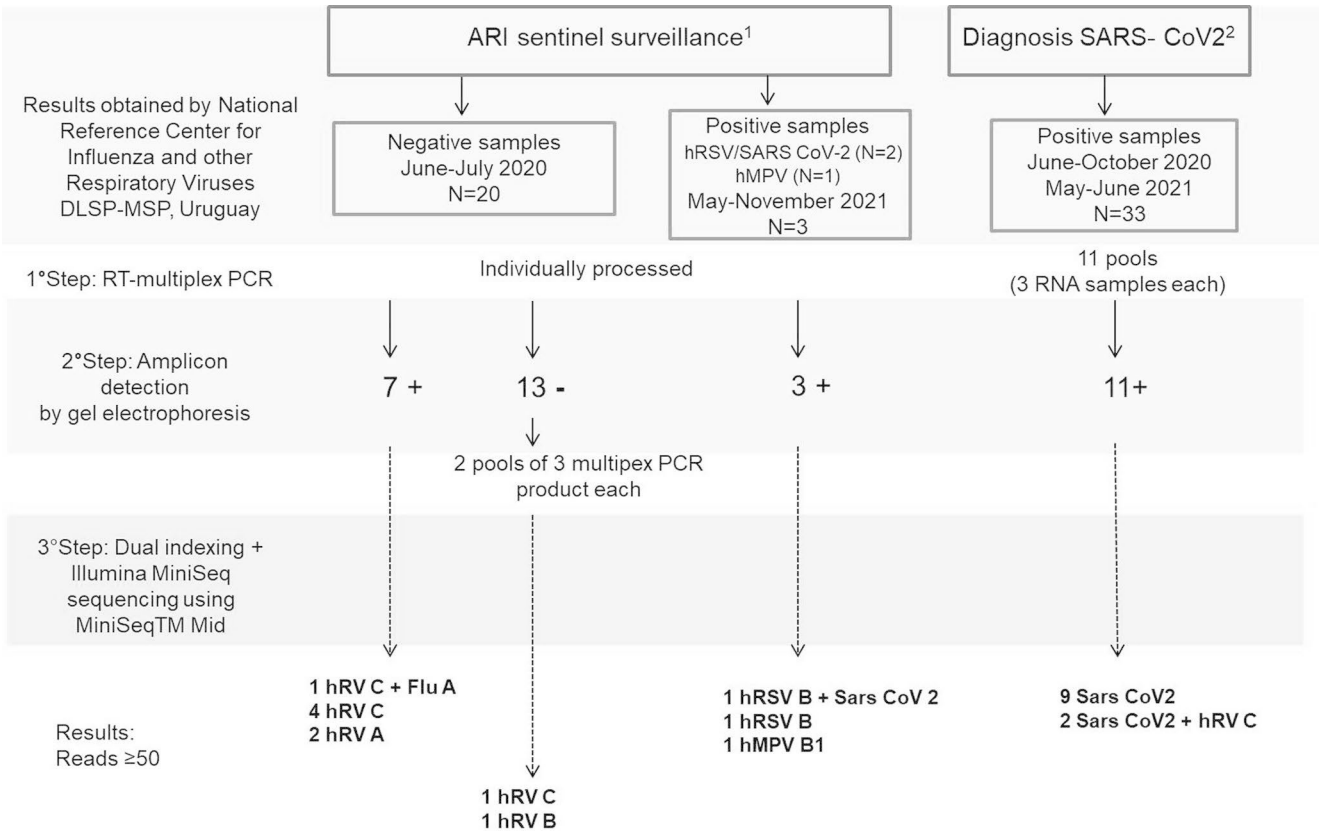

Fig. 1 Schematic diagram of sampling, methodological strategy, and results. A total of 56 samples were included and separated into two groups. Group 1: sentinel surveillance of acute respiratory infections (ARIs) diagnosed by IFI for hRSV, hMPV, hPIV, adenovirus, and influenza A and B viruses. Influenza virus and SARS-CoV-2 were also diagnosed by RT-qPCR. Group 2: diagnosis of SARS-CoV-2 by RT-qPCR. The surveillance/diagnosis results obtained by DLSP, MSP-Uruguay, the collection date, and the number of samples analyzed in this study are shown. The RNA samples were analyzed individually (group 1) or pooled (group 2). The methodological steps performed in this study are shown on the left. Viruses identified by NGS with more than 50 reads are indicated.

circulation of respiratory viruses concurrently with SARS-CoV-2 are still scarce [10–14].

In the present work we employed a methodology based on RT-multiplex PCR followed by NGS, focusing on the identification and genetic characterization of the main RNA respiratory viruses found coinfecting and cocirculating with SARS-CoV-2 during the 2020–2021 COVID-19 pandemic in Uruguay.

This study was based on a convenience sampling of combined nasopharyngeal and oropharyngeal swab samples from sentinel surveillance of acute respiratory infections (ARIs) and SARS-CoV-2 diagnosis (N = 56), which were submitted to the National Reference Center for Influenza and other Respiratory Viruses (DLSP-MSP, Uruguay) during June-October 2020 and May-November 2021. The samples that were selected included (i) surveillance-negative samples, (ii) surveillance-positive samples to compare the results of the virological surveillance and SARS-CoV-2 diagnosis, and (iii) SARS-CoV-2-positive pooled samples to identify potential coinfections (Fig. 1).

Total RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, USA). A methodology based on reverse transcription multiplex polymerase chain reaction (RT-multiplex PCR) followed by next-generation sequencing (NGS) was implemented for simultaneous identification of the main human RNA respiratory viruses: hRV, hMPV, hRSV A and B, influenza A virus, human coronavirus (hCoV) 229E, NL63, and OC43, and SARS-CoV-2. The primers used in this study mainly targeted highly conserved regions of viral genomes and are listed in Supplementary Table S1.

Complementary DNA (cDNA) was obtained by reverse transcription with 10 µl of total RNA, Superscript II® reverse transcriptase, and random primers (Invitrogen, USA). An artificial RNA sample was used as a control that included all RNA viruses at an equimolar concentration using Twist Synthetic RNA control (Twist Bioscience, USA) and RNA extracted from titrated virus stocks.

Multiplex PCR was performed with Platinum™ Taq DNA Polymerase (Invitrogen, USA) and primers with a concentration of 0.25 µM each. Each primer included P5 and P7 Illumina adapters at the 5' end of the forward and

reverse primers, and amplicons of approximately 300 bp were generated. The reaction was carried out under the following conditions: 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 35 s at 72°C. PCR products were stained with SYBR™ Safe DNA Gel Stain (Invitrogen, USA) and visualized on a 1.5% agarose gel using UV light. Amplicons with adapters were purified using AMPure XP beads (Beckman Coulter, USA) and quantified using a Qubit DNA High-Sensitivity Kit (Invitrogen, USA). A total of 100 ng of the amplicons were dual indexed by PCR with eight cycles of 98°C for 45 seconds, 62°C for 30 seconds, and 68°C for 2 minutes, using Q5 High-Fidelity DNA Polymerase (NEB). Sequencing was performed on an Illumina MiniSeq (Illumina, USA) platform using a MiniSeq™ Mid Output Reagent Kit (300 cycles, paired-end reads). Adapter/quality trimming and filtering of raw data were performed with BBDuk, and clean reads were mapped to each reference viral genome using Geneious Prime 2020.1.2 (https://www.geneious.com).

A total of 56 samples were analyzed, either individually or in pools, and were further divided into two groups. Group 1 corresponded to ARI sentinel surveillance (23/56), and group 2 to SARS-CoV-2 diagnosis (33/56). The workflow and the main results obtained are schematized in Fig. 1. The analysis of mean coverage for the artificial control RNA sample showed the detection of all target sequences of the viruses included in the panel. Although the breadth of coverage for each amplicon was 100%, differences in the number of reads obtained for the different viruses and distinct genome regions were observed (Supplementary Table S2).

In group 1, 23 samples diagnosed by sentinel surveillance were processed individually and divided into two subgroups: 20 with negative results for the respiratory panel (hRSV, hMPV, hPIV, adenovirus, influenza A and B viruses) and SARS-CoV-2 and three samples with positive results for hRSV, SARS-CoV-2, or hMPV. For the first subgroup, RT-multiplex PCR amplicons were detected by gel electrophoresis in seven samples (Fig. 1). The NGS of the amplicons allowed the identification of single viruses (hRV A and C, N = 6) and a double coinfection (hRV C and influenza A virus) (Fig. 1, Table 1, and Fig. 2a). From the remaining 13 samples of this subgroup, to further investigate the presence of respiratory viruses in samples with an undetectable signal by gel electrophoresis, we constructed two pools of three multiplex PCR products each to achieve the minimum DNA input recommended for Illumina sequencing (1 ng). Interestingly, reads for hRV C or B were obtained with both pools (Fig. 1, Table 1, Fig. 2a), showing that combining pooling of RT-multiplex PCR products with NGS in the absence of visible amplicon bands may improve the chances of virus detection.

In addition, amplicons were detected in all samples from the second subgroup (Fig. 1). hRSV was identified by NGS in two samples that were positive by indirect immunofluorescence (IFI) using a D3® Ultra 8TM DFA Respiratory Virus Screening & Identification Kit (Diagnostic Hybrids, USA) (Table 1). Analysis of the amplified region (F gene) revealed that the hRSVs detected belonged to group B (Fig. 2b). Both samples were collected in May 2021 from infants with severe acute respiratory infection (SARI) and were also diagnosed as positive for SARS-CoV-2 by real-time reverse transcription polymerase chain reaction (RT-qPCR). However, our assay identified SARS-CoV-2 in one of these samples, confirming the double coinfection in a one-month-old patient (Table 1). In addition, hMPV was detected in a positive sample diagnosed by IFI, and the analysis of the N gene revealed that this sequence clustered with viruses of genotype B1 (Fig. 2c).

Group 2 corresponded to samples submitted to DLSP-MSP for SARS-CoV-2 surveillance, which were tested by RT-qPCR following the protocol of the Pan American Health Organization (PAHO-WHO) [15]. We processed 33 SARS-CoV-2-positive samples, which were combined into 11 pools of three samples each. Fifteen samples were obtained from June to October 2020, and eighteen were obtained from May 21 to June 2, 2021. SARS-CoV-2 was detected in all of the pools, and hRV C was also identified in two pools. This result indicates that a coinfection could be detected in at least one of the pooled samples (Table 1, Figs. 1 and 2a).

The SARS-CoV-2 epidemic in South America evolved in different ways in each country. During the early stages of the pandemic in Uruguay, most cases (about 20 per day) were restricted to outbreaks associated with social events, workplaces, and health or residential care centers [16]. In late 2020, there was a gradual increase in the number of cases, leading to extensive community circulation. In May-June 2021, Uruguay reached the highest number of daily cases so far (about 3000 new infections per day) [17]. In this context, we investigated coinfections and cocirculation of the main respiratory RNA viruses.

The tool used in this study was helpful for identifying coinfections in samples from patients with SARI for whom the etiological agent had not been identified. An influenza A virus/hRV C coinfection was identified in an 89-year-old patient. This sample was submitted to DLSP-MSP in July 2020 for ARI surveillance and tested negative using a respiratory IFI panel and a protocol for influenza virus and SARS-CoV-2 qPCR. Notably, using our methodological approach, influenza A virus was identified in this sample, although the number of reads obtained for influenza virus was low compared to the number of reads for hRV C (Table 1). This result could be explained by differences in

Table 1 Viruses identified in individual samples or pools of samples from groups 1 and 2 analyzed by RT-multiplex PCR + NGS

Group1 Sample no.	Laboratory no.	Viruses identified	No. of reads obtained	Genome region	GenBank accession no. of consensus sequence	Available information
1	3635	hRV C Influenza A	94,354 198	5'UTR M	OP204100 OP287072	SARI Male 89 years old July 2020
2	3627	hRV A	127,135	5'UTR	OP204108	ILI Female 72 years old July 2020
3	3734	hRV C	152,489	5'UTR	OP204099	SARI Male 1 month old July 2020
4	3344	hRV C	34,748	5'UTR	OP204098	ILI Female 1 month old July 2020
5	3624	hRVA	50,091	5'UTR	OP204107	ILI Male 31 years old July 2020
6	3628	hRV C	19,085	5'UTR	OP204102	ILI Female 2 years old July 2020
7	3631	hRV C	234	5'UTR	OP204101	ILI Female 7 years old July 2020
8	4728	hRSV B SARS-CoV-2	35,824 25,552 424	F L RdRp	OP270256 OP292978 OP271792	SARI 1 month old IFI + hRSV SARS-CoV-2 + Ct27 (N gene) May 2021
9	4312	hRSV B	28,643 19,456	F L	OP270255 OP292977	SARI 3 months old IFI + hRSV SARS-CoV-2 + Ct39 (N gene) May 2021
10	6501	hMPV	71940	N	OP204109	SARI Female under five years old IFI + hMPV November 2021
Group 1 Multiplex PCR pool no.	Laboratory no.	Viruses identified	No. of reads obtained	Genome region	GenBank accession no. of consensus sequence	Available information
1	3737 3682 3341	hRV C	918	5'UTR	OP204103	ILI Female 38, Male 47 and Female 42 years old July 2020
2	3252 3739 3343	hRV B	310	5'UTR	OP204106	ILI Female 45, Female 47 and Female 30 years old June and July 2020
Group 2 RNA Pool no.	Laboratory no.	Viruses identified	No. of reads obtained	Genome region	GenBank accession no. consensus sequence	Available information
1	301 295 298	SARS-CoV-2	131,522	RdRp	OP271833	Adults July 2020
2	299 300 297	SARS-CoV-2	268,901	RdRp	OP271834	Adults July 2020

Table 1 (continued)

Group1 Sample no.	Laboratory no.	Viruses identified	No. of reads obtained	Genome region	GenBank accession no. of consensus sequence	Available information
3	3220 4674 433	SARS-CoV-2	48,370	RdRp	OP271835	Adults June, July and October 2020
4	356 926 274	SARS-CoV-2 hRV C	215,114 1,018	RdRp 5'UTR	OP271836 OP204104	Adults July and September 2020
5	547 589 590	SARS-CoV-2	195,155	RdRp	OP271837	Children and Adults August 2020
6	4113 4110 3960	SARS-CoV-2	170,584	RdRp	OP271838	Adults May and June 2021
7	3985 3831 3566	SARS-CoV-2	191,675	RdRp	OP271839	Children May and June 2021
8	3996 4176 4077	SARS-CoV-2	6,597	RdRp	OP271840	Children May and June 2021
9	4184 4217 4171	SARS-CoV-2	132,692	RdRp	OP271841	Children May and June 2021
10	4100 3986 3971	SARS-CoV-2 hRV C	58,772 18,225	RdRp 5'UTR	OP271842 OP204105	Children May and June 2021
11	3943 3637 3620	SARS-CoV-2	115,214	RdRp	OP271843	Children May and June 2021

ILI, influenza-like illness; SARI, severe acute respiratory infection

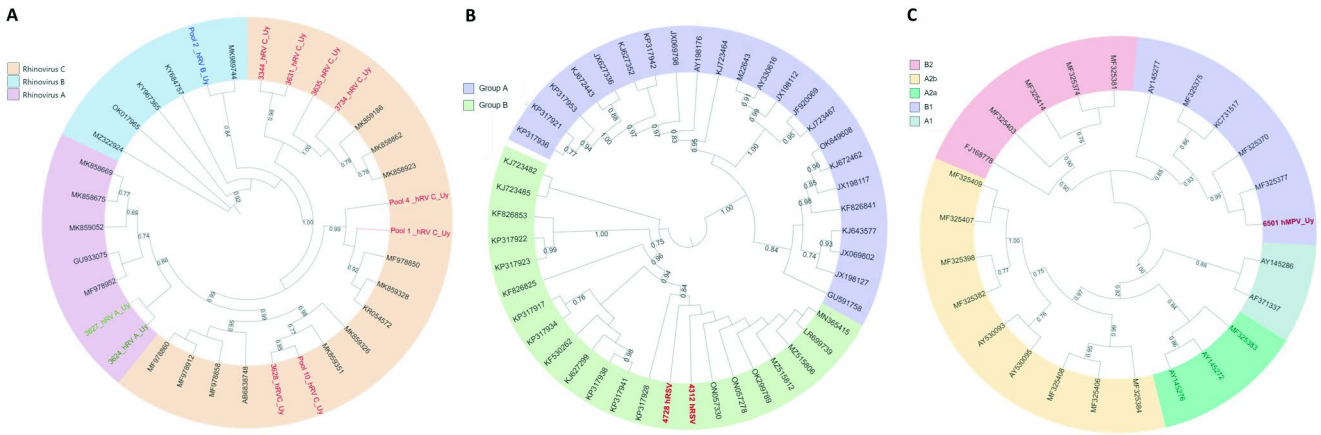

Fig. 2 Maximum-likelihood phylogenetic trees. (a) hRV, 5´UTR; (b) hRSV, F gene; (c) hMPV, N gene. Alignments were done using the ClustalW program available in BioEdit v 7.1.3.0. For comparison, reference sequences for each group of viruses were included (Supplementary Table S4). The best nucleotide substitution model for each virus and the phylogenies were inferred using the PhyML v3.0 online software. Trees were rooted at the midpoint. Statistical support of the nodes was estimated by the approximate likelihood ratio test (aLRT). Tree visualization and editing were done using iTOL v6 (https://itol.embl.de).

the target sequences and/or the sensitivity of the assays used. Sequences from more regions would be needed to obtain additional information about the genetic characteristics of the influenza viruses identified. This could be achieved by using primers that target regions of the hemagglutinin gene that would provide information about the strain or subtype. A SARS-CoV-2/hRSV B coinfection was also identified, but in a sample from an infant that was submitted to DLSP-MSP

in May 2021. This sample and another from the same period were previously diagnosed by ARI surveillance as SARS-CoV-2/hRSV B coinfections. The other coinfection could not be confirmed by our methodology, probably due to the low SARS-CoV-2 viral load (as indicated by a C_t value of 39 when tested by SARS-CoV-2 RT-qPCR) (Table 1). Our methodology also allowed further characterization of the hRSV detected as belonging to group B, providing a reliable tool for the genetic surveillance of the most prevalent viral pathogen in childhood. Yeoh et al. reported that a decrease in the detection of hRSV and influenza virus in children, around 98.0% and 99.4%, respectively, occurred in western Australia in the winter of 2020, despite the reopening of schools [18]. Conversely, Ujiie et al. reported a resurgence of hRSV infections in children in the middle of 2021, with an unusually high number of cases in Tokyo [9]. A similar scenario was observed in Uruguay according to the national surveillance carried out by the National Reference Centre for Influenza and other Respiratory Viruses, DLSP-MSP. In 2020, 852 samples from cases of influenza-like illness (ILI) or SARI were submitted to the laboratory. Among these, only five influenza A, one influenza B, and two hRSV cases were identified. By contrast, in 2021, from 644 ILI and SARI samples submitted, 99 hRSV and two influenza A cases were diagnosed (Supplementary Table S3).

Regarding SARS-CoV-2 coinfections with hRV C in adults and children, the data from 2020 and 2021 (Table 1) show that the use of pools of SARS-CoV-2-positive samples proved to be a good strategy for identifying coinfections with other viral agents in a pandemic context. According to recent reports, hRV, influenza A virus, hRSV and seasonal coronaviruses are the most common viruses found in coinfections with SARS-CoV-2, with percentages between 1 and 30%. However, the impact of coinfections on clinical outcomes is still unclear and requires further studies [19–21].

The results obtained with samples from 2020 and 2021 highlight the circulation of hRVs, mainly of type C, in adults and children (Table 1, Fig. 2a). This result agrees with reports from other countries [14, 21, 22]. It is important to emphasize that the primers used in this study for hRV detection have broad specificity, identifying members of the genus *Enterovirus* [23]. The subsequent analysis of the sequences obtained by NGS allowed us to differentiate hRV from enteroviruses. Our results represent the first molecular detection of hRV in Uruguay, and based on 5'UTR sequences, the circulation of members of the three species A, B, and C could be established (Fig. 2a). Since the 5'UTR is one of the hotspots of hRV recombination, principally between members of species A and C, other variable targets such as the capsid region, should be included in the panel to expand the genetic characterization of these viruses [24]. hRVs have not been routinely included in respiratory virus surveillance, and the implementation of our methodology may be useful for strengthening sentinel surveillance.

Although the small number of samples analyzed in our study did not allow epidemiological conclusions to be drawn, our convenience sampling, together with the novel methodological approach, was useful for confirming the virological surveillance findings and providing information on the concurrent circulation of SARS-CoV-2 and respiratory RNA viruses. Therefore, this study is a starting point for a deeper understanding of these viruses in our country, including their incidence, association with severe disease, and genetic variability. In the same way, although seasonal coronaviruses were not identified in this study, this tool is expected to be useful for their molecular surveillance.

The approach implemented in this study supplements the traditional techniques for ARI surveillance, allowing a large number of samples (individual or pooled) to be analyzed in parallel in a cost-effective way with no need for library preparation kits. This methodology has the advantage of simultaneously providing genomic information on the viruses identified (subtype, genotype), together with the potential detection of coinfections without the use of multiple fluorescence-labelled probes as used in a multiplex qPCR. It is an ideal tool for improving the surveillance of respiratory viruses, since it has the versatility of easily incorporating additional viral targets and/or new viral pathogens.

In conclusion, the RT-multiplex PCR-NGS approach proved to be helpful for investigating coinfections and cocirculation of respiratory viruses during the first two years of the COVID-19 pandemic in Uruguay. Notably, the methodology allowed us to simultaneously identify the main RNA respiratory viruses and genetically characterize the viruses detected. The findings presented here contribute to our knowledge about coinfections with respiratory RNA viruses in adults and infants. Furthermore, the results revealed the cocirculation of viruses such as hRVs that were not routinely included in sentinel diagnosis during the pandemic period. Our research serves as a basis for a planned epidemiological study, providing a useful tool to improve preventive measures in future epidemics.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-023-05717-6.

Author Contribution All authors revised and approved the manuscript. NR, YP, SF, AD, RP, NG, and JA conceived the study. NR, YP, F, LC, and AD did the experiments. GT, NR, and RP analyzed the data. AM, CT, SG, and EF participated in protocol optimization; NG, VR, LC, NM, and HC carried out diagnostic tests. JA, CM, and RP obtained financial support. NR, SF, AD, RP, YP, LC, NG, and HC wrote and revised the manuscript.

Funding Facultad de Ciencias and Comisión Sectorial de Investigación Científica (CSIC), Fundación Manuel Pérez, UdelaR

Data Availability Raw Illumina MiniSeq sequence reads were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject PRJNA815970. Assembled sequences were deposited in the GenBank database with the following accession numbers: hRV, OP204098-108; hRSV, OP270255-56 and OP292977-78; hMPV, OP204109; influenza A virus, OP287072; SARS-CoV-2, OP271792 and OP271833-OP271843.

Declarations

Conflict of Interest The authors declare no conflict of interest.

Ethics statement The "Centro Nacional de Referencia de Influenza y otros Virus Respiratorios, Departamento de Laboratorios de Salud Pública. Ministerio de Salud Pública", where the samples were diagnosed, belong to the WHO Global Influenza Surveillance and Response System (GISRS) for global surveillance of influenza and other respiratory viruses. Neither written informed consent nor explicit ethical approval were sought, as this study was only observational and carried out as part of routine virologic surveillance (anonymously, without identification of patients).

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