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Used paper tissues for pathogen identification in acute respiratory infection

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Abstract

During the Belgian winter and spring season 2022-2023, we investigated the potential of used paper tissue (UPT) as a non-invasive sampling method for the diagnosis of acute respiratory infections. Screening for respiratory pathogens was done using an in-house developed respiratory panel for simultaneous detection of 22 respiratory viruses and 7 non-viral pathogens. The method allowed the identification and typing of respiratory pathogens in symptomatic individuals, as well as in collective samples taken at a community level. Pathogens that were identified in nasal swabs could also be detected in concurrent UPT from the same patient. In all cases that tested positive on an antigen-detection rapid diagnostic test, the corresponding virus could be detected in UPT. The collection of UPT could be useful in epidemiological surveillance of SARS-CoV-2 and other coronaviruses, as well as other respiratory pathogens such as influenzavirus, respiratory syncytial virus, entero/rhinoviruses including EV D68, parainfluenzaviruses and *Streptococcus pneumoniae*. Multiple respiratory pathogens could be detected in UPTs of collectivities, confirming its applicability for community testing. This is especially interesting for screening in nursing homes, centers for the disabled, schools or other settings were taking nasal or nasopharyngeal samples is cumbersome.

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Abstract

During the Belgian winter and spring season 2022-2023, we investigated the potential of used paper tissue (UPT) as a non-invasive sampling method for the diagnosis of acute respiratory infections. Screening for respiratory pathogens was done using an in-house developed respiratory panel for simultaneous detection of 22 respiratory viruses and 7 non-viral pathogens. The method allowed the identification and typing of respiratory pathogens in symptomatic individuals, as well as in collective samples taken at a community level. Pathogens that were identified

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in nasal swabs could also be detected in concurrent UPT from the same patient. In all cases that tested positive on an antigen-detection rapid diagnostic test, the corresponding virus could be detected in UPT. The collection of UPT could be useful in epidemiological surveillance of SARS-CoV-2 and other coronaviruses, as well as other respiratory pathogens such as influenzavirus, respiratory syncytial virus, entero/rhinoviruses including EV D68, parainfluenzaviruses and *Streptococcus pneumoniae*. Multiple respiratory pathogens could be detected in UPTs of collectivities, confirming its applicability for community testing. This is especially interesting for screening in nursing homes, centers for the disabled, schools or other settings were taking nasal or nasopharyngeal samples is cumbersome.

Keywords:

used paper tissue (UPT); acute respiratory infection (ARI); respiratory pathogens; SARS-CoV-2; non-invasive sampling method

Introduction

Acute respiratory tract infections (ARTIs), including pneumonia, constitute a major disease burden worldwide, especially in young children and the elderly [1][2]. Diagnostic testing for respiratory pathogens is usually performed on samples collected by invasive methods, such as nasopharyngeal swabs, nasopharyngeal aspirates or bronchoalveolar lavages, obtained in hospital or medical practice settings. For some respiratory viruses such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), influenzavirus and respiratory syncytial virus (RSV), fast diagnosis using antigen-detection rapid diagnostic test (Ag-RDTs) can be performed on self-collected nasal swabs. Although these nasal swabs are less invasive than nasopharyngeal swabs, they can still cause some discomfort, which can pose a barrier to testing [3]. Furthermore, since some of the most vulnerable populations for ARTI outbreaks, such as residents of long-term care facilities for the elderly or mentally impaired, or infants and toddlers attending day-nurseries, are difficult to sample using these invasive methods, there is a need for less to non-invasive methods for respiratory sampling. We assessed whether paper tissues, used for nose blowing, can be used for the identification of respiratory pathogens, on an individual as well as on a community level.

materials and methods

Used paper tissues (UPT) were self-collected by individuals with clinical symptoms indicative of ARTI, including rhinorrhea, sneezing, coughing, fever, hoarseness, sore throat, loss of smell, and shortness of breath, and who had nasal discharge which could be collected by blowing or wiping the nose with a facial tissue. Collective UPT samples were gathered anonymously in a childcare facility, 3 kindergartens and 2 primary schools. Tissues that were used in a classroom or childcare group for nose blowing or wiping during the course of one day were pooled in a plastic container and delivered to the lab. UPT were stored at room temperature prior to analysis. For retrieval of respiratory pathogens from the UPT, tissues were transferred with sterile tweezers into a 100 mL disposable syringe (˜4 tissues per individual or the maximal amount of tissues that could be fitted into the syringe in case of the pooled samples). Phosphate buffered saline (PBS) was added until tissues were soaked (˜25 mL). After incubation at room temperature for 5 minutes, the plunger of the syringe was pressed to recover the eluate (˜ 10 mL) into a 15 mL Falcon tube.

Nasal swabs were self-collected in parallel with UPT by 20 individuals with symptoms of ARTI. After collection, the nasal swabs were inserted in 1 mL Universal Transport Medium (UTM) (Copan) and stored at 4°C until extraction. Nasal swabs in UTM were rigorously vortexed prior to extraction.

Nucleic acids were extracted using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit on Kingfisher Flex-96 (ThermoFisher Scientific, Europe), using 400 µL of eluate of UPT or of nasal swab UTM.

Screening for respiratory pathogens was done using the in-house developed respiratory panel (RP) of the University Hospitals Leuven for simultaneous detection of 22 respiratory viruses (influenza A, influenza B, RSV, human metapneumovirus , parainfluenzavirus (PIV) -1 to -4, adenovirus , human bocavirus , enterovirus/rhinovirus (EV/RV), EV D-68 , human parechovirus, human coronavirus (HCoV)-NL63, -229E,

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-OC43, -HKU-1, -SARS and - MERS, cytomegalovirus (CMV), herpes simplex virus (HSV) -1 and -2) and 7 non-viral pathogens (*Mycoplasma pneumoniae* ,*Coxiella burnetii* , *Chlamydia pneumoniae* , *Chlamydia psittaci* , *Streptococcus pneumoniae* , *Legionella pneumophila* and *Pneumocystis jirovecii*), as described previously [4]. This RP was developed for testing of clinical samples of hospitalized patients in specific clinical indications (lower respiratory tract infections in immunocompromised patients or in immunocompetent patients requiring intensive care admission or not responding to initial therapy), and therefore includes HSV-1, HSV-2 and CMV which are not typical respiratory pathogens but can be the cause of severe pneumonitis in immunocompromised patients. Since the current study was not focused on immunocompromised populations, these parameters were not included in our analysis. An additional SARS-CoV-2 specific RT-qPCR was carried out on samples positive for HCoV-SARS using the 2019-nCoV CDC EUA kit N1 primer probe set [5].

Typing of respiratory pathogens from UPT based on partial sequencing was done as described previously for EV/RV [6], SARS-CoV-2 [7], and influenza [8][9]. Complete genome sequencing of SARS-CoV-2 on RNA extracted from UPT was done with the nanopore technique using the ARTIC protocol as described in Wawina-Bokalanga et al. [10].

Ag-RDTs were carried out as described in the test instruction manual.

Results

As a proof of concept, used UPT of 9 individuals with symptoms of ARTI were investigated for the presence of respiratory pathogens. In 2 cases, EV/RV was detected, which could be further typed as RV C in one case and as co-infection of RV B and coxsackievirus A19 in the other case. Three samples tested positive for RSV, all three with concomitant detection of an additional pathogen (HCoV-OC43, adenovirus and *Streptococcus pneumoniae* respectively). In one sample, HCoV-OC43 was detected in combination with *Streptococcus pneumoniae* . One sample was positive for PIV-4 and one sample tested positive for SARS-CoV-2 (Cq 28.1), further typed by complete genome sequencing as variant BA.5.2.1 with a genome coverage of 99.3%. In one sample, no respiratory pathogens were detected.

Combined UPTs from 6 collectivities (one childcare center, 3 kindergartens and 2 primary schools) were collected by anonymously gathering tissues used in a classroom or childcare group over the course of one day. The presence of multiple respiratory pathogens was detected in these combined samples (Table 1).

Table 1. Identification of respiratory pathogens in combined used paper tissues from collectivities (n = 6 samples)

Collectivity	**Respiratory pathogen**	**Cq**
Childcare center	Bocavirus	33.4
	Enterovirus / rhinovirus	28.2
	Respiratory syncytial virus	27.9
	Streptococcus pneumoniae	34.5
Kindergarten A	Enterovirus / rhinovirus	26.6
	Streptococcus pneumonia	23.6
Kindergarten B	Adenovirus	33.0
	Enterovirus / rhinovirus	25.0
	Cytomegalovirus	34.7
	Streptococcus pneumoniae	23.2
Kindergarten C	Adenovirus	29.3
	Enterovirus / rhinovirus	31.3
	Cytomegalovirus	33.5
	Streptococcus pneumoniae	24.4
Primary school A	Respiratory syncytial virus	33.0
	Enterovirus / rhinovirus	26.7

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Collectivity	Respiratory pathogen	Cq
	Enterovirus D68	30.4
Primary school B	Human coronavirus OC43	32.2
	Streptococcus pneumoniae	26.2

To further investigate the potential of UPT in comparison to standard diagnostic sampling, UPTs of 20 patients with symptoms of ARTI were analyzed in parallel with self-collected nasal swabs in UTM. Sixteen of these patients performed an Ag-RDT on the same day. Results are listed in Table 2. Pathogens that were detected in the nasal swabs were also detected in the corresponding UPT, with the exception of one case where EV/RV was detected only in the nasal swab (Cq 36.6) of a patient who also tested positive for influenza B in both nasal swab (Cq 19.3) and UPT (Cq 26.6) (RP015). The Cq measured in the UPT was usually higher than in the nasal swab, although in some cases it was the other way round (Fig. 1). In all cases where the Ag-RDT tested positive, the corresponding pathogen was also detectable in the UPT. In 4 cases, both the UPT and the nasal swab tested negative with our respiratory panel.

Table 2. Identification of respiratory pathogens in used paper tissues versus nasal swabs and antigen tests (n = 20 samples).

PATIENT ID	UPT (Cq)[a]	Nasal swab (Cq)[b]	Ag-RDT[c]
RP001	SARS-CoV-2 (19.8)	SARS-CoV-2 (15.5)	FLU A: NEG[1] FLU
RP002	SARS-CoV-2 (16.8)	SARS-CoV-2 (11.7)	FLU A: NEG[2] FLU
RP003	SARS-CoV-2 (21.5)	SARS-CoV-2 (19.6)	SARS-CoV-2: POS
RP004	NEG	NEG	ND
RP005	SARS-CoV-2 (31.9)	SARS-CoV-2 (25.6)	FLU A: NEG[2] FLU
RP006	EV/RV (23.9)	EV/RV (19.5)	SARS-CoV-2: NEG
RP007	NEG	NEG	SARS-CoV-2: NEG
RP008	Influenza A (30.8)	Influenza A (31.1)	FLU A: POS[1] FLU
RP009	EV/RV (37.4) SARS-CoV-2 (21.0)	EV/RV (35.4) SARS-CoV-2 (38.6)	FLU A: NEG[1] FLU
RP010	Influenza B (27.6)	Influenza B (22.0)	FLU A: NEG[5] FLU
RP011	EV/RV (20.74)	EV/RV (22.64)	FLU A: NEG[5] FLU
RP012	Influenza A (21.5)	Influenza A (33.8)	FLU A: POS[1] FLU
RP013	Influenza B (30.4) *S. pneumoniae* (28.2)	Influenza B (22.2) *S. pneumoniae* (19.5)	FLU A: NEG[5] FLU
RP014	NEG	NEG	FLU A: NEG[2] FLU
RP015	Influenza B (26.6)	EV/RV (36.6) Influenza B (19.3)	FLU A: NEG[1] FLU
RP016	EV/RV (38.2)	EV/RV (36.9)	FLU A: NEG[1] FLU
RP017	HCoV-229E (19.6)	HCoV-229E (14.2)	ND
RP018	PIV-3 (23.6)	PIV-3 (24.1)	ND
RP019	PIV-3 (22.0)	PIV-3 (19.6)	FLU A: NEG[1] FLU
RP020	NEG	NEG	ND

Ag-RDT: antigen rapid diagnostic test; COVID-19: coronavirus disease; EV/RV: enterovirus/rhinovirus; HCoV-229E: human coronavirus 229E; HSV: herpes simplex virus; FLU: influenza; NA: not applicable; ND: not done; NEG: negative; PIV-3: parainfluenzavirus type 3; POS: positive; RV-C: rhinovirus C; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; UPT: used paper tissue.

[a] Respiratory pathogens detected in UPT by RP and SARS-CoV-2 N1 RT-qPCR, with Cq values.

[b] Respiratory pathogens detected in nasal swab by respiratory panel and SARS-CoV-2 N1 RT-qPCR, with Cq values.

[c] Lateral flow Ag-RDT test result per respiratory pathogen, with pathogen names as indicated on the test. Ag-RDT brands that were used are indicated with numbers behind the test results.

[d] Typing performed on UPT based on partial sequencing.

[1] Influenza & COVID-19 Ag Combo Rapid Test (Orientgene)

[2] SARS CoV-2 and Influenza A+B Antigen Combo Rapid Test (All test)

[3] CerTest SARS-CoV-2 Ag-Nasal Sample-Self Test (Certest Biotech)

[4] SARS-CoV-2 Antigen Rapid Test (Flowflex)

[5] SARS-CoV-2 & Influenza A/B & RSV Antigen Combo Test Kit (Fluorecare)

* viral load too low for typing

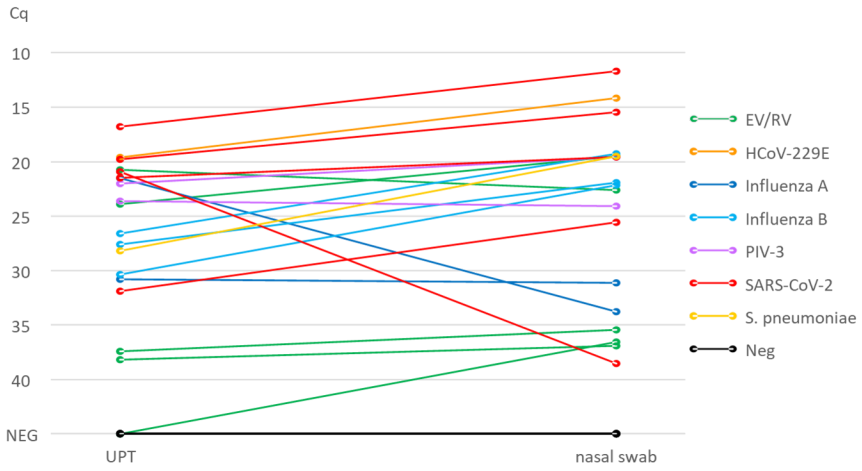

Figure 1. Respiratory pathogen load in UPT and in corresponding nasal swab, as captured by Cq values measured with the respiratory panel (for EV/RV, HCoV-229E, Influenza A, Influenza B, Streptococcus pneumoniae and PIV-3) or with the SARS-CoV-2 RT-PCR (for SARS-CoV-2). Cq values for each measurement are plotted as dots, and corresponding measurements in the same patient are connected with lines. Different respiratory pathogens are identified with colours as shown in the legend.

To assess the effect of prolonged storage of the UPT on room temperature, a large number of UPTs were collected from an EV/RV positive patient within the same day (aiming to obtain equal viral loads in all UPTs) and stored in a paper box at room temperature. Extraction and testing of a standardized UPT aliquot was performed after 1, 2, 3 , 4, 6 and 8 weeks. EV/RV Cq values were 15.9 (wk 1), 19.5 (wk 2), 16.3 (wk 3), 22.5 (wk 4), 25.2 (wk 6) and 21.2 (wk 8), showing that although Cq values fluctuated the pathogen was readily detectable in UPTs up to 2 months after sample collection.

One patient was followed over the course of a COVID-19 infection, from the time of first symptom up to complete symptom resolution, with UPT, nasal swabs in UTM and Ag-RDTs being collected daily (Fig 2). SARS-CoV-2 was detectable in UPT as of the start of symptoms, whereas the Ag-RDT only turned positive on Day 4. For as long as the Ag-RDT remained positive, SARS-CoV-2 was also detectable in UPT. In the nasal swab, SARS-CoV-2 was still detectable after Ag-RDTs turned negative, and remained detectable up to the last symptomatic day.

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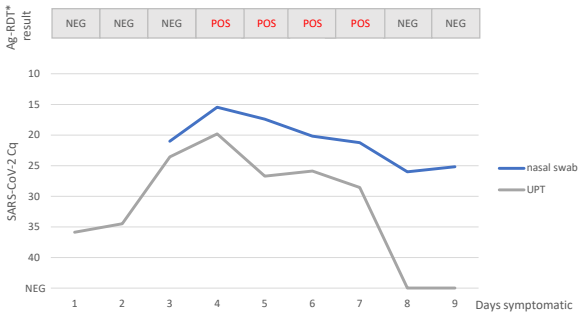

Figure 2. Evolution of SARS-CoV-2 load over the course of an infection, as captured by Cq values in UPT and nasal swab, and Ag-RDT test result.* Influenza & COVID-19 Ag Combo Rapid Test (Orientgene)

Discussion

Our method for detection of respiratory pathogens from UPT samples allowed the identification of respiratory pathogens responsible for ARTI, with adequate sample quality to allow further genetic characterization. We were able to detect respiratory viruses as well as bacteria, and a putative causative pathogen could be identified in used paper tissues of all symptomatic patients who had a positive nasal swab. All pathogens that were detected in nasal swabs were also found in concurrent UPTs from the same patient, except for one EV/RV co-infection in an influenza B positive sample. In only four of the symptomatic individuals no pathogen could be detected in UPT nor in the corresponding nasal swab. Although reported positivity rates in ARTI patients are highly variable, depending on the range of viruses and bacteria tested, the epidemiological situation and the population under investigation, our positivity rate of 80% is comparable to other studies in which both viral and bacterial respiratory pathogens were tested in nasal or nasopharyngeal swabs [11], [12]. Negative samples could be due to sampling being performed too early or too late in the course of the infection (when pathogens load is below the detection limit), or symptoms originating from other microbial infections or non-microbial causes, such as allergies.

Bacterial pathogens have already been shown to be reliably detectable from paper tissues of patients with upper respiratory tract infections [13]. In a recent study, Lagathu et al. were able to identify multiple respiratory viruses in pooled facial tissues obtained in communities of children. They compared SARS-CoV-2 Cq values between nasopharyngeal swabs and facial tissues of individual COVID-19 patients and found a higher signal from the tissues in 11 out of in 15 cases [14]. In our study, we compared Cq values for SARS-CoV-2 but also for other common respiratory pathogens such as EV/RV, influenzaviruses and *S. pneumoniae* , obtained from 20 UPT and nasal swabs, and found a high variety in Cq difference between both sample types. We also were able to detect the presence of multiple respiratory pathogens in pooled UPT samples of collectivities, confirming its applicability for community testing. This would especially be useful in schools and preschool daycare centers, since taking nasal samples from (young) children is an invasive method and

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requires training, or in elderly homes and homes for disabled people, in whom taking nasal samples is less well tolerated. Because sequencing a complete genome is possible from UPT this method can also be applied for epidemiological surveillance. We demonstrated that UPTs can be stored at room temperature for up to 8 weeks prior to analysis. This implies that UPT samples can be transported to diagnostic laboratories at low cost, even from distant locations. We did measure fluctuations in viral load between samples analyzed at different timepoints, which we hypothesize to be the result of the non-homogenous nature of the sample rather than a decline in sample quality.

Since our sample contains eluted material from entire paper tissues, the pathogen load in the sample is not only dependent on the amount of virus shedding but also on the amount of nasal discharge collected in the tissue. This makes the method less suited for (semi-) quantitative analyses. It also implies that the method cannot be used when there is very little to no nasal discharge, or when nasal discharge is difficult to collect by nose blowing or wiping with a tissue.

We were able to detect the corresponding virus in UPT of all Ag-RDT positive cases, indicating that UPTs are sufficiently sensitive to detect individuals with high virus shedding, who are most likely to be infectious. As such, UPT could provide an interesting non-invasive sampling method for screening of individuals. In the patient that was followed over the course of a COVID infection, UPT tested positive as of the start of symptoms, whereas Ag-RDTs turned positive only on day 4. This is in accordance with the notion that SARS-CoV-2 viral loads in persons with pre-existing immunity (by previous infection or by vaccination) may only rise to Ag-RDT detectable levels after several days of symptoms. Although only based on a single observation, UPT testing seems to be sensitive enough to allow detection as of the start of infection, reducing the amount of false negative test results.

Since pathogen detection was possible from combined UPTs obtained in collectivities, it can also provide a good alternative to sampling of sewage water of buildings or aircraft wastewater to obtain a community sample for pathogen screening. This would be very useful to complement the current strategy of wastewater testing of incoming aircraft for SARS-CoV-2 variant screening [15], [16].

Ethics statement

Informed consent, approved by the UZ Leuven Ethics Committee, was obtained from all individuals providing self-collected swab samples.

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Conflict of interest

The authors declare no conflict of interest.

author contributions

Annabel Rector: Conception and design of the study, data collection, data analysis and interpretation, writing of the manuscript, final approval of the version to be published.

Mandy Bloemen: Conception and design of the study, data collection, data analysis and interpretation, final approval of the version to be published.

Marc Van Ranst: Conception and design of the study, final approval of the version to be published.

Elke Wollants: Conception and design of the study, data collection, data analysis and interpretation, final approval of the version to be published.

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