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## Comparison of four nasal sampling methods for the detection of viral pathogens by RT-PCR—A GA<sup>2</sup>LEN project<sup>☆</sup>

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The aim of this study was to compare the efficacy and patient discomfort between four techniques for obtaining nasal secretions. Nasal secretions from 58 patients with symptoms of a common cold, from three clinical centers (Amsterdam, Lodz, Oslo), were obtained by four different methods: swab, aspirate, brush, and wash. In each patient all four sampling procedures were performed and patient discomfort was evaluated by a visual discomfort scale (scale 1–5) after each procedure. Single pathogen RT-PCRs for Rhinovirus (RV), Influenza virus and Adenovirus, and multiplex real-time PCR for RV, Enterovirus, Influenza virus, Adenovirus, Respiratory Syncytial Virus (RSV), Parainfluenza virus, Coronavirus, Metapneumovirus, Bocavirus and Parechovirus were performed in all samples. A specific viral cause of respiratory tract infection was determined in 48 patients (83%). In these, the detection rate for any virus was 88% (wash), 79% (aspirate), 77% (swab) and 74% (brush). The degree of discomfort reported was 2.54 for swabs, 2.63 for washes, 2.68 for aspirates and 3.61 for brushings. Nasal washes yielded the highest rate of viral detection without excessive patient discomfort. In contrast, nasal brushes produced the lowest detection rates and demonstrated the highest level of discomfort.

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### 1. Introduction

Upper respiratory tract infections are the most common cause of acute physical illness in the developed world and the observation that they are followed by acute asthma exacerbations has been known for a long period of time (Lambert and Stern, 1972; Sluder, 1919). However, it was only with the advent of reverse transcription polymerase chain reaction (RT-PCR) detection methods that it was confirmed that the presence of Rhinovirus (RV) and other respiratory viruses may be associated with 80–85% of asthma exacerbations in children, and more than 50% in adults (Johnston, 1995; Nicholson et al., 1993). It is still unknown whether the remaining 15–50% cases are exclusively due to non-viral factors, or that there are still methodological issues in viral detection (Papadopoulos

et al., 2003). Furthermore, upper respiratory tract infections in childhood are associated with complications such as otitis media (Chantzi et al., 2006), sinusitis (Pitkaranta et al., 1997), pneumonia (Tsolia et al., 2004) and acute bronchiolitis (Papadopoulos et al., 2002; Xepapadaki et al., 2004). Confirmation of a viral aetiology for respiratory infections is important both for clinical diagnosis as antiviral treatments are becoming available, and for studying respiratory viruses and their interaction with the respiratory tract (Hayden, 2004). Successful detection of a respiratory virus depends on many variables, including sampling for nasal secretions, which may considerably influence the detection rates (Ahluwalia et al., 1987; Barnes et al., 1989; Covalciuc et al., 1999; Frayha et al., 1989; Heikkinen et al., 2001, 2002; Xiang et al., 2002). Several recent studies have attempted to compare different nasal sampling methods (usually no more than two), using mainly detection methods other than PCR, without reaching a clear conclusion (Ahluwalia et al., 1987; Barnes et al., 1989; Covalciuc et al., 1999; Frayha et al., 1989; Heikkinen et al., 2001, 2002; Xiang et al., 2002). Furthermore, nasal sampling can be unpleasant, reducing cooperation especially

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in epidemiological studies that require repeated sampling; this aspect has not been studied before. We hypothesized that there might be significant differences between sampling methods in both virus detection rates and patient acceptance.

The present study aimed to compare the efficacy and degrees of patient discomfort of four different techniques for obtaining nasal secretions, for the determination of respiratory viruses by RT-PCR.

## 2. Materials and methods

### 2.1. Patients

This was a prospective multicenter study that took place in Amsterdam, the Netherlands (“center A”), Oslo, Norway (“center B”) and Lodz, Poland (“center C”). Participation in the study was offered to patients with recent ( $\leq 3$  days) symptoms of a common cold, confirmed by physician diagnosis. The study size had been calculated, based upon a power of 0.8, a significance level of 0.05, and an expected differential detection between 70% and 90% in paired samples, to be 53 patients. In total, 58 patients (60% female, age range 7–89 years, median 35 years, mean 39.2 years) were enrolled in the study, after obtaining informed consent. Centers A, B and C recruited 20, 18 and 20 patients, respectively. The study design was approved by the local Ethics Committees of the relevant Institutions. Demographic characteristics were assessed with the use of a standardized questionnaire.

### 2.2. Sampling methods

Four samples were obtained from the upper respiratory tract of each patient, in the following order: (i) nasal swab, (ii) nasal aspirate, (iii) nasal wash, and (iv) nasal brush, using one nostril for each procedure and alternating nostrils, with an interval of 5–10 min. A nasal swab sample (i) was obtained with a cotton tip (MW104, Medical Wire & Equipment, UK), rubbing the middle meatus. The cotton tips were washed twice in 1 ml of normal saline, spun for 10 min at 400 g and stored at  $-80^{\circ}\text{C}$ . (ii) A nasal aspirate was taken using a sterile mucus trap connected to gentle wall suction. If there was obvious mucus present, the trap was inserted slowly into the nostril and moved slowly in and out while sucking the mucus. A total of 0.5–1 ml of mucus and 5 ml of sterile normal saline, used to wash all the material from the tubing, was obtained. (iii) Nasal washes were performed after 2.5 ml of normal saline were instilled in one nostril (older patients were asked to avoid swallowing). The mucus was harvested 30 s later, using a sterile mucus trap connected to gentle wall suction. The aspirates and washes were placed on wet ice and stored at  $-80^{\circ}\text{C}$  as soon as possible. (iv) Nasal brushings were harvested from the nasal cavity with a brush (Cytobrush Plus, Medscand Medical, Sweden) by sampling the middle meatus. The brushes were washed twice in 1 ml of normal saline, spun for 10 min at 400 g and stored at  $-80^{\circ}\text{C}$ .

### 2.3. RNA isolation, quantitation and RT-PCR

Viral detection was performed independently in two laboratories. In the Allergy Research Center, 2nd Department of Pediatrics, University of Athens, single pathogen RT-PCRs were performed for the detection of RV, Influenza virus and Adenovirus. RNA was extracted using Trizol (Invitrogen, CA, USA), according to the manufacturer’s recommendations. Two-microliter aliquots of the isolated RNA were diluted in Tris-Cl pH 7.5 and RNA yield, concentration and purity were determined spectrophotometrically using an Eppendorf BioPhotometer (Hamburg, Germany). Reverse transcription (cDNA synthesis) was performed in 20  $\mu\text{l}$  reactions using 8  $\mu\text{l}$  RNA, Superscript III reverse transcriptase (Invitrogen)

and random hexamers according to the manufacturer’s instructions.

RT-PCRs were done in 50  $\mu\text{l}$  reactions consisting of 1x Buffer, 3 mM  $\text{Mg}^{2+}$ , 0.2 mM dNTPs, 2U of Platinum Taq DNA polymerase (Invitrogen), and 0.2  $\mu\text{M}$  of each primer. PCR for RV was done with 6  $\mu\text{l}$  cDNA and OL26 and OL27 primers (Papadopoulos et al., 2000). PCR for Influenza virus (serotypes AH1, AH3, B) was done in two rounds (nested-PCR); in the first round mixture 4  $\mu\text{l}$  of cDNA were added and 2  $\mu\text{l}$  of primary product were then transferred to 48  $\mu\text{l}$  of the secondary amplification mixture using a second primer set internal to that of the first round (Stockton et al., 1998). PCR for Adenovirus was done with 4  $\mu\text{l}$  of cDNA in a single round, according to Freymuth et al. (1997). Samples were amplified in a PTC-200 DNA Engine thermocycler (MJ Research, MA, USA), with an initial denaturation step at  $94^{\circ}\text{C}$  for 2 min and then under conditions described in Table 1.

A real-time Taqman multiplex PCR assay was performed in AMC, Dpt Medical Microbiology, Amsterdam, for RV, Enterovirus, Influenza virus, Adenovirus, RSV, Parainfluenza virus, Coronavirus, Metapneumovirus, Bocavirus and Parechovirus as described before (Molenkamp et al., 2007).

Patients positive for viral agents (“infected”) were defined as positive for any virus by any of the used methods, and negative (“uninfected”) as those negative for all the viruses and by all methods simultaneously.

The patients’ discomfort was assessed using a visual rating scale (range: 1–5). The patient was asked to choose the face that best describes how he/she was feeling with each procedure.

### 2.4. Statistics

Statistic analysis was performed by chi-square, Wilcoxon Signed Ranks, Kruskal–Wallis and Mann–Whitney tests using SPSS v.13 software. A  $p$  value  $<0.05$  was regarded as significant.

## 3. Results

### 3.1. RNA quantitation

The extracted RNA concentration was  $312.54 \pm 44.51$   $\mu\text{g}/\text{ml}$  for the aspirates,  $279.66 \pm 43.61$   $\mu\text{g}/\text{ml}$  for the brushes  $306.05 \pm 50.32$   $\mu\text{g}/\text{ml}$  for swabs and  $330.18 \pm 42.99$   $\mu\text{g}/\text{ml}$  for the washes (non-significant differences). Purity ( $A_{260}/A_{280\text{nm}}$ ) was consistent and ranged from 1.79 to 1.92.

### 3.2. Viral detection

In 48 out of 58 patients (83%), at least one type of virus was detected, by any of the four methods. Rhinovirus was found in 39 patients (67%), Adenovirus in 15 (26%), Influenza virus in 11 (19%), Coronavirus in 6 (10%), Parainfluenza virus in 3 (5%) and Bocavirus in 1 patient (2%). Detailed detection rates are presented in Table 2. The agreement between single pathogen RT-PCR and Taqman multiplex real-time PCR was 80% for RV, 94% for Influenza virus and 91% for Adenovirus. Nasal wash samples identified 88% of the infected patients, which was the highest detection rate. Aspirates detected 79% of the infected patients, swabs 77% and brushes 74%. The rate of detection of any virus in nasal washes was significantly higher than that in nasal brushes ( $p < 0.05$ ) (Table 2).

### 3.3. Individual virus analysis

Comparing the detection rates of the four methods regarding the virus type, nasal washes yield the highest detection rates for

**Table 1**  
RT-PCR conditions used in this study.

	Thermocycling conditions	No. of cycles	Final extension	Amplicon (bp)
Rhinovirus	30 s, 94 °C 30 s, 50 °C 60 s, 72 °C	40	5 min, 72 °C	380
Influenza (1st round)	40 s, 94 °C 40 s, 50 °C 65 s, 72 °C	35	8 min, 72 °C	1015 (AH1) 883 (AH3) 900 (B)
Influenza (2nd round)	40 s, 94 °C 40 s, 50 °C 65 s, 72 °C	30	8 min, 72 °C	944 (AH1) 767 (AH3) 591 (B)
Adenovirus	30 s, 94 °C 30 s, 50 °C 30 s, 72 °C	40	5 min, 72 °C	161

**Table 2**  
Detection of viruses in nasal aspirates, brushes, swabs and washes.

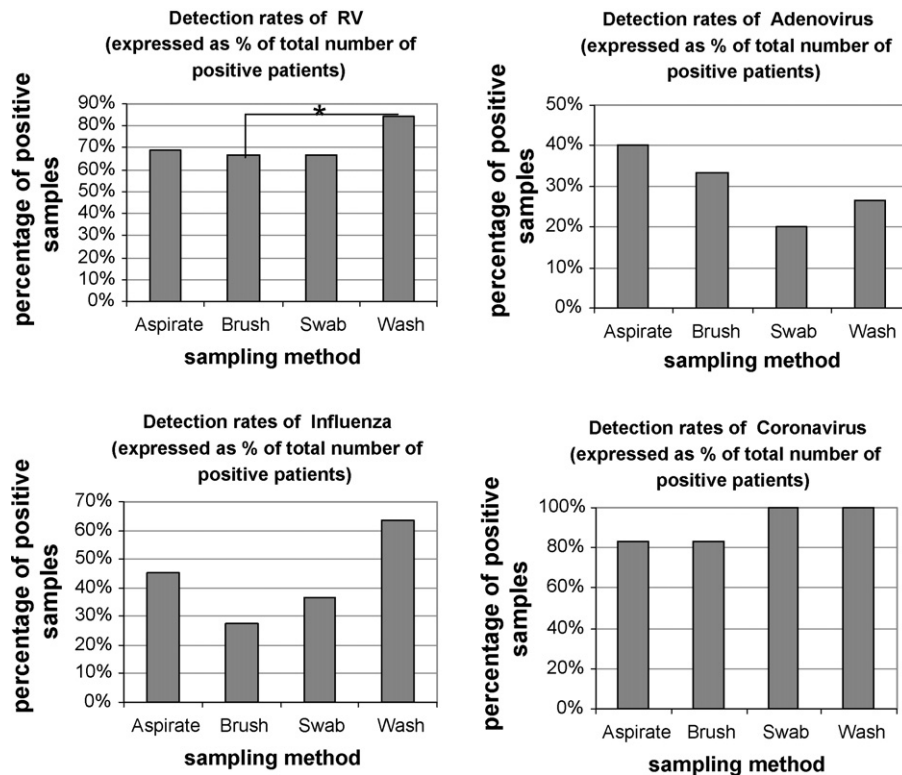
Virus	Total n	Aspirate		Brush		Swab		Wash	
		n	(%)	n	(%)	n	(%)	n	(%)
Rhinovirus	39	27	(68)	26	(65)	26	(65)	33	(83)
Adenovirus	15	6	(40)	5	(33)	3	(20)	4	(27)
Influenza	11	5	(45)	3	(27)	4	(36)	7	(64)
Coronavirus	6	5	(83)	5	(83)	6	(100)	6	(100)
Parainfluenza	3	3	(100)	3	(100)	3	(100)	3	(100)
Bocavirus	1	0	(0)	0	(0)	1	(100)	0	(0)
Any virus positive	48	36	(79)	35	(74)	37	(77)	41	(88)*

\*  $p < 0.05$  in comparison to brushes.

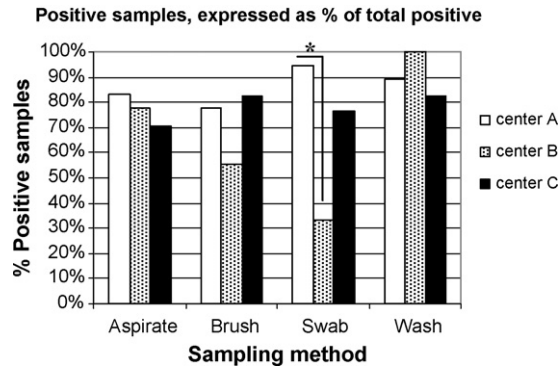
RV. There were no significant differences between the methods for other viruses, although some numerical differences were observed, possibly because of the small number of positive cases for these viruses (Fig. 1).

### 3.4. Variability between centers

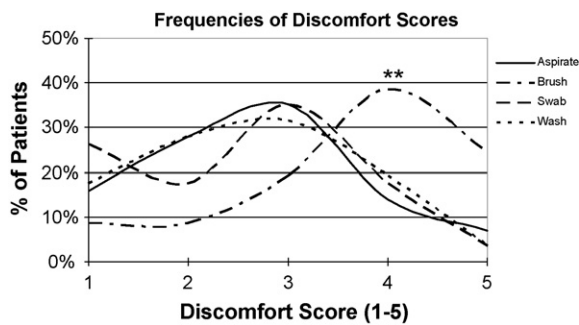
Detection rates were 92%, 85% and 72% in the 3 sampling centers. Detection rates per sampling method in each center are shown



**Fig. 1.** The rate of detection of Rhinovirus in nasal washes was significantly higher than that in nasal brushes ( $*p < 0.05$ ), while differences were not significant in the case of other viruses.



**Fig. 2.** Between-center variability in viral detection using different sampling methods. Variability was statistically significant ( $*p < 0.05$ ) only in the case of swabs (between centers A and B).



**Fig. 3.** Frequencies of discomfort scores for each sampling method. The scores for aspirate and wash are similar, whereas swab gave two peaks and brush is shifted to the higher scores. Brushes caused significant higher discomfort, when compared to the other 3 sampling methods ( $**p = 0.001$ ).

in Fig. 2. Variability was considerably high with respect to swabs (S.D. = 0.31 or 31%,  $p < 0.05$ ), less for brushes (S.D. = 0.14), and even less for aspirates and washes (S.D. = 0.06 and 0.09 respectively).

### 3.5. Discomfort

Nasal brushes caused significantly more discomfort, compared to the other three sampling methods ( $p < 0.001$ ). Washes and aspirates were comparable while swabs were perceived with the least discomfort. The mean discomfort score employing the aspirates was 2.68, the brushes 3.61, the swabs 2.54 and the washes 2.63 (Fig. 3).

## 4. Discussion

In this study, the efficacy of four nasal sampling methods, including viral detection, and discomfort in applying each method was compared. In order to increase the detection rates, only patients with common cold symptoms participated in the study. The samples were paired, as the four techniques were applied to the participants in a single visit, at the same time-point of the disease. Two PCR techniques for viral detection were employed, conventional RT-PCR and a real-time Taqman multiplex assay, targeting 10 common respiratory viral pathogens. Viral cultures have been used in the past as the gold standard in viral detection; however adoption of PCR techniques is associated with higher virus detection rates (Freythuth et al., 1999; Xiang et al., 2002). Furthermore, the multicenter character of the present study allowed the evaluation of differences in the viral detection rates due to application of the

sampling methods in different centers. With respect to this, aspirates and washes exhibited more homogeneity between centers and could therefore be preferable for multicenter studies. These two methods are more complicated, however their application is based on more standardised protocols.

In recent years, nasal or nasopharyngeal aspirates and washes have been used for obtaining nasal secretions for the detection of respiratory viruses. However, few studies have compared the viral yields from samples taken by two or more sampling methods. Among these, some found nasal aspirates and washes superior to nasal or nasopharyngeal swabs for the detection of respiratory pathogens (Ahluwalia et al., 1987; Covalciuc et al., 1999; Frayha et al., 1989; Heikkinen et al., 2001, 2002). In contrast, other comparative studies obtained adequate viral yields from nasopharyngeal swabs and nasal brushes, for the detection of viruses using immunofluorescent assays and cultures (Barnes et al., 1989; Frayha et al., 1989). Furthermore, Heikkinen et al. (Heikkinen et al., 2001, 2002) reported comparable viral detection rates between nasal swabs and nasopharyngeal aspirates (for all the viruses tested except RSV) using viral culture and immunofluorescent assays. A possible explanation for these discrepancies could be the use of immunofluorescent assays in these studies, in which pathogen detection depends on the number of cells obtained from the patient.

Some of the more recent studies have used one or more sampling methods aiming at the detection of RV (nasal swabs and washes) (Peltola et al., 2008; Wright et al., 2007), RSV (nasopharyngeal aspirate, nasal brush, and bronchoalveolar lavage fluid) (Barnes et al., 1989; Semple et al., 2007), Influenza virus (nasal aspirates) (Frisbie et al., 2004), Coronavirus (nasal brushes) (Gagneur et al., 2002), and Metapneumovirus (nasal swabs) (Heikkinen et al., 2008). Moreover, van den Hoogen (van den Hoogen et al., 2003) used nasal aspirates, throat swabs, sputum and bronchoalveolar lavage fluids for the detection of Metapneumovirus by PCR, although the design of the study did not include the comparison of the sampling methods. In a range of epidemiological studies detecting more than one virus type in nasal secretions, various sampling methods such as nasal aspirates (Kleemola et al., 2006) brushes (Falsey et al., 1996; van Bente et al., 2003a,b), swabs (Lee et al., 2007), or washes (Rohde et al., 2003; Noyola et al., 2004) have been used. But still, there is no clear evaluation of the efficacy of the sampling methods applied.

In concordance with our initial hypothesis it was shown that there are differences between sampling methods in the detection of common respiratory viruses, but also in regard to patients' discomfort. Nasal washes yielded the highest rates of viral detection without excessive patient discomfort. In contrast, nasal brushes produced the lowest rates of viral detection and demonstrated the highest level of patient discomfort. Detection rates from nasal swabs and aspirates were lower than these of washes and higher than these of brushes, however differences were non-significant. This is in agreement with previous findings, which demonstrated comparable detection rates between nasal swabs, brushes and aspirates; though a larger sample size may be needed to define the significance of these slight differences.

The differences in viral detection rates between the sampling methods are significant mainly regarding RV. For all the viruses detected, nasal washes yielded the highest detection rates, except for Adenovirus which was more frequently detected in nasal aspirates. The prevalence of Adenovirus, Influenza and Coronavirus infections is lower than that of RV, and the study was not powered to analyze differences for those viruses. However, the order of the detection rates tends to be the same in the majority and for the total of the viruses.

A possible drawback of this study is the sequential application of the procedures that may affect detection rates and/or discomfort produced after repeated sampling. One nostril was used for each

method and nostrils were alternated, with an interval of 5–10 min. Nasal swab and wash was applied in the one nostril, while aspirate and brush was applied in the other. Due to this design the samples were absolutely paired, but the effect of the repeated samplings cannot be excluded.

These findings should be taken into account in the design of epidemiological studies: inadequate sampling may lead to underestimation of viral prevalence and hence its contribution to respiratory pathology. In adopting the optimal sampling method, differences in the produced detection rates and patients discomfort should be considered in addition to cost, availability of facilities, trained personnel and time required to collect the samples.

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