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Comparison of nasopharyngeal flocked swabs and aspirates for rapid diagnosis of respiratory viruses in children

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Abstract

Background: The quality of clinical specimens is a crucial determinant for virological diagnosis.

Objectives: We compared the viral diagnostic yield for influenza A and respiratory syncytial virus (RSV) from the recently developed nasopharyngeal flocked swabs (NPFS) with nasopharyngeal aspirates (NPA) collected in parallel from 196 hospitalized children with acute respiratory infection during the peak period of influenza A and RSV activity in Hong Kong. Specimens were tested by RT-PCR for influenza A and RSV and viral load determined. They were also tested by direct immunofluorescence (DIF) for influenza A and B, RSV, parainfluenza types 1–3 and adenovirus.

Results: Both NPA and NPFS had excellent sensitivity (100%) for detecting influenza A by RT-PCR but NPA was slightly more sensitive than NPFS for detecting RSV by both RT-PCR (100% vs. 92.3%) and DIF (87.2% vs. 84.6%) and for detecting influenza A by DIF (90.2% vs. 82.9%). Viral load for influenza A in NPA and NPFS was not significantly different but that for RSV was higher in NPA.

Conclusion: NPA remains the optimal specimen for diagnosis of respiratory infections by RT-PCR and DIF. However, collection of NPFS is easier to perform in an out-patient setting, was more acceptable to parents and less likely to generate aerosols than NPA engendering potentially less infection control hazard.

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1. Introduction

Acute respiratory infections are the most common illnesses of otherwise healthy adults and children and most of these infections are caused by viruses (Treanor, 2002). Furthermore, novel emerging viral respiratory infections (e.g. SARS) have the potential to cause explosive disease outbreaks with huge impact on economies and society in general (Peiris et al., 2004). Rapid viral diagnosis leads to optimized clinical care, reduced antibiotic use, helps infection control and is cost effective (Woo et al., 1997). Antigen detection

methods have been commonly used for rapid respiratory viral diagnosis but PCR-based methods are now becoming more widely used. In all these methods the type and quality of the clinical specimen is of utmost importance. Nasopharyngeal aspirates (NPA) are generally considered the best specimens for rapid detection of respiratory viruses (Ahluwalia et al., 1987; Zambon, 1998; Macfarlane et al., 2005). However, obtaining a NPA is unpleasant to the patient, requires specialized equipment and a skilled operator for specimen collection and therefore difficult to obtain in an out-patient or field setting. Nasal swab specimens have been found to be less productive than NPA in some studies (Macfarlane et al., 2005) but not in others (Heikkinen et al., 2002). Nasopharyngeal swabs (NPS) have been reported by some to have compara-

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ble positivity rates to NPA (Frayha et al., 1989) although this is still controversial (Ahluwalia et al., 1987). Compared to NPA, conventional NPS swabs usually yield fewer epithelial cells for direct antigen detection by IF assay (Ahluwalia et al., 1987).

Recently a flocked-nasopharyngeal swab was designed with the aim of improving the yield of nasopharyngeal epithelial cells and enhancing diagnostic yield (Copan Diagnostics, Corona, CA). A study showed that flocked swabs yielded more cells and provided better clinical specimen when compared with nasal swab (Daley et al., 2006; Chernesky et al., 2006). However, there are still no direct comparisons between nasopharyngeal flocked swabs (NPFS) and NPA for the rapid diagnosis of respiratory viruses by direct immunofluorescence assay and PCR. We conducted a prospective study in children with acute respiratory disease comparing NPFS with NPA for the detection of influenza A and respiratory syncytial virus (RSV) by PCR and direct immunofluorescence (DIF) assays. We also compared the viral nucleic acid load in parallel NPA and NPFS specimens using quantitative real time PCR.

2. Materials and methods

2.1. Patients and specimens

One hundred ninety-six hospitalized children under 18 years of age with acute respiratory tract infections at Queen Mary hospital from February to May 2007 were recruited. The study protocol was approved by the ethics committee of Queen Mary Hospital and written informed consent was obtained prior to recruitment. NPA and NPFS were collected in parallel from each patient. The NPA specimen was collected from one nostril and the NPS specimen from the other. Initially, children were randomized to have either NPA or NPS performed first. However, early interim analysis revealed that there was little difference in epithelial cell yield in relation to the collection order. Thus subsequently, the order of sampling was left to the preference of the individual nurse.

The NPFS was collected as follows. The distance between the patient's nares and earlobe was measured to estimate the length of insertion. The swab was gently inserted up the nostril towards the pharynx for that distance until resistance was felt and was then rotated 3 times to obtain epithelial cells. The swab was then withdrawn and put in 2.5 ml viral transport medium. NPA was collected using a vacuum suction trap kit into another 2.5 ml viral transport medium. The specimens were kept cool and delivered to the laboratory within 3 h of collection

In the laboratory, both NPA and NPFS were divided into two aliquots and processed in an identical fashion. One aliquot was used for the DIF test and the other was used for PCR assays and in the case of NPA, also for culture. The original volume of NPA was recorded and this was used for

calculation of absolute amount of virus present in the sample when quantitative real time PCR was done.

2.2. Direct antigen detection by immunofluorescence

Immunofluorescence on the clinical specimens was done as previously described using IMAGENTM respiratory screen and typing reagents (Oxoid Ely Ltd., UK) (Chan et al., 2002). All specimens positive by DIF were scored according to fluorescent intensity and numbers of positive cells per 40 high power fields. All the specimens found positive in the respiratory screen with a pooled IF reagent were further identified using antibody reagents to the individual virus (influenza A or B, RSV, parainfluenza type 1, 2, 3 and Adenovirus) using the IMAGENTM typing kit.

2.3. PCR

Total nucleic acid was extracted by using NucliSens easy-MAG instrument (bioMerieux, Netherlands) according to the manufacturer's instruction. Nucleic acid was recovered in 55 μ l elution buffer and was kept at -80°C until use.

The influenza A and B and RSV reverse transcriptase quantitative PCR (RT-qPCR) test was carried out as previously described (Peiris et al., 2003). In brief, 12 μ l of the eluted RNA was used for generation of cDNA using the Invitrogen Superscript II Kit with random primer. 2 μ l of cDNA was amplified in LightCycler with a total volume of 20 μ l reaction containing FastStart DNA Master SYBR Green I Mix reagent kit (Roche Diagnostics GmbH, Germany), 0.5 μ M of each primer and MgCl_2 (for influenza A: 3 mM; influenza B: 4 mM and RSV: 5 mM). The primers and PCR conditions are summarized in Table 1. Fluorescence was monitored once each cycle at the end of the annealing phase.

For adenovirus amplification, 5 μ l of nucleic acid template was used to amplify a PCR product corresponding to hexon gene using primers and PCR conditions in Table 1. The reagents used were 1 \times FastStart DNA Master SYBR Green I Mix (Roche Diagnostics GmbH, Germany), 3.0 mM MgCl_2 and 0.5 μ M of each primer.

PCR products were subjected to melting curve analysis at the end of the assay ($65\text{--}95^{\circ}\text{C}$; 0.1°C per s) to confirm specificity. For each quantitative assay, a reference standard was prepared using pCRII-TOPO vector (Invitrogen, San Diego) containing the corresponding target viral sequences. A series of 5 \log_{10} dilution equivalent to $1 \times 10^1\text{--}1 \times 10^6$ copies per reaction were prepared to generate calibration curves and run in parallel with the test samples.

2.4. Viral culture for respiratory viruses

MDCK, LLC-MK2, HEp-2C and RD cell monolayers in culture tubes were inoculated with 200 μ l of the nasopharyngeal aspirate-virus transport medium suspension and virus isolation was carried out as previously described (Lo et al., 2005).

Table 1
Primer sequences and cycling condition

q-PCR	Target	Primers	Cycling condition (ramp rates of 20 °C/s)	Reference
Influenza A	M gene	F- CTTCTAACCGAGGTGCAAACG R- GGCATTTTGGACAAAKCGTCTA	Denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 3 s, 72 °C for 12 s	
Influenza B	Nonstructure protein gene 8	F-GGGATATACGTAATGTGTTGT R-GCACTGCCTGCTGTACACTT	Denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 55 °C for 5 s, 72 °C for 20 s	Gruteke et al. (2004)
RSV	L gene	F- TTTCCACAATATYTAAGTGTCAA R- TCATCWCCATACTTTTCTGTCAA	Denaturation at 95 °C for 10 min, followed by 50 cycles of 95 °C for 10 s, 45 °C for 5 s, 72 °C for 7 s	Templeton et al. (2004)
Adenovirus	Hexon gene	F- GCCGCAGTGGTCTTACATGCACATC R- CAGCACGCCGCGGATGTCAAAGT	Denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 3 s, 72 °C for 12 s	Mitchell et al. (2003)

Table 2
Profiles of test results for influenza A and respiratory syncytial virus by RT-PCR, direct immunofluorescence (DIF) and culture from nasopharyngeal aspirates (NPA) and nasopharyngeal flocced swabs (NPFS)

Virus	Culture	NPA PCR	NPFS PCR	NPA DIF	NPFS DIF	Number positive
Influenza A	+	+	+	+	+	34
	+	+	+	+	–	3
	+	+	+	–	–	3
	–	+	+	–	–	1
	+	+	+	+	+	28
	+	+	–	+	+	2
RSV	+	+	+	–	–	3
	–	+	+	+	+	2
	–	+	+	+	–	1
	–	+	–	+	+	1
	–	+	+	–	–	2

3. Results

3.1. Diagnostic yield by NPA and NPFS specimens

Paired NPA and NPFS were collected from one hundred ninety-six pediatric patients recruited during February to May 2007 which is the peak influenza A and RSV season in Hong Kong. There were 113 males and 83 females with the mean

age 6.3 months, range 1–89 months. RT-PCR was done on all specimens for influenza A and RSV. DIF was done on all specimens for influenza A and B, RSV, parainfluenza type 1, 2, 3 and adenovirus. Virus culture was done for all NPA specimens. RT-PCR or PCR was done for other viruses to only to resolve discrepant results.

The profile of test results from the NPA and NPFS specimens by RT-PCR and DIF for influenza A and RSV is shown

Table 3
Diagnostic yield from NPA and NPFS specimens for the diagnosis of influenza A and RSV by RT-PCR and direct immunofluorescence (DIF)

True positive ^a	Specimen/method	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Influenza A = 41	Culture	97.6	100	100	99.4
	NPA/RT-PCR	100	100	100	100
	NPFS/RT-PCR	100	100	100	100
	NPA/DIF	90.2	100	100	97.5
	NPFS/DIF	82.9	100	100	95.7
RSV = 39	Culture	84.6	100	100	96.3
	NPA/RT-PCR	100	100	100	100
	NPFS/RT-PCR	92.3	100	100	98.1
	NPA/DIF	87.2	100	100	96.9
	NPFS/DIF	84.6	100	100	96.3

^a A true positive result is defined as a patient with two independent tests (different tests on same or different specimens or same test on two different specimens) were positive for the same virus.

(Table 2). A true positive for influenza A or RSV was defined as a patient with two independent tests (different tests on same or different specimens or same test on two different specimens) positive for the same virus. Both NPA and NPFS had excellent sensitivity (100%) for detecting influenza A by RT-PCR. However, NPA was slightly more sensitive than NPFS for detecting RSV by RT-PCR (100% vs. 92.3%) and DIF (87.2% vs. 84.6%) and for detecting influenza A by DIF (90.2% vs. 82.9%) (Tables 2 and 3).

3.2. Viral load

The mean of viral load of RSV RNA was significantly higher in NPA compared to NPFS specimens (mean 4.2×10^9 and 4.52×10^8 ; $p=0.002$). For influenza A virus, the mean viral loads for NPA and NPFS was 2.25×10^{11} and 6.82×10^9 but these differences were not statistically significant ($p=0.31$) (Table 4). The scatter-plots of the viral load in NPA and NPFS for influenza A and RSV specimens are shown in Fig. 1. Viral load was also done for influenza B and adenovirus in specimens positive by DIF for these viruses but there were too few positive specimen to make meaningful comparisons.

3.3. Direct immunofluorescence tests

The morphology of DIF staining of paired NPA and DIF specimens that were positive for influenza and RSV were comparable (data not shown). Similarly Giemsa stained NPA and NPFS stained specimens showed similar morphology of cells collected by these two methods. Of the 80 specimens found to be IF positive for a respiratory virus, 74 were positive in both NPA and NPFS specimens. Six specimens were positive only in the NPA; three of these had influenza A, and one each had adenovirus, parainfluenza type 3 and RSV, respectively. These were confirmed by Q-PCR as true positives. The mean number of positive cells per 40 high power fields of the NPA in these 6 specimens was low, being a mean of 5.5 positive cells for the three influenza positive specimens and one positive cell for each of the other viruses. In contrast, the overall mean number of cells positive with the IMAGENTM respiratory screen reagent in positive NPA and NPFS specimens was 18 and 23, respectively indicating that the specimens found false negative by

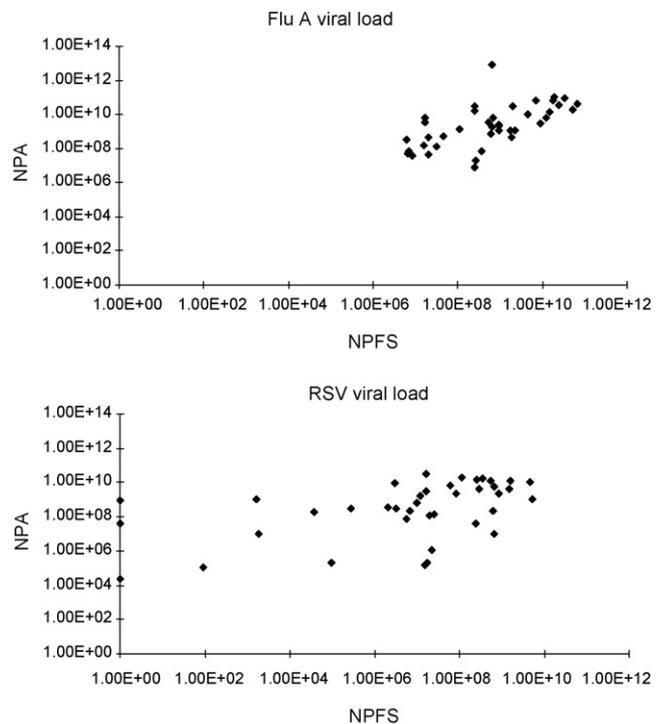


Fig. 1. Influenza A and RSV viral load in parallel NPA and NPFS specimens.

NPFS likely had lower numbers of positive cells in the specimen.

The overall mean (\pm S.D.) numbers of epithelial cells (log10) per 40 high-power fields (excluding squamous epithelial cells) found in the NPA and NPFS specimens were 1.94 ± 0.611 and 2.034 ± 0.52 ($p=0.12$). The overall mean numbers of respiratory epithelial cells (log10) per 40 high power fields for the DIF positive and negative specimens was 2.10 ± 0.54 and 1.8 ± 0.61 , respectively ($p=0.0002$). Similarly, mean numbers of respiratory epithelial cells (log10) per 40 high powered fields in NPFS specimens positive and negative for virus by DIF was 2.2 ± 0.43 and 1.90 ± 0.57 , respectively ($p=0.004$).

4. Discussion

We compared the NPFS and NPA collected in parallel from 196 hospitalized pediatric patients for diagnosis of res-

Table 4 Comparison of the numbers of DIF positive cells, DIF intensity and viral load in NPA and NPFS specimens in patients with influenza A and RSV infections

Positive specimens ^a	Type of specimens	Direct immunofluorescence (DIF)			RT-PCR	
		Mean of total respiratory epithelial cells/40× hp	IMAGEN TM respiratory screen (mean of total positive cells/40× hp)	Mean of DIF intensity	Mean of viral load (total genome copies per specimen)	Ratio of viral load in NPA/NPS
RSV = 39	NPA	264.8	25.1	2.6	4.20×10^9	9.3
	NPFS	242.6	26.5	2.9	4.52×10^8	
Influenza A = 41	NPA	224.8	14.7	2.8	2.25×10^{11}	32.9
	NPFS	268.9	26.7	2.7	6.82×10^9	

^a True positives influenza A or RSV patients are defined as those with two tests positive for the same virus (see Section 3).

piratory infections by DFA tests and for diagnosis of influenza A and RSV by RT-PCR. The reason for focusing on influenza A and RSV for the RT-PCR study was that these viruses were known to be the predominant respiratory virus infections occurring during the period of the study.

Specimens from either method yielded epithelial cells of good morphology and in adequate numbers for immunofluorescent diagnosis of a panel of 7 respiratory infections. This is difficult to achieve with other types of swabs including conventional NP swabs. However, 6 of 80 specimens found to be weakly positive by DFA from NPA specimens were negative on NPFS. Nevertheless, NPFS specimens appear to be a reasonable alternative to NPA in situations where NPA specimen collection is not feasible.

NPA and NPFS appear to be equivalent for the diagnosis of influenza A by qualitative RT-PCR. Although the mean viral load appears to be higher in NPA, these findings were not statistically significant. However, NPA specimens were superior to NPFS for the detection of RSV by RT-PCR and this is reflected in a 9.3-fold higher viral load in these specimens ($p=0.002$). The viral load data from specimens positive for influenza B and adenovirus were too small to be meaningfully analyzed (data not shown).

The choice of specimen must be balanced with the availability of facilities, cost, expertise available and the potential infection control risk. The collection of NPFS was less invasive for children compared with NPA and therefore more acceptable to parents. Further, the collection of a NPFS which is similar to NS is easy and convenient and it requires no additional devices. Therefore, although NPFS has a slightly lower sensitivity compared to NPA, the NPFS is a suitable alternative for NPA for rapid respiratory diagnosis by DIF antigen detection or RT-PCR in situations where equipment for obtaining NPA is not available. In addition, when compared to collection of NPA which requires the use of suction, NPFS collection may generate less aerosol associated cross-infection hazard.

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References

- Ahluwalia G, Embree J, McNicol P, Law B, Hammond GW. Comparison of nasopharyngeal aspirate and nasopharyngeal swab specimens for respiratory syncytial virus diagnosis by cell culture, indirect immunofluorescence assay, and enzyme-linked immunosorbent assay. *J Clin Microbiol* 1987;25:763–7.
- Chan KH, Maldeis N, Pope W, Yup A, Ozinskas A, Gill J, et al. Evaluation of the Directigen FluA + B test for rapid diagnosis of influenza virus type A and B infections. *J Clin Microbiol* 2002;40:1675–80.
- Chernesky M, Castriciano S, Jang D, Smieja M. Use of flocced swabs and a universal transport medium to enhance molecular detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *J Clin Microbiol* 2006;1084–6.
- Daley P, Castriciano S, Chernesky M, Smieja M. Comparison of flocced and rayon swabs for collection of respiratory epithelial cells from uninfected volunteers and symptomatic patients. *J Clin Microbiol* 2006;44:2265–7.
- Frayha H, Castriciano S, Mahony J, Chernesky M. Nasopharyngeal swabs and nasopharyngeal aspirates equally effective for the diagnosis of viral respiratory disease in hospitalized children. *J Clin Microbiol* 1989;27:1387–9.
- Gruteke P, Glas AS, Dierdorp M, Vreede WB, Pilon JW, Bruisten SM. Practical implementation of a multiplex PCR for acute respiratory tract infections in children. *J Clin Microbiol* 2004;42:5596–603.
- Heikkinen T, Marttila J, Salmi AA, Ruuskanen O. Nasal swab versus nasopharyngeal aspirate for isolation of respiratory viruses. *J Clin Microbiol* 2002;40:4337–9.
- Lo JY, Tsang TH, Leung YH, Yeung EY, Wu T, Lim WW. Respiratory infections during SARS outbreak, Hong Kong, 2003. *Emerg Infect Dis* 2005;11:1738–41.
- Macfarlane P, Denham J, Assous J, Hughes C. RSV testing in bronchiolitis: which nasal sampling method is best? *Arch Dis Child* 2005;90:634–5.
- Mitchell S, O'Neill HJ, Ong GM, Christie S, Duprex P, Wyatt DE, et al. Clinical assessment of a generic DNA amplification assay for the identification of respiratory adenovirus infections. *J Clin Virol* 2003;26:331–8.
- Peiris JSM, Tang WH, Chan KH, Khong PL, Guan Y, Lau YL, et al. Children with respiratory disease associated with metapneumovirus in Hong Kong. *Emerg Infect Dis* 2003;9:628–33.
- Peiris JSM, Guan Y, Yuen KY. Severe acute respiratory syndrome. *Nat Med* 2004;10(Suppl.):S88–97.
- Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *J Clin Microbiol* 2004;42:1564–9.
- Treanor JJ. Respiratory virus infections. In: Richman DD, Whitley RJ, Hayden FG, editors. *Clinical virology*. 2nd ed. Washington, DC: ASM Press; 2002. p. 7–26, Chapter 2.
- Woo PC, Chiu SS, Seto WH, Peiris M. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. *J Clin Microbiol* 1997;35:1579–81.
- Zambon M. *Laboratory diagnosis of influenza*. Oxford: Blackwell Science; 1998. p. 291–313.