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1 **Increased detection of respiratory viruses in paediatric outpatients with acute**

2 **respiratory illness by real-time polymerase chain reaction using nasopharyngeal flocked**

3 **swabs**

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20

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22 Abstract (64)

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25

1   **Abstract** (64 words)

2   Detection of respiratory viruses by realtime multiplexed PCR (M-PCR) and of RSV by M-

3   PCR and immunofluorescence(IF) was evaluated using specimens collected by

4   nasopharyngeal flocked swab(NFS) and nasal wash(NW). In children with mild respiratory

5   illness, NFS collection was superior to NW collection for detection of viruses by M-PCR

6   (sensitivity 89.6% vs 79.2%, P=0.0043). NFS collection was non-inferior to NW collection in

7   detecting RSV by IF.

8

9   **Main text** (1509 excluding acknowledgement)

10   Respiratory viruses are major causes of infant and childhood acute respiratory infection(ARI)

11   with respiratory syncytial virus (RSV) contributing significantly to the disease burden(3, 5,

12   11). Nasopharyngeal aspirates (NPA) and nasal washes (NW) have been the preferred

13   sampling methods for the diagnosis of respiratory viruses (7, 12, 13). However

14   nasopharyngeal flocked swab (NFS) is increasingly recognized as an alternative(1). Possible

15   advantages of NFS over NW and NPA include simplicity of use, improved standardisation in

16   different age groups and between operators, and better acceptability in a wider range of

17   settings. However, there are few published data on the sensitivity of NFS when using realtime

18   multiplex polymerase chain reaction(M-PCR) assays for virus detection(10). We report on a

19   study designed to assess diagnostic performance of NFS relative to NW for detection of RSV

20   by both immunoflourence antibody tests (IF) and M-PCR and for other respiratory viruses by

21   M-PCR only.

22

23   In an outpatient health facility serving a rural population in the Kilifi District of coastal

24   Kenya, children (<13 years old) presenting during the peak of a RSV season in 2009 were

25   screened for virus-associated ARI. Children were eligible if identified as having one or more

1   of the following symptoms: difficulty in breathing, nasal discharge, blocked nose, cough, or

2   fast breathing for age, unless the symptoms were deemed severe enough to require hospital

3   referral. Informed consent was sought from the parent/guardian of each child. Ethical

4   approval for the study was obtained from the Kenya National Ethical Review Committee.

5   Three trained field assistants participated in this study. For each child a field assistant

6   collected a NFS specimen from one nostril followed immediately with a NW sample from the

7   other nostril. Thereafter the caretaker (and children aged 3 years and above) responded to

8   simple questions on their preferred specimen collection method. The two specimens were

9   stored in a cool box, with ice packs, and transferred within one hour to a refrigerator at ~4^{0}C.

10   Samples were transported in a cool box at the end of every day to the laboratory at KEMRI-

11   Wellcome Trust Research Programme in Kilifi town.

12

13   NFS specimens were collected as described elsewhere(4) using a commercially available

14   device that has a fine nylon flock on the tip of a flexible plastic rod (Cat # 503CS01; Copan,

15   Italia). Briefly, the swab was gently passed up the nostril towards the pharynx for a distance

16   equal to that between the patient's nares and earlobe, rotated 2-3 times, held in place for 5

17   seconds then withdrawn gently and put in 2 ml of viral transport medium – locally prepared

18   as described elsewhere(8, 15). For NW, normal saline (3-10ml according to age) was squirted

19   into the patient's nasal cavity using a soft rubber bulb and immediately sucked out. Fluid

20   escaping from the other nostril was collected in a suitable receptacle. The NW process was

21   repeated in the other nostril if less than 1ml of fluid was retrieved(7, 12). The two procedures

22   were performed while the child was in a sitting position, head slightly tilted backward, with

23   or without support from their caretaker.

24

1   NW samples were screened for RSV by commercial IF kit (Cat # 3125; Millipore Light

2   Diagnostics, Temecula, CA 92590 USA) as previously described(12) and if found positive

3   the paired NFS sample was also screened by IF. Slides for IF were prepared using a cytology

4   centrifuge (Cytospin 3, Thermo Shandon Ltd, Cheshire UK) (13). For all pairs of NW/NFS

5   samples nucleic acid was extracted using QIAamp Viral RNA mini kit (Cat # 52906; Qiagen,

6   UK) and tested by M-PCR method, using the ABI-7500 platform (Applied Biosystems, Inc;

7   California, USA), as described elsewhere(6) for 16 respiratory pathogens, namely RSV A and

8   B, adenovirus, rhinovirus, human metapneumovirus(hMPV), human coronavirus (NL63,

9   OC43, 229E), parainfluenza (PIV 1, 2, 3, 4), influenza (A, B, C) and *Mycoplasma*

10 *pneumoniae (Mpn)*. Specimens were assigned positive for a particular pathogen if the cycle

11 threshold (Ct) value was ≤35.0, otherwise they were considered negative(2). The laboratory

12 technicians were blinded to specimen pairing.

13

14 The sensitivity for detection of RSV by IF on NFS collections was determined using NW IF

15 results as the reference. For M-PCR data, a sample was considered a true positive if either of

16 the specimens was positive and comparisons made using McNemar's chi-square test. The

17 Binomial Exact method was used to determine 95% confidence limits for prevalence and

18 sensitivities (one-sided 97.5% reported if sensitivity was 100%). The mean (95% CI) of the

19 Ct values by specimen collection method was calculated and comparisons made using paired

20 t-test for each virus. Each comparison was limited to sample pairs for which either had Ct

21 values ≤35.0: 'undetermined' Ct values (negatives) were coded as 40 for this analysis.

22 Statistical analyses were done in STATA 11.1 (Stata-Corp LP, College Station, TX, USA).

23

24 A total of 299 children had paired NW and NFS samples collected between 28th January 2009

25 and 17th April 2009. The median age (interquartile range) was 1.8 (0.9 to 4.2) years, with

1   infants (<1 year of age) accounting for 89 (29.8%) of the samples. Male participants were

2   145 (48.5%).

3   There were 43(14.4%) NW specimens positive for RSV by IF: all 43 were also positive by IF

4   on the paired NFS collections. The number of RSV positives detected increased to 64

5   (21.4%) and 70 (23.4%) by M-PCR on NW and NFS, respectively. Overall 199 (66.6%)

6   children had at least one virus detected from either NW or NFS by M-PCR with 12 being

7   detected from NW only and 30 from NFS only. The proportion of individuals positive for at

8   least one virus was higher in NFS compared to NW (187/299, 62.5% (95% CI; 56.8 – 68.0)

9   and 169/299, 56.5% (50.7 – 62.2)), respectively, McNemar's chi-square test, P=0.008).

10   Rhinovirus was the most frequently detected virus (79, 26.4%), followed by RSV (73,

11   24.4%), PIV (36, 12.0%), adenovirus (22, 7.4%), human coronaviruses (22, 7.4%) and hMPV

12   (15, 5.0%). Influenza (A) virus was detected in one patient - in both NFS and NW

13   specimens. There was no detection of influenza B and C, human coronavirus 229E and

14   OC43, and Mpn. The sensitivities of the M-PCR for detection of respiratory viruses on NW

15   and NFS are shown in Table 1. A total of 172 viruses were detected from both NW and NFS

16   collections while 26 viruses were detected from NW only and 52 from NFS only by M-PCR.

17   The sensitivity of NW and NFS in detection of respiratory viruses by M-PCR was 198/259,

18   79.2% (95% CI; 73.6 – 84.1) and 224/250, 89.6% (85.1 – 93.1), McNemar's chi-square test,

19   P=0.0043.

20

21   A total of 74 RSV infections were detected by either IF or M-PCR assay. One participant had

22   both samples positive by IF but all negative by M-PCR. The sensitivity for detection of RSV

23   was higher for NFS (94.6% (86.7 – 98.5) and NW (87.8% (78.2 – 94.3) by M-PCR assay

24   relative to NW IF (58.1% (46.1 – 69.5%), p<0.001). Though there was no statistically

25   significant difference in M-PCR RSV sensitivity using NFS compared with NW, the mean

1   RSV Ct values were significantly lower (i.e. an indication of higher viral load) in NFS

2   collections relative to NW; and this was the case for rhinovirus and adenovirus (p-values

3   <0.05.) However, these differences were in the 1 – 2 Ct range (data not shown).

4

5   Of the 275 caretakers and 153 participants who responded to questionnaire on acceptability

6   of the specimen collection methods, 60.0% and 71.9% preferred NFS, 35.3% and 22.2%

7   preferred NW, while there was no preference for either method by 4.7% and 5.9%,

8   respectively. The 3 trained field assistants participating in this study preferred using NFS

9   over NW in 80.2% of the 268 collections for which data were recorded. In all instances

10   above there was evidence of a statistically significant preference for NFS over NW (H_0=50%,

11   Pearson's Chi-square P <0.02).

12

13   We found no evidence of inferiority of the NFS compared to the NW for the detection of

14   RSV by IF. Relative to IF, the use of M-PCR significantly increased the proportion of RSV

15   positive cases (from 14% to ~22% of 299 participants) but with no marked difference

16   between collection device. The sensitivity of NFS was significantly higher than NW (89.6%

17   versus 79.2%. P=0.0043) for the detection of at least one of the 16 respiratory pathogens

18   tested in the children with ARI managed as outpatients. Individually, the detection of

19   rhinovirus and adenovirus using NFS had a statistically higher sensitivity relative to the NW

20   and this was reflected in lower (improved) Ct values in NFS relative to NW. This could be

21   attributed to greater collection variability associated with NW and a dilution effect of saline

22   in NW. NFS have been previously shown to yield adequate numbers of respiratory epithelial

23   cells for detection of viruses(4). This evidence suggests NFS is a suitable alternative

24   sampling device for detection of viruses as reported elsewhere (4, 9). NFS sensitivity

25   estimates of above 90% for a range of viruses have been reported in a study comparing NFS

and NPA using M-PCR assays(4). However, conflicting findings were reported when per-nasal (shallow) flocked swabs and NPA were compared in immuno-compromised patients(14). Posterior nasopharyngeal sampling seems to be a prerequisite to achieving comparable sensitivity to NPA or NW.

We conclude that NFS collection offers a suitable alternative to NW collection based on performance and acceptability for detection of RSV by IF and for detection of common respiratory viruses in general by M-PCR.

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3
4

Table 1: Sensitivity of real-time multiplex PCR for detection of respiratory viruses on nasal wash vs. posterior nasopharygeal flocked swab collections

(n=299)	No. of viruses detected by:				Sensitivity (95% CI*) for:		
Respiratory virus	Both	NW only	NFS only	Either NW/NFS[£]	NW	NFS	P value[$]
Rhinovirus	50	8	21	79	73.4 (62.2-82.7)	89.9 (81.0-95.5)	0.024
RSV	61	4	9	74[&1]	87.8 (78.2-94.3)	94.6 (86.7-98.5)	0.146
RSV A	*26*	*4*	*4*	*34*	*88.2 (72.5-96.7)*	*88.2 (72.5-96.7)*	*1*
RSV B	*35*	*0*	*5*	*40*	*87.5 (73.2-95.8)*	*100 (91.1-100)*	*0.063*
PIV	25	7	5	37[&2]	86.5 (71.2-95.5)	81.1 (64.8-92.0)	1
Adenovirus	7	3	12	22	45.5 (24.4-67.8)	86.4 (65.1-97.1)	0.035
Corona virus	16	3	3	22	86.4 (65.1-97.1)	86.4 (65.1-97.1)	1
hMPV	12	1	2	15	86.7 (59.5-98.3)	93.3 (68.1-99.8)	1
Total[¶]	172	26	52	250	79.2 (73.6-84.1)	89.6 (85.1-93.1)	0.0043

KEY: NW, nasal wash; NFS, nasopharyngeal flocked swab; RSV, respiratory syncytial virus; PIV, parainfluenza virus 1, 2, 3 & 4; hMPV, human metapneumovirus; CI: confidence interval; * one-sided 97.5% CI reported if sensitivity was 100%; [£] Virus positive by either NW or NFS was considered the true positives for the sensitivity analysis; $, Exact McNemar's significance probability values comparing sensitivities for NW and NFS; [&1] includes one co-infection of RSV A and B; [&2] includes one co-infection of PIV3 and 4; [¶] shows all viruses detected which includes one influenza A infection detected in both NFS and NW.