Added value of an oropharyngeal swab in detection of viruses in children hospitalized with lower respiratory tract infection

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Abstract (word count=48): Paired nasopharyngeal and oropharyngeal swabs collected from 533 children hospitalized with LRTI were assessed by multiplex RT-PCR. Oropharyngeal swabs increased the number of viral infections detected by 15%, compared to collection of a nasopharyngeal swab alone. This advantage was most pronounced for detection of influenza, parainfluenza, and adenovirus.

Main text (word count=1481, excluding acknowledgements)

Samples from the nasopharynx (NP) or oropharynx (OP) are often used to diagnose respiratory tract infections. Using modern molecular diagnostics, several studies have described the performance of a NP flocked swab sample in detecting respiratory viruses [1, 3, 8, 11]. OP swab samples have been found to be consistently less sensitive than NP samples for a variety of viruses; however, maximum sensitivity is attained by using multiple types of samples [6, 7, 9]. Through a standardized multi-site study we aim to describe the etiology of respiratory tract infection in children in developing countries. Before beginning this study we evaluated the added diagnostic value of an OP swab over and above diagnoses made by a NP swab.

Children aged 1 day – 12 years admitted with a lower respiratory tract infection (LRTI) to Kilifi District Hospital, Kenya between October 2009 and August 2010, were eligible for participation. LRTI was defined as a history of cough and/or difficulty breathing and at least one accompanying respiratory sign (ie, indrawing, nasal flaring, central cyanosis, head nodding, or tachypnea). Written informed consent was obtained from the parent or guardian of each participant. Paired NP and OP samples were collected from each child on the day of admission. A NP flocked swab (Copan Diagnostics, Inc., Italy) was passed into the posterior nasopharynx via the nostril, rotated 2-3 times, withdrawn and placed in a vial of transport medium (25mg/ml veal infusion, 5mg/ml bovine factor V, 50mg/ml...
gentamicin and 250µg/ml amphotericin B; prepared in-house). A polyurethane foam-tipped OP swab (Sigma-swab, Medical Wire & Equipment, England) was inserted into the mouth, swabbed over the tonsils and posterior pharynx, withdrawn and placed in a vial of transport medium (as above). Samples were stored at 4°C until analyzed. All samples were analyzed within 48 hours of collection.

Nucleic acid was extracted from samples using either a Qiagen viral RNA miniprep kit (Qiagen, UK; sample volume = 140µL) or a total nucleic acid extraction kit (Roche Applied Science, Germany) with a MagNA Pure LC32 automated NA extractor (sample volume = 200µL). Paired samples were extracted using the same method. To ensure co-extraction of both DNA and RNA, no DNase-step was included. To allow for extraction of nucleic acid from any intracellular pathogens such as Mycoplasma pneumoniae, the samples were not spun down to pellet cellular debris prior to extraction.

Extracted samples were tested by a modified multiplex (MPX) RT-PCR assay in triplexes for 16 respiratory pathogens -- respiratory syncytial viruses (RSV) A and B, adenovirus, rhinovirus, human metapneumovirus (HMPV), coronaviruses (NL63, OC43, 229E), parainfluenza viruses (PIV) 1-4, influenza viruses (A, B, C), and Mycoplasma pneumoniae[4]. The complete list of primer and probe sequences is shown in Table 1. The primers were obtained from either Sigma-Genosys (Sigma Aldrich, UK) or MWG (Eurofins MWG Operon, UK). The hydrolysis probes using black hole quencher (BHQ) molecules were obtained from MWG (Eurofins MWG Operon, UK) and the hydrolysis probes using TAMRA or minor-groove binder non-fluorescent quenchers (MGBNFQ) molecules were obtained from ABI (Applied Biosystems, UK). Reverse transcription was carried out for 20 minutes at 50°C. Platinum taq polymerase was activated at 95°C for 5 minutes, followed by 40 cycles of PCR performed at 95°C for 15s and 60°C for 30s. The
assays were run using the Qiagen Quantifast Multiplex RT-PCR kit (Qiagen, UK) in
triplex sets and analysed on an ABI 7500 (Applied Biosystems, UK) using SDS 3.2
software. The control materials were either RNA supplied by the West of Scotland
Specialist Virology Centre (Gartnavel Hospital, Glasgow, UK) or PCR products
generated in-house. The positive controls consisted of viral RNA or PCR products for:
influenza viruses A, B and C; parainfluenza viruses 1-4; human coronaviruses 229E,
OC43 and NL63; HRV; human metapneumoviruses A and B; and RSV A and B. The
control material for adenovirus and *Mycoplasma pneumoniae* consisted of plasmids
cloned with PCR target sequences. Specimens were deemed to be “positive” for a
particular pathogen if the cycle threshold value was less than 35. Samples that were
positive for influenza A by MPX PCR were tested for 2009 H1N1 influenza with a real
time RT-PCR assay (CDC, USA) [2].

To estimate sensitivity, the gold standard for the presence of a pathogen was defined as
a positive result in either the NP or the OP swab. All 95% confidence intervals were
estimated using exact methods. The added value of an OP swab was calculated as the
ratio (number of infections detected by either swab / number of infections detected by a
NP swab) minus one and expressed as a percentage. Statistical analyses were
performed using STATA 11.0 (Stata Corp, College Station, Texas).

Paired NP and OP swabs were collected from 533 children admitted to the hospital with
signs and symptoms of lower respiratory tract infection. The median age (interquartile
range) of participants was 18 months (4 to 21 months) and 297 (56%) were boys. A virus
was detected in 339 (64%) NP samples and 268 (50%) OP samples. Overall, the OP
sample increased the number of children detected to have a viral infection by 8.8%
(Table 2; 8% [22 children] in those aged <24 months, 10% [5 children] in those aged 24-
59 months, and 18% [3 children] in those aged ≥60 months). Collection of an OP sample increased the total number of different viruses detected by 15.3% (Table 2; 13% [45 viruses] in those aged <24 months, 27% [15 viruses] in those aged 24-59 months, 24% [4 viruses] in those aged ≥60 months). Co-infection with two or more viruses was detected in 66 (12%) NP samples and 46 (9%) OP samples. The viruses most commonly detected in children with co-infection were rhinovirus, parainfluenza virus, and adenovirus. The added value of the OP sample varied by virus from 9% (rhinovirus) to 31% (adenovirus). None of the samples tested positive for *Mycoplasma pneumoniae*.

The primary aim of the study was to assess the added diagnostic yield from an OP swab; therefore, the study was not powered to determine whether sensitivity of detection differed between the two collection methods. Despite this, the 95% confidence interval (CI) around the sensitivity estimates suggest that the NP sample was more sensitive for detection of RSV, parainfluenza viruses, coronaviruses, HMPV, and rhinovirus. The sensitivity of a NP swab for all viruses assessed was 86.7% (95% CI: 83.3, 89.6) and the sensitivity of the OP swab was 66.2% (95% CI: 61.8, 70.4).

Other than supportive care, no specific treatment is available for most of the viruses studied. However, accurate detection of influenza infection can be important for case management. In the setting of pandemic influenza, the practice of sampling both the NP and OP has been recommended but the value of double sampling has not been clearly established [5, 12]. OP swabs were more sensitive than nasal swabs (type of swabs not specified) for detection of the 2005 H5N1 influenza virus [5]; however, a study conducted during the 2009 influenza epidemic in Thailand found that Dacron-tipped OP and nasal swabs had comparable sensitivity for detection of H1N1 by RT-PCR [10]. In the present study, 6 (18%) influenza infections, including 3 (25%) of 12 infections with the 2009
H1N1 influenza strain, were detected only in the OP sample, suggesting that double sampling is beneficial for maximal detection of this treatable but sometimes fatal illness.

It is not known whether maximal detection of respiratory viral infection is achieved through sampling of multiple anatomical sites or collection of multiple samples from the same site. We elected to sample from different anatomical sites (NP and OP) because of limited data suggesting differential detection of some pathogens (eg, influenza) from different sites. Additional studies would be required assess whether collection of an additional NP sample, rather than an OP sample, would have resulted in a similar increase in viral detection. The majority of extractions were done using a Qiagen kit, with 7% of samples done by MagNA Pure; re-analysis excluding the latter did not materially alter the findings (results not shown). The types of NP and OP swabs used in this study were selected based on availability and design, with both purporting to offer improved specimen uptake and release. Additional studies would be required to assess how swab design and material affects sensitivity.

Selection of a sampling method for detection of respiratory viral infection must balance epidemiological sensitivity against the feasibility, costs, and time required for specimen collection. The collection of an OP swab is relatively quick and simple and our experience is that it is well accepted; clinical staff, along with most patients and parents, are familiar with the procedure. Reduction in the cost of consumables can be achieved by placing the OP swab and NP swab into the same vial for transport to the lab and testing. For clinical settings or for comprehensive studies of the etiology of lower respiratory tract infection, collection of an OP swab in addition to an NP swab increases the detection of viral infections.
This work was supported by a Wellcome Trust grant (084633) awarded to DJN and by the Bill and Melinda Gates Foundation through the PERCH project (Pneumonia Etiology Research for Child Health). All authors report no conflict of interest. This study was approved by the Kenyan National Ethical Review Committee (SSC 1536). We offer sincere thanks to the clinical and laboratory staff for their dedication and hard work in collecting and processing these specimens, to Bill Carmen (West of Scotland Specialist Virology Centre, Scotland) for providing real-time PCR methods, and to Pat Cane (Health Protection Agency, United Kingdom) for oversight of molecular techniques. This paper is published with the permission of the Director of the Kenya Medical Research Institute.
REFERENCES


<table>
<thead>
<tr>
<th>Virus Target</th>
<th>Target Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Probe sequence</th>
<th>Product size (bp)</th>
</tr>
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<td>Influenza A Matrix</td>
<td>AAGCACAGACACAAAAYTCGTACCCTCTT</td>
<td>TCTACGTYTGCACTCCYGCYCT</td>
<td>FAM-TYACGCTCACCGTGCCCAGTG-BHQ1</td>
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<td>Influenza B NS</td>
<td>ATGATCTTACAGTGAGAGATAAGAAG</td>
<td>CGAATTGGCTCTTGATATGCTT</td>
<td>CYS-ATGCCCATCGATCTCCTCAAYTCACTCT-BHQ2</td>
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<tr>
<td>Influenza C Matrix</td>
<td>GGCAGAGCACTATGTGAAA</td>
<td>TCCAGTGCYTCTTACATTCTCTT</td>
<td>VIC-TCTCCCTTCTTGTTTTTTGAAA-MGBNFQ</td>
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<td>GCYGTACCTCAGTCCAATTTCA</td>
<td>TCCAATGATTGCTGAAAATTGCT</td>
<td>VIC-CACATATTAGAAACCTTCT-MGBNFQ</td>
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<td>HMPV B Fusion</td>
<td>GCYGTACCTCAGTCCAATTTCA (Common with A)</td>
<td>GTTATCCCTGCATTGCTCTGAAAATTCT</td>
<td>VIC-CGCACAACATTAGAAACCTTCT-MGBNFQ</td>
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<td>CCTGATCCTCAGTACCCTAACAGA</td>
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<td>PIV 3 HN</td>
<td>CCGGGATATAYTAYAAGGCAA</td>
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<td>FAM-TGGATCAGGCTTCCTCAAAAYCTTCTT-MGBNFQ</td>
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<tr>
<td>PIV 4 Fusion</td>
<td>CAGAYACATCAGTCCAATTTCAA</td>
<td>TGACTACCTGACTGCCCACAARA</td>
<td>CYS-COMATCAGAGCTCAGAATYCAAA-GTCT-BHQ2</td>
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<td>CAGTCAAATTGGGCTGATGCAA</td>
<td>AAAGGGCTATAAAAGAGAAATAGGTTGCTTCT</td>
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<td>CAGTGAAGGCTATCCGACTAGGT</td>
<td>CCTTCTGCTGCTCTTAAATGATTACCC</td>
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<td>HCV NL63 1a</td>
<td>AGCTACCTTCTATTGAAAGCAGTATTTATTAA</td>
<td>AGAAGATCTAATGTTATCTAAAATACCG</td>
<td>VIC-ATTGGAAGGCTTCTCAAGCTAGAGT</td>
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<td>AAGATGCAATTTCAATATTCACAGA</td>
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<td>RSV B NP</td>
<td>AAGATGCAATTTCAATATTCACAGA</td>
<td>TTCTGACACATGATTAGGAG</td>
<td>FAM-CACATCACAGGAGAGAGAGAGAGTAGT</td>
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<tr>
<td>Rhirovirus 5' UTR</td>
<td>TGAGAACGGCTGTGAGAGAC</td>
<td>CAAAGTACGTGCCTTACAAC</td>
<td>VIC-TCTGACAGCACCGTGGTGTTGTCCA-BHQ2</td>
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<tr>
<td>Adenovirus Matrix</td>
<td>GGCAGTGAGGGTCTTCTTATTACCT</td>
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<td>VIC-TCTGACAGCACCGTGGTGTTGTCCA-BHQ2</td>
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<td>Mycoplasma pneumoniae</td>
<td>CACCATCATCCTGACAGTAC</td>
<td>CACCATCATCCTGACAGTAC</td>
<td>CYS-CCTGGCTGGACAGGCTACTCTC-BHQ2</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Viral detection by multiplex PCR of paired nasopharyngeal (NP) flocked swabs and oropharyngeal (OP) swabs collected from children aged 1 day - 12 years admitted to Kilifi District Hospital with lower respiratory tract infection (n=533)

<table>
<thead>
<tr>
<th>Virus</th>
<th>NP+</th>
<th>Only NP+</th>
<th>Only OP+</th>
<th>% Increase using OP swab*</th>
<th>% Sensitivity NP (95% CI)</th>
<th>% Sensitivity OP (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV A&amp;B</td>
<td>83</td>
<td>24</td>
<td>13</td>
<td>12 (7.20)</td>
<td>89 (82, 94)</td>
<td>80 (72, 87)</td>
</tr>
<tr>
<td>PIV 1-4</td>
<td>22</td>
<td>27</td>
<td>10</td>
<td>20 (10, 34)</td>
<td>83 (71, 92)</td>
<td>54 (41, 67)</td>
</tr>
<tr>
<td>Influenza A-C</td>
<td>21</td>
<td>6</td>
<td>6</td>
<td>22 (9, 42)</td>
<td>82 (65, 93)</td>
<td>82 (65, 93)</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>12</td>
<td>10</td>
<td>3</td>
<td>14 (3, 35)</td>
<td>88 (69, 98)</td>
<td>60 (39, 79)</td>
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<tr>
<td>Adenovirus</td>
<td>29</td>
<td>22</td>
<td>16</td>
<td>31 (19, 46)</td>
<td>76 (64, 86)</td>
<td>67 (55, 78)</td>
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<tr>
<td>HMPV</td>
<td>34</td>
<td>23</td>
<td>7</td>
<td>12 (5, 24)</td>
<td>89 (79, 96)</td>
<td>64 (51, 76)</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>53</td>
<td>51</td>
<td>9</td>
<td>9 (4, 16)</td>
<td>92 (85, 96)</td>
<td>55 (45, 64)</td>
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<td>Any virus</td>
<td>238</td>
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<td>30</td>
<td>8.8 (6.0, 12.4)</td>
<td>91.9 (88.6, 94.5)</td>
<td>72.4 (67.6, 76.9)</td>
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<td>All viruses</td>
<td>255</td>
<td>163</td>
<td>64</td>
<td>15.3 (12.0, 19.1)</td>
<td>86.7 (83.3, 89.6)</td>
<td>66.2 (61.8, 70.4)</td>
</tr>
</tbody>
</table>

* CI – confidence interval; RSV – respiratory syncytial virus; PIV – parainfluenza virus; HMPV – human metapneumovirus

% Increase = \frac{(NP+ + OP+) + (Only NP+) + (Only OP+)}{(NP+ + OP+)} \times 100