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Short communication

Most human metapneumovirus and human respiratory syncytial virus in infant nasal secretions is cell free

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Abstract

Background: Nasopharyngeal secretions aspirated from infants with bronchiolitis (NPA) are a valuable resource for the study of virus dynamics and local inflammatory responses, however samples are small and difficult to manipulate.

Objectives: To improve yield of NPA from infants. To establish if removal of the cellular component of NPA affects quantification of human metapneumovirus (hMPV) or human respiratory syncytial virus (hRSV) genome.

Study design: Weight of NPA collected into traps from 30 infants was compared with that collected in trap plus catheter and washed through with saline from another 30 infants. hMPV ($n = 33$) and hRSV ($n = 30$) genome was measured by reverse-transcribed real-time polymerase chain reaction (RT-RT-PCR) in paired whole and cell-free NPA collected by the improved method.

Results: The improved method of NPA collection gave near two-fold greater weight ($p = 0.002$) of NPA (mean = 0.52 g (S.D. = 0.30 g)) than the traditional method (0.32 g (S.D. 0.19)). There was strong agreement and no significant difference between viral load measured in whole and cell-free fractions of NPA for both viruses (samples (n), correlation coefficient (cc) and significance (p)); hMPV ($n = 33$, cc = 0.938, $p < 0.001$) and hRSV ($n = 30$, cc = 0.977 and $p < 0.001$).

Conclusions: The majority of hRSV and hMPV in nasal secretions is not associated with cells. Removal of the cellular component of NPA does not interfere with quantification of hRSV and hMPV.

Keywords: Viral-load quantification; hRSV; hMPV; Nasopharyngeal aspirate; Human infants

1. Introduction

Bronchiolitis is the clinical diagnosis and histopathological description of a seasonal respiratory tract infection in human infants. A spectrum of disease severity is observed ranging from coryza with cough through to failure of gas exchange requiring mechanical ventilation. The commonest causal pathogen is human respiratory syncytial virus (hRSV) followed by human metapneumovirus (hMPV) (Garcia et al., 2006). Nasopharyngeal respiratory secretions collected from infants with bronchiolitis by direct aspiration (nasopharyngeal aspirate, NPA) are a valuable resource for the study of virus dynamics and local inflammatory responses. The cellular and cell-free fractions of respiratory secretions can be used for different purposes such as immunocytochemistry and cytokine quantitation. NPA samples are difficult to handle, as they are viscous, tenacious and small (Bont et al., 2001).

We describe a method for improved collection and preparation of NPA from infants and quantification of hMPV and hRSV virus genome load by reverse-transcribed real-time polymerase chain reaction (RT-RT-PCR). We show that these methods provide ample quantities of respiratory secretions providing nucleic acids for use in quantitative assays. We demonstrate that removal of the cellular component of NPA

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for use in other studies does not alter quantification of hMPV or hRSV. We deduce that the majority of both viruses in nasal secretions is not associated with cells.

2. Method

2.1. Participants

Ethical approval was given by The Liverpool Children's Local Research Ethics Committee (LREC ref. 01/02/RE). Infants were prospectively recruited following admission to The Royal Liverpool Children's Hospital (Alder Hey) with a clinical diagnosis of bronchiolitis over the winter season of 2004/2005. Bronchiolitis was diagnosed by attending paediatricians when infants (children < 2 years old) presented with tachypnea (>50 breaths/min), subcostal recession, and bilateral inspiratory crackles on auscultation. All infants in this report were admitted to general paediatric wards for feeding difficulties and or supplemental oxygen. None were ventilated or sedated at time of sampling. Written consent was obtained from parents. This report forms part of the study "Respiratory Syncytial (RSV) Bronchiolitis: Clinical and Immunological Associations in Infancy" (UK National Research Register N0461108820).

2.2. Improved method for collection of NPA

NPA was collected within the first 24 h of admission by MGS assisted by nursing staff as follows. Infants were swaddled in a small blanket, placed supine in a cot and held by the nurse. Nasal secretions from both nostrils were aspirated without lavage using a soft size 10F catheter with two distal lateral eyes and a proximal side port for finger-tip suction control (CARETIP^{TM} atraumatic-neonatal, Meddis Ltd. UK) connected to a conical trap (Tracheal suction set, Unomedical Ltd. UK) by medical vacuum (20 mmHg) while advancing and withdrawing the catheter tip to a depth of approximately 5 cm.

The collection trap and catheter containing NPA were immediately taken to a laboratory and weighed. One aliquot of 2 ml of sterile normal saline (0.9% NaCl in H_2O) was repeatedly gently aspirated (10 mmHg) through the catheter to the trap (typically 3×), mixing and mobilising the secretions. One fresh 1 ml aliquot of saline was then aspirated through the catheter to remove residual secretions, producing a mixture of NPA and 3 ml of saline in the conical trap. The collection set and NPA solution was reweighed.

The NPA solution was decanted through saline-wetted nylon monofilament gauze with a 60 μm pore size (Sefar Nitex 03-48/31, Sefar Inc., Switzerland) to remove bulk mucus. The volume of NPA filtrate was recorded. The empty collection set was weighed again permitting calculation of total weight of NPA collected.

In preliminary experiments which used the traditional method for collecting NPA the suction catheter was discarded at the bedside and only the trap containing NPA was retained, weighted and 3 ml of saline directly added.

2.3. Comparison of viral load in whole and cell-free fractions of NPA

A 500 μl aliquot of NPA filtrate was put aside as "whole sample". The remaining NPA filtrate was subject to centrifugation at 300 × *g* for 5 min at +4 °C and the supernatant carefully removed, split into 500 μl aliquots as "cell-free sample". All aliquots stored at −70 °C.

2.4. Nucleic acid extraction, reverse transcription and RT-PCR

Nucleic acid was extracted from 140 μl of each pair or "whole" and "cell-free" NPA samples using QIAamp^{TM} viral RNA MiniKit^{TM} (Quiagen Ltd., UK). Ten microlitre of nucleic acid solution was reverse transcribed in the presence of hMPV or hRSV specific primers as previously described (Table 1) (Greensill et al., 2003). cDNA solutions were stored at −70 °C.

One microlitre of cDNA solution was subject in duplicate to RT-PCR using novel primers designed by BE specific to highly conserved regions of the hMPV L gene and hRSV N gene using an Opticon 2^{TM} thermal cycler (Genetic Research Instrumentation Ltd., UK). Various RT-PCR optimisation assays were performed (data not shown).

Each RT-PCR reaction consisted of 20 mM Tris–HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM of each primer pair, 250 pM dNTPs, 0.15% Triton X-100, 20 pg BSA, 1 unit platinum Taq Polymerase (Cat. No. 10966-026, Invitrogen), 0.5× SYBR Green I (Cambridge Bioscience, UK), in a final reaction volume of 20 μl.

PCR conditions consisted of an initial denaturing step at 95 °C for 10 min, followed by 35 cycles of denaturation

Table 1
Primers used for reverse transcription and RT-PCR (all read 5′–3′)

Primers	Sequence
hMPV RT L6 (Peiris et al., 2003)	CAT GCC CAC TAT AAA AGG TCA G
hMPV PCR BE-L forward	TCC ATG GAA ACA ATC TTA TT
BE-L reverse	CCC CAG TCT TTC TTG AAA AT
hMPV template	TCC ATG GAA ACA ATC TTA TTG AGT CTT TAT CAG CAG CAT TAG CAT GTC ATT GGT GTG GGA TAT TAA CAG AAC AAT GCA TAG AAA ATA ATA TTT TCA AGA AAG ACT GGG G
hRSV RT N1 (Cane and Pringle, 1991)	GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC
hRSV PCR BE-N forward	TGG GTG GTG AAG CAG GAT TCT A
BE-N reverse	CAG CAT TGC CTA ATA CTA CAC T
hRSV template	TGG GTG GTG AAG CAG GAT TCT ACC ATA TAT TGA ACA ACC CAA AAG CAT CAT TAT TAT CTT TGA CTC AAT TTC CTC ACT TCT CCA GTG TAG TAT TAG GCA ATG CTG