Establishing a biologically relevant epithelial airway model of polymicrobial-Respiratory Syncytial Virus infection (RSV) and nontypeable *Haemophilus influenzae* (NTHi) biofilm colonization

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Introduction/Aim:
Infections of the respiratory airway rarely involves a single pathogen aetiology. Modelling the human respiratory epithelium has been a challenge due to its complex structure multiple cell types. Using juvenile epithelial brushings, we generated a polarised epithelial layer using the Air-Liquid Interface (ALI) method and infected or inoculated two common but significant respiratory pathogens, namely Respiratory Syncytial Virus (RSV) and nontypeable *Haemophilus influenzae* (NTHi) to assess the impact on respiratory airway epithelial cells of sequential infection by the virus on NTHi biofilms and *vice versa*, characterise their behaviour through various functional, immunological, bacterial and viral endpoints.

Methods:
Red fluorescent tagged RSV (A2 strain, rrRSV-BN1, 1 x 10^6 pfu) and Green fluorescent tagged NTHi (86-028 NP, 2.5 x 10^7 CFU) were inoculated individually, concurrently or sequentially onto differentiated and polarised human airway epithelial cells. RSV infection and/or NTHi colonisation were allowed to proceed for 7 days before three washes of 0.2ml 1X PBS were performed on the apical epithelial surface. Washes are used to determine bacterial and viral loads via qPCR, as well as cytokine profiles determined via multiplex. Apical and basal insert surfaces were fixed with Hoechst 33342, excised, mounted cell surface up with ProLong Gold antifade mountant and overlayed with coverslips. Five confocal microscopy field-of views of the inserts were obtained on a C2+ system (Nikon), and 1.2µm Z-stacks thickness used to reconstruct 3-dimensional images of the epithelium. Biofilm structural analysis was performed using the COMSTAT program. Image stacks were examined for: biomass thickness (µm), surface area (µm^2), and surface area to biomass ratio.

Results:
Red fluorescence from RSV absent in epithelial cells inoculated four days earlier with NTHi when examined through confocal microscopy (at Day7-post primary inoculation/infection).

Event	Infection Groups					
	1 (NTHi only Control)	2 (RSV only Control)	3 (NTHi, RSV)	4 (RSV, NTHi)	5 (Concurrent NTHi+RSV)	6 (-ve Control)
Day 0 - Inoculation/Infection	GFP tagged-NTHi 86-028NP+pRSM2211 ($2.5x10^7$ CFU)	RFP tagged-rrRSV-BN1 ($1x10^6$ PFU)	GFP tagged-NTHi 86-028NP+pRSM2211 ($2.5x10^7$ CFU)	RFP tagged-rrRSV-BN1 ($1x10^6$ PFU)	GFP tagged-NTHi 86-028NP+pRSM2211 ($2.5x10^7$ CFU) + RFP tagged-rrRSV-BN1 ($1x10^6$ PFU)	20µl 1XPBS
Day 4-Post 1° Inoculation/Infection			RFP tagged-rrRSV-BN1 ($1x10^6$ PFU)	GFP tagged-NTHi 86-028NP+pRSM2211 ($2.5x10^7$ CFU)		
Day 7-Post 1° Inoculation/Infection						
Confocal Microscopy:	Green fluorescence (NTHi) on epithelial cells.	Red fluorescence (RSV) in susceptible epithelial cells	Only Green fluorescence (NTHi) on epithelial cells	Green fluorescence (NTHi) on epithelial cells. Red fluorescence (RSV) in susceptible epithelial cells	Green fluorescence (NTHi) on epithelial cells. Red fluorescence (RSV) in susceptible epithelial cells	No Green or Red Fluorescence observed
Z-Stacks [NTHi Biofilm Thickness (µm)]	50-100	Absent	50-100	50-100	50-100	Absent
Density of NTHi Biofilm	+++	Absent	+++	++	++	Absent
Density of RSV infected epithelial cells	Absent	+++	+	+++	+	Absent

Conclusion:

Prior NTHi colonisation of respiratory epithelium elicits protection against a secondary RSV infection, but not *vice versa*.

Grant Support:

N/A