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ORIGINAL ARTICLE

# Preceding human metapneumovirus infection increases adherence of *Streptococcus pneumoniae* and severity of murine pneumococcal pneumonia

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*Background:* Coinfection with respiratory virus and *Streptococcus pneumoniae* has been frequently reported in several epidemiologic studies. The aim of this study was to explore the effect of preceding human metapneumovirus (hMPV) inoculation on subsequent pneumococcal infection.
*Methods:* Hep-2 and A549 cells were infected with hMPV then inoculated with *S. pneumoniae*. Bacterial adhesion was measured using colony forming unit and cytometric-fluorescence assays. *In vivo* bacterial adhesion was examined in hMPV-infected mice after inoculation of fluorescence-conjugated *S. pneumoniae*. Pulmonary inflammation (bacterial titers, cytokine levels, and histopathology) of hMPV-infected mice was investigated after inoculation with *S. pneumoniae*.
*Results: In vitro* results of bacterial infection with *S. pneumoniae* on A549 and Hep-2 monolayer cells showed that even though cellular adherence was variable among different serotypes, there was significantly enhanced bacterial adherence in A549 cells with preceding hMPV infection. In addition, *in vivo* study of hMPV-infected mice showed increased adhesion of *S. pneumoniae* on the bronchial epithelium with delayed bacterial clearance and exacerbated histopathology. Furthermore, mice with preceding hMPV infection showed repressed recruitment of airway neutrophils with decreased expression of neutrophil chemoattractants during pneumococcal infection.

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*Conclusion:* These results suggest that hMPV-infected airway cells, especially the lower airway epithelium, express increased adherence with *S. pneumoniae*. Furthermore, hMPV-infected mice showed impaired recruitment of airway neutrophils, possibly leading to delayed bacterial clearance and exacerbated pulmonary inflammation, after secondary infection with pneumococcal isolates.


## Introduction

*Streptococcus pneumoniae* is a common cause of invasive disease and respiratory tract infections and is among the most common pathogens of community-acquired pneumonia in children.[1–3] Human metapneumovirus (hMPV) is a new member of the genus *Metapneumovirus*. hMPV was first identified in respiratory specimens obtained from young children with various respiratory syndromes,[4] and this virus is genetically and clinically similar to respiratory syncytial virus (RSV). hMPV and RSV are among the most common pathogens causing lower airway infection during infancy.[5]

*S. pneumoniae* can be easily isolated from the nasopharyngeal airway of healthy children.[6] Colonization of *S. pneumoniae* is common in children, and invasion into the respiratory tract requires bacterial adherence to the epithelial lining of the airway. Reports have shown that preceding or even simultaneous infections with a respiratory virus, including influenza virus, RSV, parainfluenza virus, rhinovirus, and adenovirus, occur during pneumococcal disease.[1–3] The concept of a respiratory virus predisposing to a bacterial infection was first considered during the influenza pandemic in 1918. Decreased pulmonary clearance of *S. pneumoniae* and significant lethal synergism have been observed after influenza infection in animal models,[7,8] which could be mediated by the neuraminidase on the capsid of the influenza virus.[8,9] Enhanced adherence and decreased pulmonary clearance of *S. pneumoniae* were also observed in several *in vitro* and *in vivo* studies of RSV.[10,11] It has been suggested that viral G protein expressed on infected epithelial cells might facilitate the adherence of *S. pneumoniae*.[12] It has also been observed that increasing expression of platelet-activating factor receptor on respiratory epithelial cells after RSV infection can enhance pneumococcal adhesion.[13]

Several recent publications have reported coinfection with hMPV and pneumococcus that resulted in exacerbated pneumococcal pneumonia in mice.[14–17] However, a detailed effect of hMPV infection on subsequent pneumococcal disease has not been studied. The aim of this study was to clarify how hMPV infection causes further exacerbation of pneumococcal pneumonia while focusing on its effect on bacterial adhesion and invasion. To our knowledge, this is the first report to clarify that preceding hMPV infection indeed augments *in vitro* and *in vivo* pneumococcal adhesion, which explains the underlying mechanism of decreased pulmonary clearance of *S. pneumoniae* in hMPV-infected mice.

## Materials and methods

### Cell cultures, hMPV, and *S. pneumoniae*

Hep-2 (ATCC CCL-23) and A549 cells (ATCC CCL-185) were used for adhesion studies. The hMPV strain CAN97-83 was obtained with kind permission from Dr Roberto P. Garofalo (University of Texas Medical Branch, Galveston, TX, USA). hMPV was propagated in LLC-MK2 cells and viral titer was determined by a cell-based immunoassay.[18] *S. pneumoniae* serotype (ST) 3 and ST14 were attained from clinical samples and resuspended to a concentration of $10^8$ colony-forming units (CFU)/mL.[12] To prepare the fluorescein isothiocyanate-labeled *S. pneumoniae* (FITC-SP), the vital bacteria were mixed with FITC and resuspended in a solution at a final concentration of $10^8$ CFU/mL.[10]

### CFU-based adhesion assay

A549 cells and Hep-2 cells were infected with hMPV at 1.0 multiplicity of infection (MOI) for 1 hour and incubated for 48 hours. The cells were then infected with *S. pneumoniae* at 100 MOI and incubated for 30 minutes. After washing the unbound bacteria, cells were then detached with cell dissociation solution (Sigma-Aldrich, St Louis, MO, USA) and resuspended. CFU of the original bacterial suspension (total CFU) and of the suspension after the adherence assay (final CFU) were estimated by the CFU-counting method.[10] The bacterial concentration (CFU/mL) was calculated as CFU $\times$ dilution $\times$ 10 (10 $\mu$L/spot). Adherence percentages were calculated as final CFU/total CFU $\times$ 100.

### Cytometric fluorescence adhesion assay and microscopic visualization of adhesion of FITC-SP

A549 cells in plates were infected with hMPV at 1.0 MOI for 1 hour and incubated for 48 hours. FITC-SP ST14 at 25 MOI was then added after removing medium from hMPV-infected or uninfected monolayers cells. The plates were spun to facilitate attachment of bacteria, then incubated for 30 minutes. Cells were detached with cell dissociation solution and fixed with formaldehyde. Cells adhered with FITC-SP were counted and analyzed with a FACSCalibur instrument using CellQuest (BD Bioscience, San Jose, CA, USA) software.

To visualize the adhesion of FITC-SP on cells, A549 cells were inoculated with hMPV and subsequently with FITC-SP ST14 as described above. Cells were then fixed and observed under inverted confocal microscopy (TCS SP2 Leica Microsystems, Buffalo Grove, IL, USA).