An Improved Toolkit for *In Vitro* hMPV Characterization

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Background

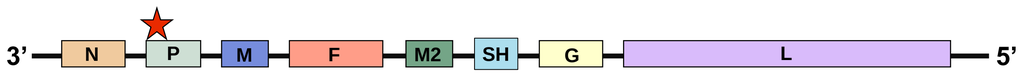
- Human metapneumovirus (hMPV) is a leading cause of acute viral respiratory infections and is associated with considerable morbidity. Young, elderly, and immunocompromised individuals are most at risk for developing severe disease.
- Nucleotide analysis of hMPV's fusion (F) and glycoprotein (G) genes class hMPV into four distinct clades: A1, A2, B1, and B2.
- Due to the slow and inconsistent growth of hMPV *in vitro*, limited reagents and assays exist for its characterization.
- Herein, we describe several advances in virus detection, quantification, and growth methods for the generation of an improved toolkit for *in vitro* characterization of multiple hMPV strains across each of the four clades.

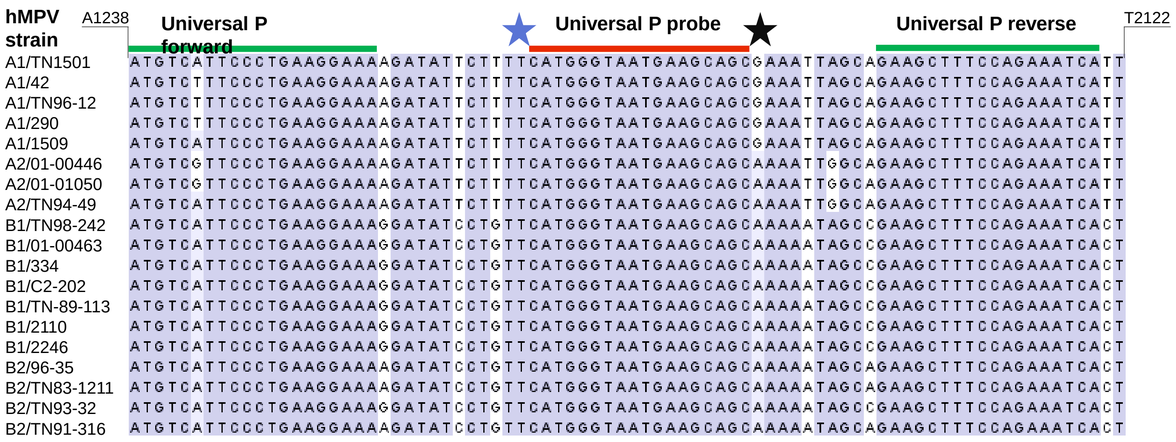
Methods

- Complete genome next-generation sequencing (NGS) of 19 hMPV clinical isolates was performed, followed by nucleotide alignment using A plasmid Editor (ApE) and Jalview. A universal RT-qPCR primer-probe set with the highest possible nucleotide sequence identity across all hMPV strains was designed to detect the phosphoprotein (P) gene. The probe contained a 5' fluorescein (FAM) dye along with a ZEN internal quencher and a 3' Iowa Black (IB®) FQ quencher (IDT, Newark, NJ).
- All 2D cell-based experiments were performed in rhesus (LLC-MK2s) kidney epithelial cells at multiplicity of infection (MOI) ranging from 0.001 to 0.014. Plaque assays for hMPV clinical isolates were developed with a microcrystalline cellulose (MCC) overlay using the A1/TN1501, A2/TN94-49, and B1/TN98-242 strains.
- Virus growth conditions were optimized from existing methods [1,2] for clinical isolates A1/TN1501, A2/TN94-49, A2/01-00446 and A2/01-01050.
- Differentiated primary human airway epithelial 3D tissues (EpiAirway™, MatTek Corporation; Ashland, MA) with an airway-liquid interface (pHAEC-ALI) were infected with the A1/1509, A2/TN94-49, A2/01-00446, A2/01-01050, B1/334 and B2/TN96-35 strains. RT-qPCR using the universal primer probe set was used to determine viral RNA amplification.

Results

Universal primer probe set design against genetically distinct hMPV genogroups





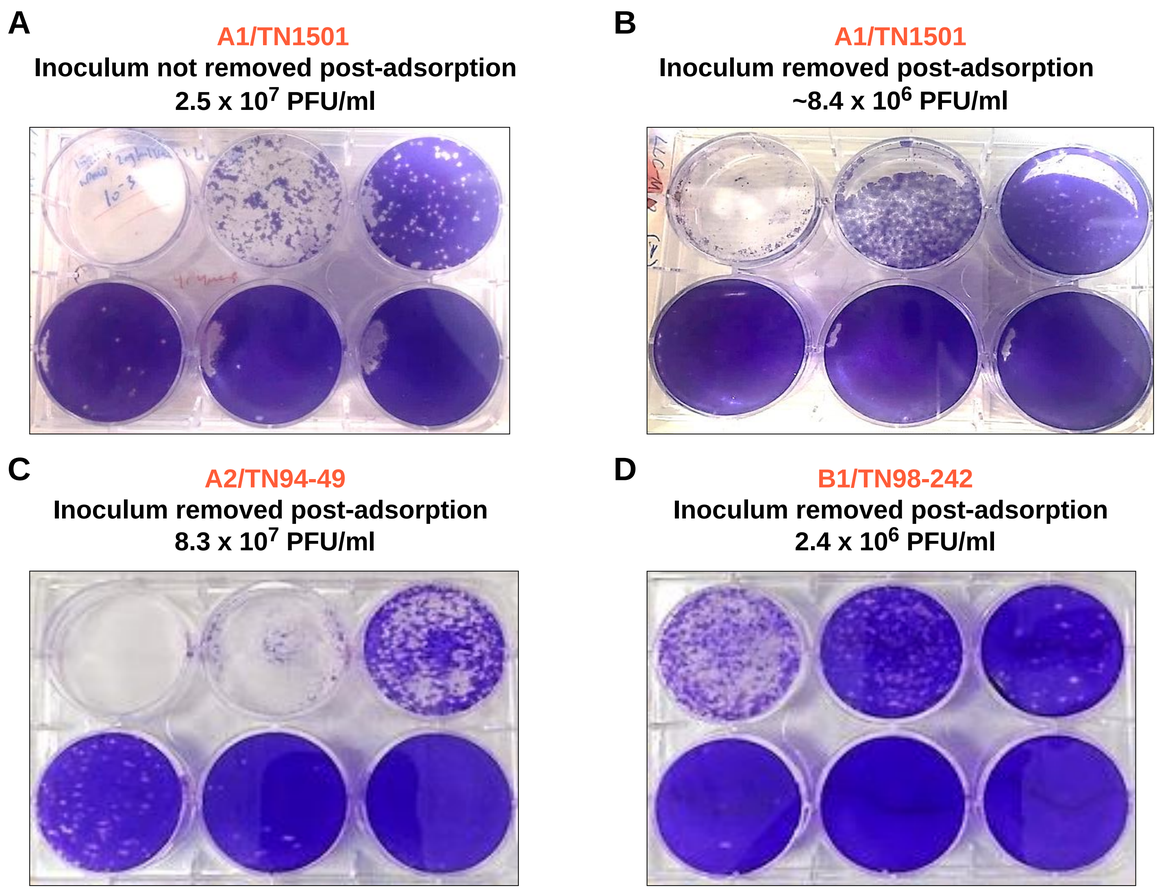
The location of the universal primer probe set within the hMPV genome is indicated by a red star. The nucleotide sequence of all hMPV clinical isolates was determined using complete genome NGS. The blue and black stars represent FAM and IBFQ quencher, respectively. Nucleotide numbering of the hMPV P gene is based on accession number KC562223.1.

Sequence of universal primer probe targeting the hMPV P gene	
Universal P forward	**5'- ATG TCA TTC CCT GAA GGA AA - 3'**
Universal P probe	**5'- FAM-CAT GGG TAA TGA AGC AGC-ZEN-IBFQ - 3'**
Universal P reverse	**5'- TGA TTT CTG GAA AGC TTC - 3'**

Nucleotide sequences of the universal primer probe set targeting the hMPV P gene. Phosphoprotein = P, fluorescein = FAM, Iowa Black = IB

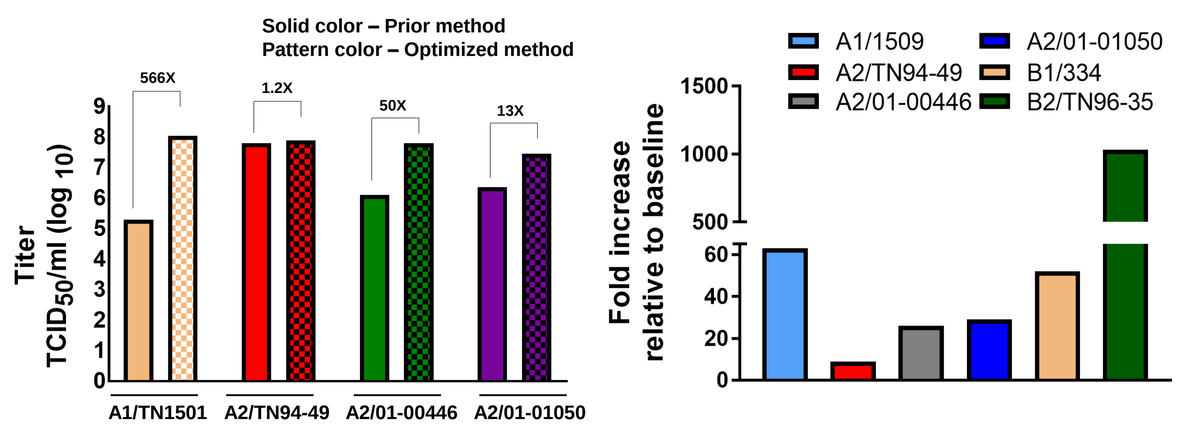
Results

Development of a plaque assay for characterizing hMPV clinical isolates



LLC-MK2 cells (1 x 10^6) were seeded day prior in 6-well plates. Virus preps were diluted 10-fold and adsorption occurred for 4 hours at 37ºC with periodic rocking. Post-adsorption, inoculum remained on **(A),** or was aspirated off **(B,C and D)** and the monolayer washed with OPTI-MEM prior to addition of the semi-solid overlay (0.6% MCC supplemented with 0.025 M HEPES, 0.12 M $NaHCO_3$, 0.05% BSA and 1.5 µg/mL TPCK-trypsin). Plates were incubated at 37ºC for 7 days. Cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Crystal violet was removed, and plates were gently washed with deionized water.

Optimization and amplification of hMPV growth in both 2D and 3D cell culture systems



Optimized Method	
TPCK-trypsin	Reduced concentration - 0.3 µg/mL
TPCK-trypsin	Daily spiking - 0.3 µg/mL
Glutamax™	Daily spiking - 200 mM

(A) hMPV titers obtained for prior [2] and optimized methods using a 2D system. Optimizations were based off methods from [1] with modifications to cell type, MOI and virus harvesting period, depending on the strain. LLC-MK2 cells (5.2 x 10^6) were seeded day prior in 10 cm^2 dishes. Virus was absorbed at a MOI of 0.001. Post-adsorption, virus growth media was added (OPTI-MEM supplemented with 0.3 µg/mL TPCK-trypsin, and 200 mM Glutamax™). Dishes were spiked daily with 0.3 µg/mL TPCK-trypsin and 200 mM Glutamax™. Virus was harvested when 40-50% of the monolayer remained. **(B)** Virus was adsorbed on the apical surface with 4.7 x 10^4 PFU. Post-adsorption, baseline was determined by sacking a tissue and quantifying viral RNA. At 4 days post-infection all other tissues were harvested, and viral RNA quantity was determined as described above. Fold increase was determined relative to baseline.

Conclusions

- A molecular virology toolkit for *in vitro* characterization of genetically distinct hMPV strains was developed.
- These improvements will contribute to the advancement of hMPV virology and the development of direct-acting antivirals targeting this virus.
- Enanta is actively optimizing lead nanomolar hMPV inhibitors to move forward in development.

References, acknowledgements and disclosure

- 1. Hackett, B.A., *CRITICAL EVENTS IN HUMAN METAPNEUMOVIRUS INFECTION: FROM ENTRY TO EGRESS*, in *Theses and Dissertations--Molecular and Cellular Biochemistry*. 2013, University of Kentucky.
- 2. Williams, J.V., et al., *The cotton rat (Sigmodon hispidus) is a permissive small animal model of human metapneumovirus infection, pathogenesis, and protective immunity.* J Virol, 2005. **79**(17): p. 10944-51.

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