Measles Virus Infection of Primary Respiratory Epithelial Cells Derived from Rhesus Macaques

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Proposal:

The goal of this project is to further investigate an *in vitro* cell culture system using respiratory epithelial cells derived from rhesus macaques. These primary tracheal and nasal epithelial cells (rmTECs/NECs) will be infected with either wild-type (Bilthoven) or vaccine (Edmonston) strains of measles virus (MV). Currently, the mechanism of MV infection in the respiratory system is relatively unknown. Assessment of the role of an additional unknown epithelial receptor (EpR) in MV infection and differences in MV infection in differentiated versus undifferentiated epithelial cells will be examined. The techniques that will be used are cell culture, real time RT-PCR, titering of infectious virus ($TCID_{50}$ assay), and immunofluorescence assay. These studies will bring greater appreciation and understanding of the basic respiratory infection pathways of measles virus infection and development of an aerosolized vaccine against measles.

Abstract:

Measles remains a leading vaccine-preventable cause of child mortality globally. Although a live-attenuated vaccine against measles virus (MV) is available, measles has been difficult to control. MV is a respiratory infection typically spread by aerosol droplets which target respiratory epithelial cells as initial sites of viral entry and replication. Primary tracheal and nasal epithelial cells (rmTECs/NECs) derived from rhesus macaques serve as an ideal system to study

MV infection in the respiratory tract, because: 1) rmTECs/NECs are polarized and differentiated to mimic respiratory epithelium *in vivo* and 2) rhesus macaques are the only susceptible host to MV infection other than humans. We have optimized a method for culturing well-differentiated polarized rmTECs/NECs and shown that both WT and vaccine strains of MV successfully infect cells from both apical and basolateral surfaces. Though no significant difference in viral infection was observed with an increased duration of infection, viral titers maintained high. Evidence of infection was characterized by observations of changes in cell morphology and titering of infectious virus in the supernatant. A working *in vitro* model of the respiratory system is important in bringing greater appreciation and understanding for the development of a respiratory vaccine against measles.

Introduction:

Measles virus (MV) is of the *Morbillivirus* genus in the *Paramyxoviridae* family. It is a highly contagious disease characterized by symptoms including fever, cough, conjunctivitis, and the appearance of a generalized rash[i]. Measles is a leading vaccine-preventable cause of childhood mortality in developing countries and remains one of the most contagious diseases despite a working live-attenuated vaccine (LAV). In the past year (2009-2010), measles outbreaks were documented in over 30 African countries as a result of poor vaccine coverage[ii]. Though efforts to eradicate measles have increased, high vaccine coverage is difficult to sustain in developing countries[iii]. Despite a working vaccine, mechanisms of MV infection are relatively unknown. Respiratory epithelia are believed to be the initial target site of MV infection and replication, but viral entrance and spread require further investigation. To evaluate MV infection pathways, we have optimized an *in vitro* respiratory system using primary tracheal and nasal epithelial cells derived from rhesus macaques (rmTECs/NECs). Following differentiation and polarization,

rmTECs/NECs were infected with either wild-type MV (Bilthoven) or vaccine strain MV (Edmonston). Direction of infection was examined by infecting polarized cells from the apical or basolateral surface and supernatant harvested from the infection were titered by the $TCID_{50}$ assay and observed for syncytia. Virus has been known to preferentially bud from the apical plasma membrane surface, so investigation of viral titers were studied by titering supernatant collected from the apical surface of cells infected from both directions. In this side-by-side study, comparing rmTECs/NECs derived from the same monkey, we have investigated the effect of MV infection from both apical and basolateral surfaces, differences in viral growth curves between Bilthoven and Edmonston strains of MV, and observed changes in the viral growth curves with increased duration of MV infection.

Hypothesis:

We hypothesize that longer duration of MV infection will result in higher levels of viral titers in both rmTECs/NECs particularly for wild-type infections. Previous experiments have shown a delayed increase in viral titers when comparing infections between vaccine and wild-type strains. A side-by-side comparison where both rmTECs/NECs are harvested from the same animal will reduce possibilities that differences observed are due to cell population variability between different animals.

Methods:

Cell Differentiation and Polarization

Primary tracheal and nasal epithelial cells derived from rhesus macaques (rmTECs/NECs) frozen down from previous monkey necropsies were thawed and grown in a transwell cell culture system. Transwell-Clear (Corning Costar, Corning, NY) supported membranes were each coated with rat tail collagen type I (diluted in 0.02 N glacial acetic acid) and incubated overnight at 4°C,

and washed three times with phosphate-buffered saline (PBS) before cell plating[iv]. rmTECs/NECs were grown in TEC Plus media, and both apical and basolateral media were changed every 2 days for 10 days or until optimal resistance was reached[v]. This form of the rmTEC/NEC system is the LLI (liquid-liquid interface). Trans-epithelial resistance (TER) was measured with the Millipore Millicell-ERS (Millipore, Bedford, MA) every 2 days and on reaching a TER of > 3.00 kΩ-cm^2, the apical media was removed to create the form, ALI (air-liquid interface) and media from the basolateral surface was replaced with TEC MM (a cocktail of TEC Basic, 2% NuSerum, and 1% Retinoic Acid)[vi]. Resistance measurements indicating successful formation of tight junctions between cells continued until optimal resistance was reached before infecting cells. Cells remained in ALI for 16 days before infection.

Infection and Harvesting of Cells and Supernatant

Once cells are observed to be differentiated (from observations of beating movement due to ciliated cells and immunofluorescence staining for ciliated cells and goblet cell markers) and polarized from TER readings, infection of rmTECs/NECs were initiated. Stock viral samples of Ed-MV and Bil-MV were titered using cell lines (Vero, VeroSLAM) using an 8-point log-fold serial dilution form of the end-point $TCID_{50}$ assay. Viral titering of stock samples provide information about the virulence of the virus in plaque forming units (pfu), an estimation of the number of infectious viral particles capable of infecting cells (forming plaques). Once the pfu of the stock virus is known, the multiplicity of infection (MOI) is calculated: the ratio of the number of virus particles to the number of cells per well. In this experiment, both Ed-MV and Bil-MV infections were at MOI= 4.5.

Following infection, cells were incubated in 37°C for 1, 4, 8, or 12hrs and at each time point post-infection, cells were washed with 1x PBS, replenished with fresh DMEM complete growth

media, and supernatant containing infectious virus was harvested at subsequent 24hr time points post-infection (hpi) over an 8-day time course ending at 192hrpi. Supernatant and cells harvested from the cell culture were frozen down in eppendorf tubes and transwell membrane inserts, respectively at -80°C.

$TCID_{50}$ and Viral Titering Assay

Vero cells and VeroSLAM cells (kidney cell line) were infected with infectious supernatant collected at the prescribed hpi and titered using a 6-point log-fold serial dilution containing four replicates per sample beginning with an undiluted sample. Infected Vero cells were incubated at 37°C for 1 week and viral titers were read by observed cytopathic effect (CPE) characterized by syncytia (cell-to-cell fusion of nuclei), an indication of MV infection[vii]. CPE readings are log transformed into log$TCID_{50}$ values and graphed using PRISM, graphing software, to better visualize and characterize the fold change of MV infection over time.

Results:

Increased Duration of MV infection does not appear to significantly increase viral titer

Though a test to show statistical significance was not applied general observations of the viral growth curves showed that duration of infection from 1h to 8 or 12hrs were clustered together. Though, Bil-MV infection appeared to show slightly higher viral titers comparing 1h to 12h infection. Almost a log-fold increase is observed due to an increase in duration of viral infection (see figure 3).

Bilthoven and Edmonston- MV can infect rmTECs/NECs from apical and basolateral surfaces

Viral growth curves show results from titering apical supernatant because previous experiments (see Figure 1) have shown that basolateral supernatant from apical and basolateral infections produce baseline levels of virus. To determine if rmTECs/NECs favored MV entry from a

particular direction (apical/basolateral surface), these polarized cells were infected from both directions. Though viral titers for cells infected basolaterally showed lower viral titers, infection was observed. Peak viral titers differed between rmTECs/NECs, but among the same cells, peak titers were similar despite infection with a different strain of MV (see Figure 2 ,4).

High Viral Titers are Maintained After Onset of Infection

For rmTECs/NECs infected with Ed-MV or Bil-MV, it appears that viral titers remain high following initial increase in viral titers (48hpi- Ed-MV, 72hpi- Bil-MV). A drop in viral titers is observed late in the time course around 144hpi for Ed-MV and 192hpi for Bil-MV. High titers are maintained despite the heat-sensitive nature of MV.

Discussion:

Measles viruses (MV), along with other *Morbillivirus* are special types of *paramyxoviruses* that bind specifically to host cell protein receptors instead of sialic acid residues (located on apical and basolateral plasma membranes)[viii]. MV-Hemmaglutanin (H) protein and MV-Fusion (F) protein are important for viral infection and entry into host cells. Additionally, wild-type and vaccine strains of MV require specific receptors for entry into host cells. Wild-type (Bilthoven) MV use CD150 (or SLAM) while the vaccine strain (Edmonston) MV uses CD46 or CD150 for entry into host cells. Speculation remains that an unknown epithelial cell receptor (EpR), located on the basolateral surface plays a role in MV entry.[ix] Though past experiments have shown that even among polarized cell lines, MV has been shown to preferentially enter either apically or basolaterally, or both surfaces. Though direction of infection appears to be cell-type dependent, results have shown that MV preferentially buds or is released into the apical surface more efficiently than into the basolateral surface.[x] A possible

explanation for this effect can be explained by the observation of a larger population of CD46 receptors located on the apical surface, therefore resulting in higher viral titers of apical supernatant.

In our experiment, we observed MV infection of primary epithelial cells from both apical and basolateral surfaces. For both strains of MV and cell types, it appeared that apical infection resulted in higher viral titers over the time course. This might be a false-positive association since apical compartments in the clear-membrane inserts used to grow up rmTECs/NECs can hold less volume that the basolateral surface. Because of this design, despite careful calculations to add equal volumes of virus into each compartment, the issue of dilution arises. However, this error does not discount our observations since it has been shown that MV preferentially buds apically. Quantitatively, our data may overestimate the fold difference between apical and basolateral infection as indicated by viral titers of the apical supernatant.

Another observation to entertain is effect of duration of infection on viral titer. Our hypothesis that increased viral infection would result in increased viral titers was not entirely correct. Viral titers appeared to show a slight increase for Bil-MV infection of rmTECs/NECs, while Ed-MV infections maintained similar levels. Bil-MV infections also appeared to reach peak titers at a later time point than Ed-MV infected cells. An increased amount of time required for peak viral infection may be a possible explanation for life-long immunity against MV after wild-type infection. High viral titers are maintained several days post-infection which is remarkable considering that MV loses its virulence with at warm temperatures and host cells are not replenished.

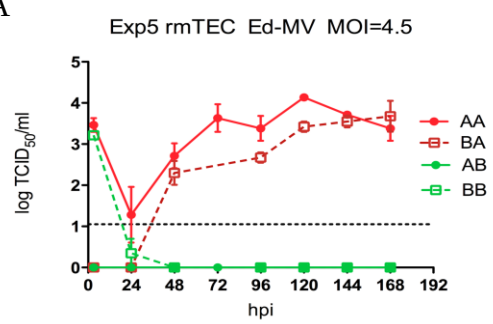
Future Directions:

Re-infect rmTECs/NECs with Ed-MV or Bil-MV using the data gathered from this experiment. Try infecting rmTECs/NECs for 1h with Ed-MV and 12h for Bil-MV for optimal infection results. Set-up an experiment to determine if the EpR is a factor in MV infection of Vero/VeroSLAM cells by blocking the CD46 receptor by assessing viral titer. Attempt to characterize the types of MV protein present cell infected with MV using cell lines and eventually primary epithelial cells by Western Blot.

Figures:

Figure 1: rmTEC/NEC Edmonston-MV Infection Viral Growth Curves

A



B

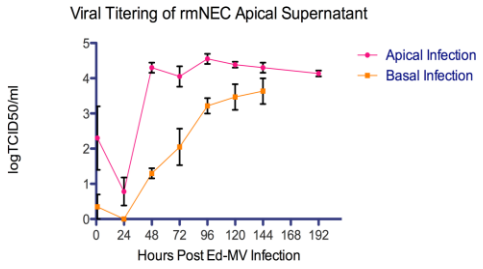


Figure 1: (A) rmTEC infection with Ed-MV at MOI= 4.5. Viral titers show that basolateral supernatant containing infectious virus (AB, BB) showed baseline levels of virus with infection from both sides. Apical supernatant showed highest viral titers (AA, BA). **(B)** rmNEC infection viral growth curve showing apical supernatant from apical and basolateral infection.

Figure 2: Viral Growth Curves for rmTECs/NECs Infected from Apical and Basolateral Surfaces with Increasing Viral Infection

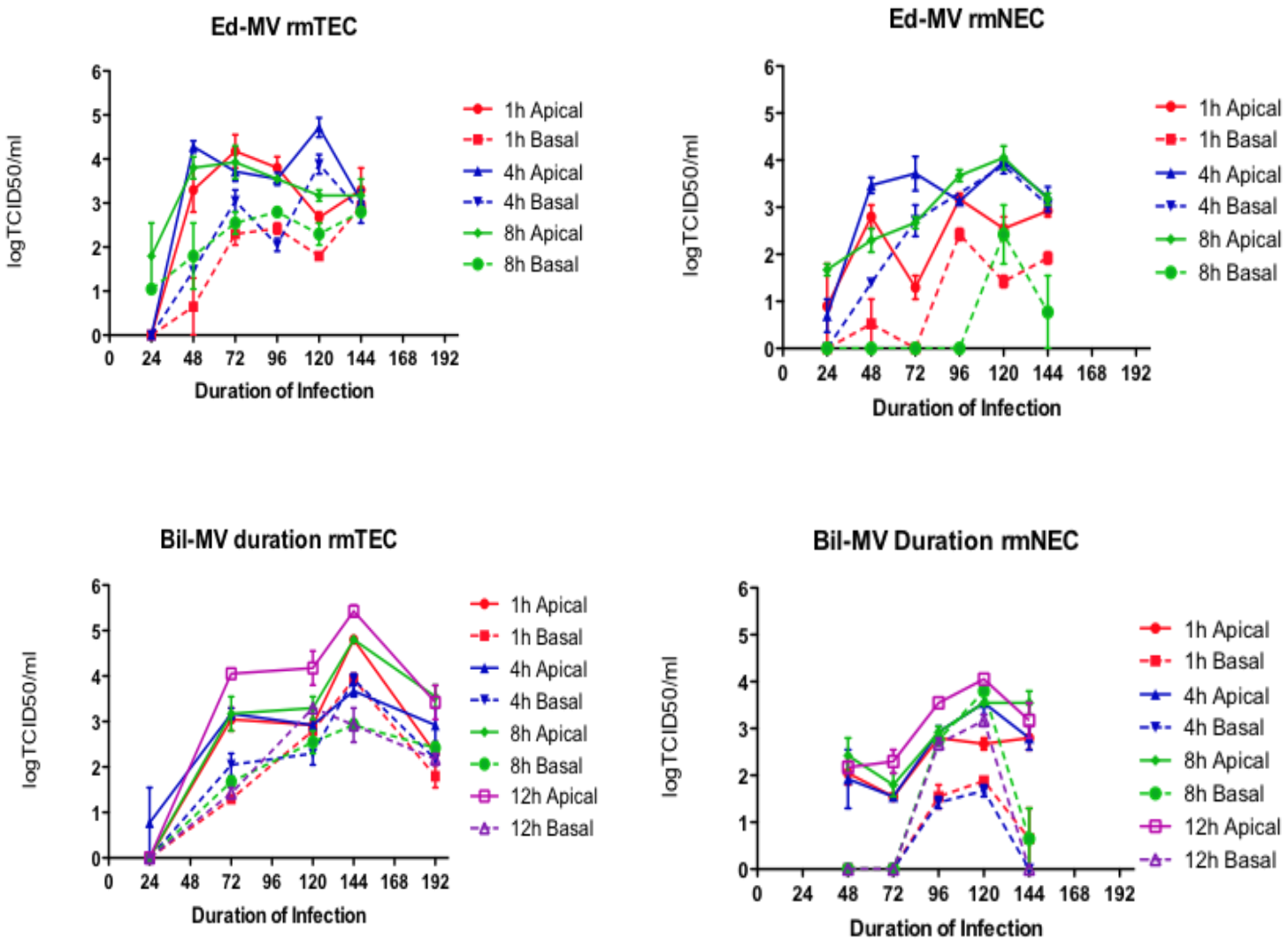


Figure 2: Overview of growth curves for rmTECs/NECs infected with either Bilthoven or Edmonston-MV for increasing duration of time. Titers remain high following infection. No significant difference is observed for Ed-MV infection, but Bil-MV infection appear to have about a log-fold increase comparing 1h to 12h viral infection.

Figure 3: Increased Duration of MV Infection Does Not Significantly Affect Viral Titers of Primary Epithelial Cells

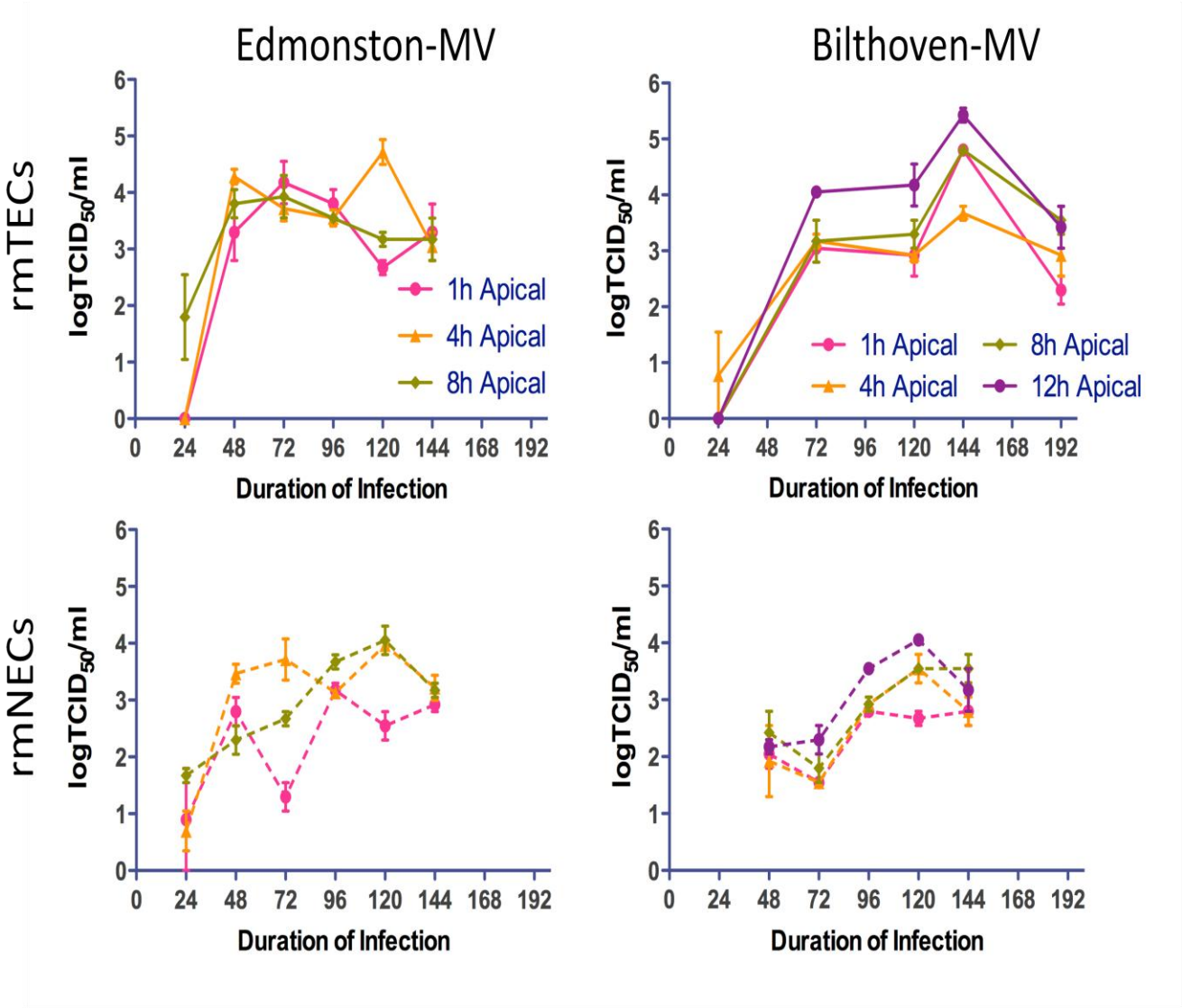


Figure 3: Increased duration of viral infection did not significantly affect the production of infec.ous virus. Both Ed-MV and Bil-MV showed successful infection of rmTECs/NECs. Apical supernatant from cells infected apically were titered since measles preferentially buds apically. Viral titers remained steady throughout the time course suggesting successful infection of cells. Ed-MV and Bil-MV supernatant containing infectious virus were titered using cell lines: Vero/hSLAM VeroSLAM cells, respectively. MOI= 4.5 for Bil-MV and Ed-MV.

Figure 4: MV Enters Respiratory Epithelial Cells from Both Apical and Basolateral Surfaces

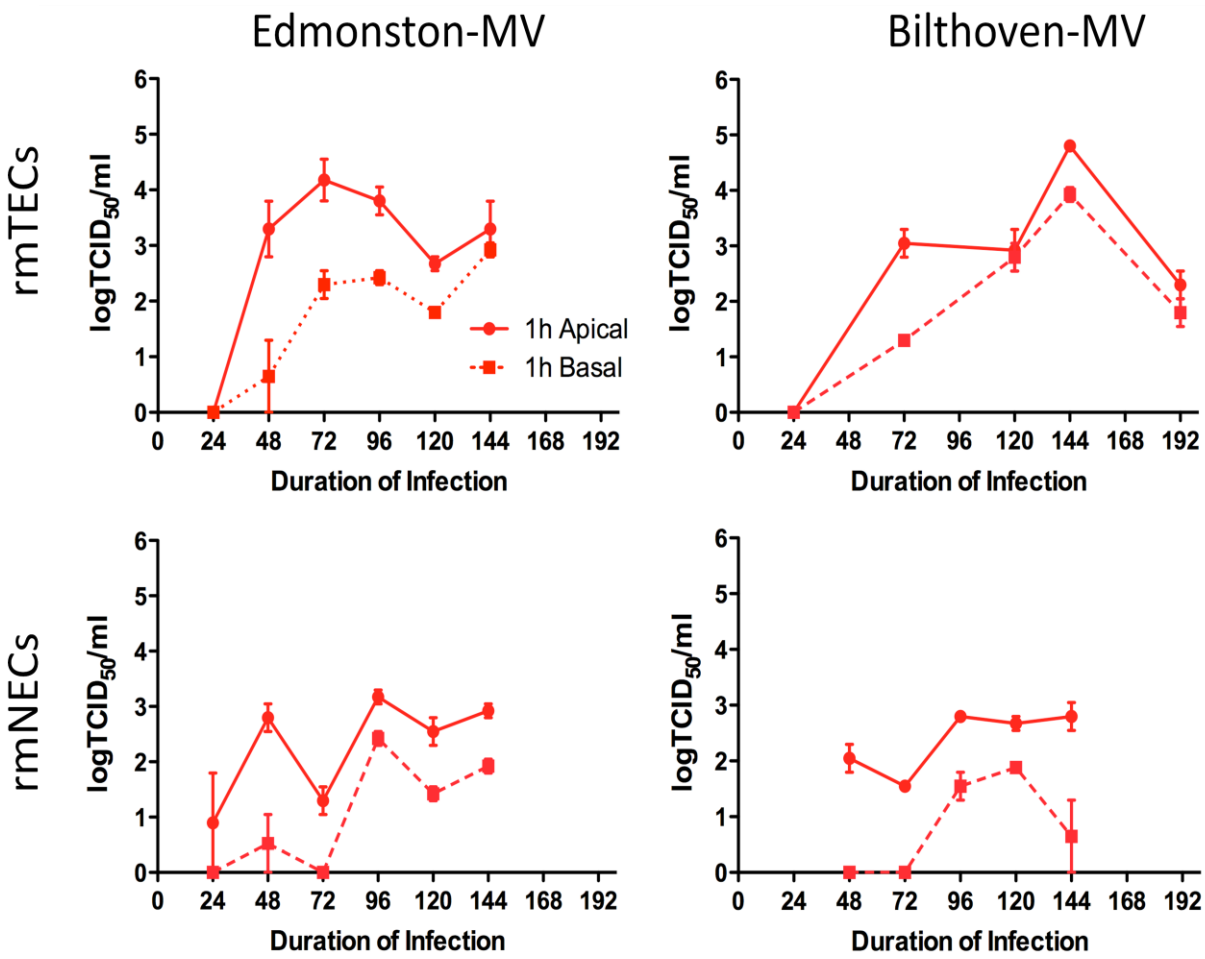


Figure 4: Viral growth curves for rmTECs/NECs infected with Bil-MV and Ed-MV showed higher viral titers for cells infected apically. Both strains of MV maintained high viral titers over the time course.

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Keywords: Measles Virus Infection, Primary Respiratory Epithelial Cells, Bilthoven, Edmonston

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