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INVESTIGATING STREPTOCOCCUS PNEUMONIAE AND ADENOVIRUS CO-INFECTIONS OF LUNG EPITHELIAL CELLS

A Thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

by

MARK NICHOLAS CALABRO

B.S., Ohio University, 2005

2021

Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

November 19, 2021

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Mark Nicholas Calabro ENTITLED Investigating *Streptococcus pneumoniae* and Adenovirus Co-infections of Lung Epithelial Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Calabro, Mark Nicholas. M.S., Microbiology and Immunology Graduate Program, Wright State University, 2021. Investigating *Streptococcus pneumoniae* and Adenovirus Co-Infections of Lung Epithelial Cells

Co-infection is common among viruses and bacteria in the human respiratory system. Adenovirus (AdV) and *Streptococcus pneumoniae* are clinically relevant respiratory pathogens that cause morbidity and mortality in a variety of patient populations with the highest morbidity occurring among immunocompromised individuals, but also prevalent in infants and the elderly. Acute respiratory distress syndrome may become severe in healthy individuals when co-infection with *S. pneumoniae* and AdV occurs due to synergistic effects of the pathogens on the host. I hypothesized that *S. pneumoniae* infection decreases AdV transduction of airway epithelia. To test this hypothesis, we utilized the polarized immortalized airway epithelial cell line Calu-3. Calu-3 cells were inoculated with *S. pneumoniae* followed by the addition of recombinant adenovirus, AdVLacZ. Then, the effect of bacteria on AdV transduction into Calu-3 cells was determined by quantifying β-galactosidase. Cell-associated viral genomes were measured by quantitative polymerase chain reaction. These results showed that *S. pneumoniae* infection decreased significantly AdV transduction in Calu-3 cells, but there was no significant difference in quantity of cell-associated AdV genomes.

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DEDICATION

This thesis is dedicated to my family. To my wife Megan, son Alexander, and daughter Annalise, thank you for your patience, understanding, and love during my graduate school career. To my mother-in-law Patti DeLotell and father-in-law Tim DeLotell, thank you for being there to help Megan and the kids while I was away at school. To my parents Mary and Nick Calabro, thank you for your encouragement and support.

I. INTRODUCTION AND BACKGROUND

Adenovirus

Adenovirus (AdV) is a non-enveloped icosahedral virus that contains double stranded DNA (12). There are 50 strains of adenovirus. (28) AdV can invade and infect several cell types in humans, and infections can lead to many clinical illnesses. Human AdV is highly communicable and usually causes mild symptomatic illness but can also lead to severe and even fatal infections (1). AdV is commonly responsible for upper and lower respiratory tract infections, gastrointestinal (GI) tract infections, and conjunctivitis (2, 3). These infections are more likely to be seen epidemically in children (lacking humoral immunity), closed communities (military recruits), and those with impaired immune systems (e.g., patients with *Human immunodeficiency virus 1* infection, organ transplant recipients) (6). Immunocompromised patients are highly susceptible to viral disease, leading to significant morbidity and mortality. This project focuses on respiratory illness caused by adenovirus.

Adenovirus as a Gene Therapy Vector

Because of their high transmissibility, recombinant adenovirus vectors have become popular and often investigated for gene therapy. However, the toxicity and immune response to AdV-mediated gene therapy has become a daunting obstacle to overcome (10, 11).

Adenovirus Vector Vaccines

Pharmaceutical companies have developed adenovirus vector vaccines to combat the COVID-19 pandemic. They include the Johnson & Johnson (Jannsen), AstraZeneca, Gamaleya, and CanSino Biologics vaccines (34).

Transmission

Transmission of adenovirus occurs by close personal contact and by respiratory droplets in the air spread by coughing and sneezing. Touching an object or surface with adenoviruses on it, then touching your mouth, nose, or eyes before washing your hands can also spread the virus.

Pathogenesis of Respiratory Infection

Human adenovirus causes common cold or flu-like symptoms, fever, sore throat, acute bronchitis, and pneumonia.

Risk Factors

People with decreased immune function are more susceptible to human adenovirus.

Diagnosis

The main diagnostic test for human adenovirus is real-time polymerase chain reaction (PCR) assay.

Prevention

Vaccination against adenovirus is currently only given to military recruits. The military adenovirus vaccination program was re-instated March 2011. Barr Labs Inc. manufactures the vaccine, which is given as one dose of two oral tablets containing live Adenovirus Type 4 and

Type 7 (29). Implementation of good health practices like hand hygiene, cough etiquette, and staying home when sick can also help control the transmission of adenovirus.

Streptococcus pneumoniae

Streptococcus pneumoniae is a gram-positive alpha-hemolytic commensal organism (4, 13). The pneumococcal polysaccharide capsule has more than 90 distinct serotypes. *S. pneumonia* can cause infection in the middle ear, sinus infection, pneumonia, bacteremia, and meningitis. Pneumonia with empyema, bacteremia, and meningitis are invasive infections (30). Pneumococcal bacteria are the most common cause of pneumonia. Young children and the elderly are most susceptible to infection (5).

Virulence factors

S. pneumoniae has several virulence factors that help it evade the immune system. The capsular polysaccharide inhibits opsonization by blocking fixation of complement leading to activation of the alternative pathway of complement. Pneumococcal surface protein C (PspC) recruits Factor H to the pathogen membrane to inactivate bound C3b. This prevents formation of the membrane attack complex (MAC) (33). The capsule also blocks detection of two cell wall components, peptidoglycan, and lipopolysaccharide (LPS), by pattern recognition receptors (PRRs) found on macrophages, neutrophils, and dendritic cells. These receptors recognize microbial-associated molecular pattern (MAMPs), also referred to as pathogen-associated molecular patterns (PAMPs) that are part of the pathogen (33). The capsule also serves as a platform for antigenic variation. Over 90 serotypes of *S. pneumoniae are* known. Infection with one serotype can lead to type-specific immunity. This protects from reinfection with that specific

serotype, but not with a different serotype. Thus, each serotype of *S. pneumoniae* presents as a distinct pathogen to the adaptive immune system (33).

Symptoms

Pneumonia:

Symptom onset typically appears 1-3 days after exposure. Fever, chills, productive cough, dyspnea, tachypnea or tachycardia, hypoxia, and malaise.

Meningitis:

Stiff neck, fever, headache, photophobia (eyes being more sensitive to light), and confusion.

Bacteremia:

Confusion or disorientation; Shortness of breath; High heart rate; Fever. shivering, or feeling very cold; Extreme pain or discomfort; Clammy or sweaty skin (30).

Transmission

Transmission of *S. pneumoniae* occurs through direct person-to-person contact with respiratory secretions like saliva or mucus (27). Certain percentages of the population carry *S. pneumoniae* as commensal flora of the oral pharynx.

Risk Factors

Pediatric populations with increased risk factors for contracting pneumococcal disease include children under the age of 2, who have certain illnesses (sickle cell disease, HIV infection, diabetes, immune compromising conditions, nephrotic syndrome, or chronic heart, lung, kidney, or liver disease). Children with cochlear implants or cerebrospinal fluid (CSF) leaks (escape of the fluid that surrounds the brain and spinal cord (27).

Adults over the age of 65 are at increased risk of contracting pneumococcal disease. Some adults 19 through 64 years old are also at increased risk for pneumococcal disease, including those with chronic illnesses (chronic heart, liver, kidney, or lung disease; diabetes; or alcoholism). Immunocompromised patients (HIV/AIDS, cancer, or damaged/absent spleen) carry a higher risk of disease as do patients with cochlear implants or cerebrospinal fluid (CSF) leaks and adults who smoke cigarettes (27).

Prevention

The Center for Disease Control and Prevention's Advisory Committee on Immunization Practices (ACIP) recommends routine vaccination of infants with a 4-dose series of PCV13 (Pneumococcal Conjugate Vaccine 13 capsular subtypes) and of adults aged 65 years or older with 1 dose of PPSV23 (Pneumococcal Polysaccharide Vaccine 23 capsular subtypes) (26). Table 1 shows an overview of approved pneumococcal vaccines in the United States.

Trade Name	Abbreviation	Manufacturer	Doses In routine Series	Approved Ages	Comments
Pneumovax 23	PPSV23	Merck	1	≥ 2 years	Inactivated polysaccharide
Prevnar 13	PCV13	Pfizer	4	≥ 6 weeks	Inactivated, Adj. CRM197 conjugate

Table 1 Approved Pneumococcal Vaccines in the United States (29)

Good health practices like hand hygiene, cough etiquette, and staying home when sick can also help control the transmission of *S. pneumoniae*.

Diagnosis

Definitive diagnosis of *S. pneumoniae* typically relies on culture isolation of the bacteria from sterile body sites. For example, blood cultures from a patient with invasive pneumonia (30). Culture confirmation of pneumonia should include antibiotic susceptibility reporting. A urinary antigen test for the C-polysaccharide antigen of *S. pneumoniae* is available to test for community acquired pneumonia in adult patients. Abbott's BINAXNOW *Streptococcus pneumoniae* Antigen Card can test for the detection of *S. pneumoniae* in urine as well as the cerebral spinal fluid (CSF) of patients with meningitis (32).

Treatment

Mild respiratory illness can be self-resolving with over-the-counter drugs for symptomatic management for outpatient populations. Treatment guidelines set forth by the Infectious Disease Society of America are complex, considering patient age, immune status, co-morbidities, setting, and prognosis (30). Empiric treatment of ear infections and pneumonia in

children consists of penicillin and cephalosporins. Data show that pneumococcal bacteria are resistant to one or more antibiotics in 30% of cases (30).

Adenovirus Entry into Host Cells

Adenovirus enters the host cell through the Coxsackievirus and Adenovirus Receptor (CAR) or more specifically, the eight-exon encoded alternatively spliced isoform, $CAR^{Ex8.}$ The receptor may fulfill a critical role in adenovirus/S*treptococcus pneumoniae* co-infection. CAR receptors are involved in cell-cell adhesion, protein trafficking, and viral infection (24). CAR^{Ex8} has previously been shown to be located on the apical surface of epithelial airway cells where it is able to mediate AdV infection from the apical surface (22). Moreover, the CAR^{Ex8} isoform is up-regulated by the pro-inflammatory cytokine IL-8 and smoke (23). *S. pneumoniae* can infect the same airway epithelial cells as adenovirus. A more thorough look at CAR could give insight into the effect of adenovirus/S*treptococcus pneumoniae* co-infection.

Bacterial/Viral Co-Infections

Bacterial-viral co-infections are common, especially in children under five and in immunocompromised individuals (6, 15-17,19). While one study showed that co-infections with AdV and *S. pneumoniae* are one of the most prevalent compared to other AdV and bacteria co-infections (1), another study has suggested that the combined number of cases reported is modest (15-17). It is possible the combined number of cases may be under reported because viral and bacterial respiratory infections are common, and they can display similar symptoms (sore throat/postnasal drip, bronchitis/cough, congestion/running nose, earaches, and development of pneumonia) (14, 21). The detection of one does not necessarily indicate the absence of the other (5, 18). Normally, the human immune system can eliminate minor bacterial and viral respiratory

infections, but a single infection may change an individual's immunity and pathology (5, 19), which could leave an individual more susceptible to subsequent infection. Despite the occurrence of co-infection, not much is understood about the effects of viral/bacteria co-infections and what their synergistic effects may be. While it has been shown that AdV plays a role in the adherence of *S. pneumoniae* to the airway epithelial cells, how *S. pneumonia* affects AdV infection is completely unknown (8). Understanding the effect of one pathogen on another may result in better understanding of how the body deals with co-infections, as well as novel therapeutic approaches for treating these infections.

A model is required to examine the effects *Streptococcus pneumoniae*/Adenovirus co-infection on lung epithelia. This thesis explores a model system to investigate co-infection by the addition of *Streptococcus pneumoniae* followed by recombinant human adenovirus.

II. MATERIALS AND METHODS

Bacterial Culture

Bacterial cultures were manipulated in a certified biological safety cabinet using aseptic technique and Biosafety Level 2 conditions. Bacterial culture and co-infection experiments were carried out in the Microbiology Laboratory at Wright State University.

Initiation of Bacterial Growth:

Streptococcus pneumoniae strain (ATCC49619) was ordered from American Type Culture Collection (ATCC; Manassas, VA). Bacterial culture was initiated using the protocol supplied by ATCC. Briefly, 0.5 mL of Todd Hewitt Broth (Item No. 249240; BD Biosciences) with 0.02% Yeast Extract (Item No. 11929; BD Biosciences) was added to the freeze-dried culture shipment and mixed. The entire suspension was transferred to a sterile Pyrex screw cap culture tube (100 mm X 13 mm, 9 mL Fisher 14-933A) containing 5 to 6 ml of Todd Hewitt Broth with 0.02% Yeast Extract (THY). Additionally, several drops of the suspension were transferred to a 5% blood agar plate, for streaking and pure culture solation. Cultures were incubated at 37°C with an atmosphere of 5% CO_2 for 24 hours. Additional test tubes were inoculated by transferring 0.5 mL of the primary culture to additional secondary culture tubes. The blood agar plate cultures were observed for expected colonial morphology of *S. pneumoniae*.

Preparation of *Streptococcus pneumoniae* Bacterial Stocks:

The initial bacterial cultures described above were subcultured onto new 5% blood agar plates, streaking for isolation, and new 9 ml screw cap culture tubes containing 8 ml THY broth. The blood agar plates were incubated overnight 16-18 hours in a candle jar at 37°C. The broth culture tubes were capped tightly and incubated at 37°C and grown to mid-log growth phase, defined as 0.2- 0.6 OD at 600 nm. Optical density was measured by Thermo Scientific Genesys 20 Spectrophotometer using the tube reader insert for the instrument. The broth cultures of *S. pneumoniae* were then aliquoted into 2 ml cryogenic tubes (Nalgene, Thermo Scientific) containing 20% Glycerol (Amresco). The vials were frozen at -80°C in an ultra-low freezer.

Bacterial Growth for Co-infection

Streptococcus pneumoniae strain (ATCC49619) was grown on BD Trypticase Soy Agar II with 5% sheep blood agar plates from Beckton Dickinson (Item No. B21261X; Fisher Scientific) overnight ~16-18 hours in a candle jar at 37°C. The morning of the co-infection experiment, the jar was opened, and 8-10 individual colonies were transferred using a sterile loop into a 9 ml screw cap culture tube containing 8 ml of THY Broth. Three culture tubes were inoculated this way. Tubes were capped tight, mixed gently, and incubated at 37°C to mid-log growth phase (determined as 0.2- 0.6 OD at 600 nm measured by Thermo Scientific Genesys 20 Spectrophotometer using the tube reader insert for the instrument. In early experiments, the bacteria were also counted on a hemacytometer before co-infection. A 10 ul sample of the bacteria was used to charge one side of the hemacytometer. The number of cells occupying four mini-squares within the central square were counted, averaged, and multiplied by the hemacytometer coefficient to calculate the concentration of bacteria per milliliter.

A *S. pneumoniae* dose-response curve was tried in later experiments. A milliliter of sample of the mid log-phase culture was spun down in a mini-centrifuge for three minutes. The supernatant was aspirated with a pipette taking care not to disturb the bacterial pellet. The pellet was gently resuspended in one ml of phosphate buffered saline (PBS) +/+ (containing Mg^{+2} and $Ca^{+2)}$. A dilution series was made as follows: 100 ul of the original bacterial PBS +/+ microtube was added to a microtube containing 900 ul of PBS +/+ and mixed by inversion and pipetting; 100 ul from this dilution were taken from the dilution of the original sample and mixed in a microtube with 900 ul PBS +/+. Undiluted cultures are referred to as "High" and first and second dilutions as "Medium" 10^{-1} and "Low" 10^{-2} concentration of *S. pneumonia*, respectively.

<u>Bacterial Viable Cell Counts:</u>

To check the number of viable *Streptococcus pneumonia* used in co-infection experiments, a viable cell count was performed during each experiment. From the tubes used above for co-infection, an additional dilution series was made in PBS +/+ as follows: 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-6}. Dilutions were mixed by gentle inversion and pipetting. One blood agar plate was divided into quadrants for each serial dilution concentration from a single sample. Plates were "spotted" by pipetting 10ul of the dilution using a Ranin AutoRep repeater pipette making 8-10 replicate "spots".

The spots were allowed to absorb before the plates were incubated in a candle jar at 37°C overnight (16-18 hours). The number of colonies per 10 ul spot was counted the following morning and averages calculated for each sample. This average was multiplied by the dilution factor to calculate the colony forming units per milliliter (CFU/ml). Typically, 10^{-5} dilution spots were counted yielding an estimate of 10^{7} CFU/ml *Streptococcus pneumoniae* for the undiluted culture.

Epithelial Cell Culture

To evaluate the hypothesis, the proposed model of experimentation was implemented by inoculating Calu-3 (ATCC® HTB-55™) cells with *Streptococcus pneumoniae* strain (ATCC49619) followed by transduction with Ad5CMVCytoLacZ (University of Iowa-3554). Cell cultures were manipulated in a certified biological safety cabinet using aseptic technique and good laboratory practice under Biosafety Level 2 conditions.

Growth Medium

Calu-3 (ATCC® HTB-55™) cells were grown in 75 cm^2 treated tissue culture flasks with angled necks and vented caps with 10m L of culture media. Culture medium used: RPMI 1640 (Gibco, ThermoFisher Scientific; Item No. 31800089) with 25 mM HEPES (Gibco, ThermoFisher Scientific; Item No. 15630080) added for extra buffering capacity. Penicillin-Streptomycin (10,000 U/ml) (Gibco, ThermoFisher Scientific; Item No. 15140122) was added to culture medium to help prevent bacterial contamination. Medium was supplemented with 10% Fetal Bovine Serum (Gibco™, ThermoFisher Scientific; Item No. 26400044). The pH was adjusted to 7.35 as measured using a Metler Toledo pH meter. The pH Meter's calibration curve was run each time before measuring media pH. Culture media was vacuum filter-sterilized using a 0.22 um Millipore Steritop filter (Fisher Scientific; Item No. S2GVT05RE) in cell culture hood.

Growth Conditions

Calu-3 cells were incubated at 37°C with an atmosphere of 5% CO_2 in a humidified Sanyo incubator.

Cell Culture Maintenance

Calu-3 cell media was changed every 2-3 days, culture flasks were observed for morphology and confluence using an inverted tissue culture microscope (Motic model AE2000).

Passage of Cells

Calu-3 cells were allowed to grow to around 80% confluence in $75cm^2$ flasks. Typically, this growth took five days. To passage cells, an aliquot of Trypsin-EDTA (Gibco, ThermoFisher; Catalog No. 25200056) was warmed in 37° C water bath for five minutes. Calu-3 culture media was removed using a serologic pipette. Cells were rinsed gently with five ml of PBS -/- (without Ca^{+2} or Mg^{+2}). After PBS -/- was added, the flask was gently rocked by hand, and PBS -/- was aspirated. Two ml of Trypsin EDTA was added into the flask. The flask was incubated at 37°C with five percent CO_2 for 5-10 minutes. After incubation, the flask was gently rocked and observed under the inverted tissue culture microscope for cell dissociation. Trypsinization was stopped by adding eight ml of Calu-3 culture media to the flask with a serological pipette. Cells were gently mixed by pipetting up and down. Calu-3 cells were passed into three new flasks by adding three ml of the trypsinized cells to seven ml of fresh Calu-3 culture medium. Volumes of trypsinized cells and fresh media were adjusted to achieve desired cell concentrations.

Seeding Calu-3 cells onto Transwell Plate Inserts

Calu-3 cells were studied using an *in vitro* experimental model developed in the laboratory. Using 24-well standard tissue culture treated plates (Corning Falcon, Catalog No. 353226) with a transwell insert PET membrane, 0.4 um pore size (Corning, Catalog No. 3470) enabled the establishment of an air/liquid interface that resembles how the cells grow *in vivo* as

lung epithelial cells. This provided a polarized epithelial layer with an apical air interface and basolateral liquid culture media interface.

The above-mentioned cell culture maintenance and passaging methods were followed. Instead of passaging cells to new culture flasks, the trypsinized cells were transferred into a 15 ml conical tube (Falcon) and centrifuged for five minutes at room temperature at a speed of 152 RCF, acceleration 5/10, brake 5/10 in an Eppendorf 5810 R centrifuge. The supernatant was aspirated in the tissue culture hood and the cells were re-suspended gently in five ml fresh media.

A hemocytometer was used to count the total number of CaLu-3 cells added to each 0.4 µm transparent PET membrane insert to ensure consistent amounts added.

Ten microliters of the cell suspension was used to charge one side of the hemacytometer. Cells in the four corners of the hemacytometer were counted, averaged, and multiplied by the hemacytometer coefficient to obtain the concentration of cells per milliliter. The concentration was adjusted with media to 8.0×10^5 cells/ml. The number of Calu-3 cells used to seed each well is 2.0×10^5 suspended in 250 µl culture media.

The Calu-3 cell suspension was gently mixed after the concentration was adjusted. Using a manual Ranin AutoRep repeater pipette, 250 µl of the cell suspension was gently pipetted onto 0.4 µm pore containing transparent PET membranes that was inserted into a multi-well 24 well tissue culture treated polystyrene plates. After cells were seeded, 500 µl of fresh Calu-3 media was added to the well (basolateral surface) of the tissue culture plate using Ranin AutoRep repeater pipette. The plate lid was put on and secured with parafilm. The plate wells were observed using the tissue culture microscope. Plates were incubated under the conditions described above.

After an overnight incubation of about 16-18 hours, the apical media was aspirated. Taking great care to not disturb the cells bound to the membrane. The plate wells were observed using the tissue culture microscope. Plates were then placed back into the incubator at 37°C with five percent CO_2 humidified atmosphere. During the incubation, the basolateral media was replaced every two to three days. Cells were checked microscopically for morphology and confluence. Growth of a polarized epithelia on the membranes took about 10-12 days before using the wells for co-infection experiments.

Trans-Epithelial Electrical Resistance (TEER, or TER)

Tight junction formation of the Calu-3 cells was evaluated by measuring Trans-Epithelial Electrical Resistance (TEER, or TER) using a EVOM2 Voltomhmeter, according to the manufacturer's protocol. To check TEER, 250 ul of Calu-3 media was carefully pipetted at an angle against the side of the transwell insert onto the apical surface of the cells. This was done using the Ranin AutoRep repeater pipette with a micro-pipette tip attached to the repeater syringe tip. The basolateral media was aspirated and replaced with 500 ul of fresh Calu-3 media. The TEER electrode was then submerged into each transwell, the reading was allowed to stabilize over several seconds, and recorded. After measurement, the apical media was carefully aspirated. Plates were sealed with parafilm, checked under the inverted microscope, and placed back into the incubator. The TEER was measured several times during cell growth on the membranes. Within 24 hours of beginning the co-infection experiment, TEER was measured to ensure that the cell tight junctions are intact. A threshold TEER reading of 1000 Ohms or greater was used to establish tight junction integrity. Cell morphology and confluence will be assessed using an inverted tissue culture microscope from Motic (model AE2000). Polarized epithelia growth took 10-12 days before co-infection experiments.

Adenovirus

A recombinant, replication-deficient Adenovirus was purchased from University of Iowa Carver College of Medicine's Viral Vector Core- 3554 Ad5CMVCytoLacZ (hereon referred to as Adenovirus LacZ). The recombinant virus offers episomal gene expression and can infect dividing and non-diving cells. The viral vector was kept frozen at -80°C in an ultra-low freezer. All viral vector work was carried out at Biosafety Level 2 conditions using good laboratory practice inside a biological safety cabinet. The viral solution for co-infection was prepared as follows: 0.75 ul of concentrated viral stock per transwell was used. The viral stock was diluted with sterile PBS +/+ to give a volume of 20 ul diluted viral stock per transwell infected. This was done in a sterile microcentrifuge tube and mixed by pipetting and vortexing. Yielding multiplicity of infection (MOI) of 125 viral particles per Calu-3 cell.

Co-infection Procedure

Co-infection experiments were carried out in the biological safety cabinet of the Microbiology Laboratory at Wright State University.

The 24-well plate enabled testing several conditions between the addition of adenovirus and *S. pneumoniae* in replicate. Conditions as described in the table below.

Initial Infection for 4 hours	PBS	Broth	*Streptococcus pneumonia*	PBS	*Streptococcus pneumonia*
Followed by: For 1 hour	Adenovirus	Adenovirus	Adenovirus	PBS	PBS

Table 2 Experimental Conditions

Initial infection was carried out by adding 50 ul of *S. pneumoniae* or PBS +/+ or THY broth as controls. The 24-well plate was resealed with parafilm and incubated at 37°C with humidified air supplemented with five percent CO_2 for four hours.

Secondary infection was carried out by adding 20 µl of the diluted Adenovirus stock solution or PBS +/+ as control. The 24-well plate was resealed with parafilm and incubated at 37°C with humidified air supplemented with five percent CO_2 for one hour.

After the secondary infection incubation time was finished, 250 µl of PBS +/+ was gently pipetted onto the apical surface of the Calu-3 cells and aspirated by pipette. This served as a wash step. The 24-well plate was finally resealed with parafilm and incubated at 37°C with humidified air supplemented with five percent CO_2 overnight (16-18 hours).

Preparation of Calu-3 Cells for Co-Infection

Within 24 hours of experimentation, the TEER of Calu-3 cells was checked to ensure tight-junction integrity. In the morning of co-infection experiments, fresh antibiotic-free, serum-free culture media was prepared. Two hundred fifty microliters of PBS +/+ was used to gently rinse the apical surface of the Calu-3 cells to remove any media. Basolateral media was aspirated and replaced with antibiotic-free, serum-free media. Plates were sealed with parafilm, checked using the tissue culture microscope, and transferred to the tissue culture incubator in the Microbiology Laboratory at Wright State University.

Preparation of Bacteria for Co-Infection

For sample conditions requiring addition of bacteria, 50 µl of the *Streptococcus pneumoniae* suspended in THY broth was pipetted onto the apical surface of the Calu-3 cells.

This was done using a Ranin AutoRep repeater pipette with a sterile syringe tip. Bacteria was cultured for co-infection as described above.

Preparation of Adenovirus for Co-Infection

For sample conditions requiring Adenovirus, 20 µl of the diluted viral stock was pipetted onto the apical surface of the Calu-3 cells. Yielding an MOI of 125 viral particles per Calu-3 cell. This was done using a Ranin AutoRep repeater pipette with a sterile syringe tip.

Preparation of PBS+/+ for Co-Infection

For sample conditions requiring PBS+/+ used as a control, 50 µl was used for initial infection, or 20 µl for the secondary infection.

Beta Galactosidase Assay and Bradford Total Protein Assay

After the co-infection incubation finished, the Calu-3 cells were lysed for total protein assay followed by a beta galactosidase assay to measure adenovirus transduction.

Sample Lysis

One hundred twenty microliters of Tropix Lysis solution (Applied Biosystems) diluted at a ratio of 1:5 total volume with deionized H_2O was added to the transwell inserts. Using an Eppendorf Research Plus 200 µl adjustable micropipette the cells were gently scraped from the PET membrane, mixed, and transferred to a sterile micro-centrifuge tube. To ensure maximum sample recovery, the transwell insert membranes were cutout using a scalpel and placed into the same micro-centrifuge tube as the sample lysate. The tubes were vortexed, then centrifuged for five minutes at room temperature at 17,000 x g in a Thermo Scientific Sorvall Legend Micro 17. Sample supernatant was transferred to a new micro-centrifuge tube.

Bradford Assay

BioRad protein assay dye was diluted at a ratio of 1:5 final volume with deionized H_2O.

Six microliters of the well-mixed sample lysis from above was pipetted into a plastic one ml cuvette. Five hundred microliters of the diluted dye added to the plastic cuvette using a Ranin AutoRep repeater pipette. Sample absorbance was measured at 595 nm on Eppendorf BioPhotometer plus. 6132. Protein concentration was calculated using the standard curve established in the lab: y = 0.0474 (ABS) (1000-fold dilution) - 0.0278

Beta galactosidase Assay

β-Galactosidase assay was performed using Applied Biosystems Galacton-Plus™ Substrate and reaction buffer and Light Emission Accelerator-II. Luminescence was read on a BioTek Synergy™ H1. The luminescence was normalized to the total protein concentration of the sample to quantify adenovirus transduction of the Calu-3 cells.

A volume of two µl of the above well-mixed sample lysate was pipetted into 96-well flat-bottom optically clear microplate. Galacton Plus was diluted at a ratio of 1:100 with the Galacto Reaction Buffer Diluent. Using a Ranin AutoRep repeater pipette, 100 µl of the diluted Galacton Plus was pipetted into the sample microplate wells. The plate was then covered with a lid, placed inside a Styrofoam box, and incubated for 60 minutes at room temperature in the dark. After incubation, 150 µl of Tropix accelerator II was added to the sample wells and the plate was immediately read for luminescence using the BioTek Synergy™ H1.

DNA Isolation and Quantitative PCR Analysis

DNA isolation from Calu-3 on transwell inserts was accomplished by using Qiagen DNeasy-Blood-Tissue following the manufacturer's protocol. Samples to quantify the amount of dsDNA in each of the sample using a Nanodrop One (thermo-fisher). Employing the data from the Nanodrop, an adjustment can be assessed for each dsDNA sample concentration to ensure equal loading of template. qPCR was performed using Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR thermocycler (Life Technologies, ThermoFisher) following the manufacturer's protocol. QuantaBio PerfeCTa SYBR Green SuperMix Low ROX Catalog Number: 95096 qPCR master mix was used in 10 ul reaction volume.

Component	Volume for 10 µl Reaction	Final Concentration
PerfeCTa TA SYBR Green SuperMix Low ROX (2X)	5.0 µl	1X
Forward Primer	0.5 µl	100nM
Reverse Primer	0.5 µl	100nM
Template	4.0 µl	48ng

Table 3 QPCR Mix

IDT Ready Made Primers for human GAPDH Housekeeping gene control.

Primer	IDT Catalog Number	DNA Sequence (5' to 3')
GAPDH Forward Primer	51-01-07-12	ACC ACA GTC CAT GCC ATC AC
GAPDH Reverse Primer	51-01-07-13	TCC ACC ACC CTG TTG CTG TA
Adenovirus Hexon Forward		ACGCCTCGGAGTACCTG AG
Adenovirus Hexon Reverse		GTGGGGTTTCTGAACTT GT

Table 4 Primer Sequence

	Temperature	**Time**
Initial Denaturation and Activation	95° C	10 Minutes
QPCR Cycling 40 Cycles		
Denaturation	95° C	10 Seconds
Primer Binding, Elongation, Data Collection	59° C	1 minute
Melt Curve		
Denaturation	95° C	1 minute
Start	59° C	30 Seconds
Data Collection	0.05° C	1 second
End	95° C	30 Seconds

Table 5 QPCR method

III. RESULTS

The experimental conditions were as follows:

1. A mock bacterial infection of cells with PBS for four hours followed by AdLacZ for one hour.
2. A mock bacterial infection of cells with sterile Todd Hewitt broth with yeast extract for four hours followed by AdV for one hour.
3. An initial infection of cells with *S. pneumoniae* for four hours followed by AdLacZ for one hour.
4. Negative control (cells not infected with either *S. pneumoniae* or AdVs)
5. An Initial infection of cells with *S. pneumoniae* for four hours followed by PBS for one hour was a positive control for bacterial infection but a negative control for Adv.

Comparing the results can help determine if there is a difference in initial infection of lung epithelial cells with *S. pneumoniae* and subsequent infection with adenovirus. The hypothesis is that there will be a decrease in viral transduction of Calu-3 epithelia during co-infection compared to adenovirus infection alone. Each sample was measured in duplicate to ensure validity of the results. These cells were treated in wells on four different plates where each plate contained multiple samples for each experimental condition (replicates). Conditions 1, 2, and 3 are the conditions of interest, so they are the only experimental conditions that were included in the statistical analysis. There are four response variables of interest: protein concentration, luminescence, the ratio of these two measures (luminescence: protein concentration), and cell-associated viral genomes as measured by quantitative PCR (qPCR).

Protein Concentration

Protein quantitation is often necessary before processing protein samples for further testing and is often used to normalize measurements since cell numbers can vary from well-to-well. Protein quantitation was performed using a Bradford assay as described in Materials and Methods section. The protein concentration was then used to normalize the luminescence values of the Beta galactosidase assay. Ideally, no significant differences amongst the conditions would be present. Denoting equal extraction efficacy. However, there is strong evidence to suggest there is a significant mean difference between the protein concentrations for condition 2 (Broth, Adenovirus) and condition 3 (*S. pneumoniae*, Adenovirus) (p value = 0.0033) (Figure 1). No other significant differences were detected.

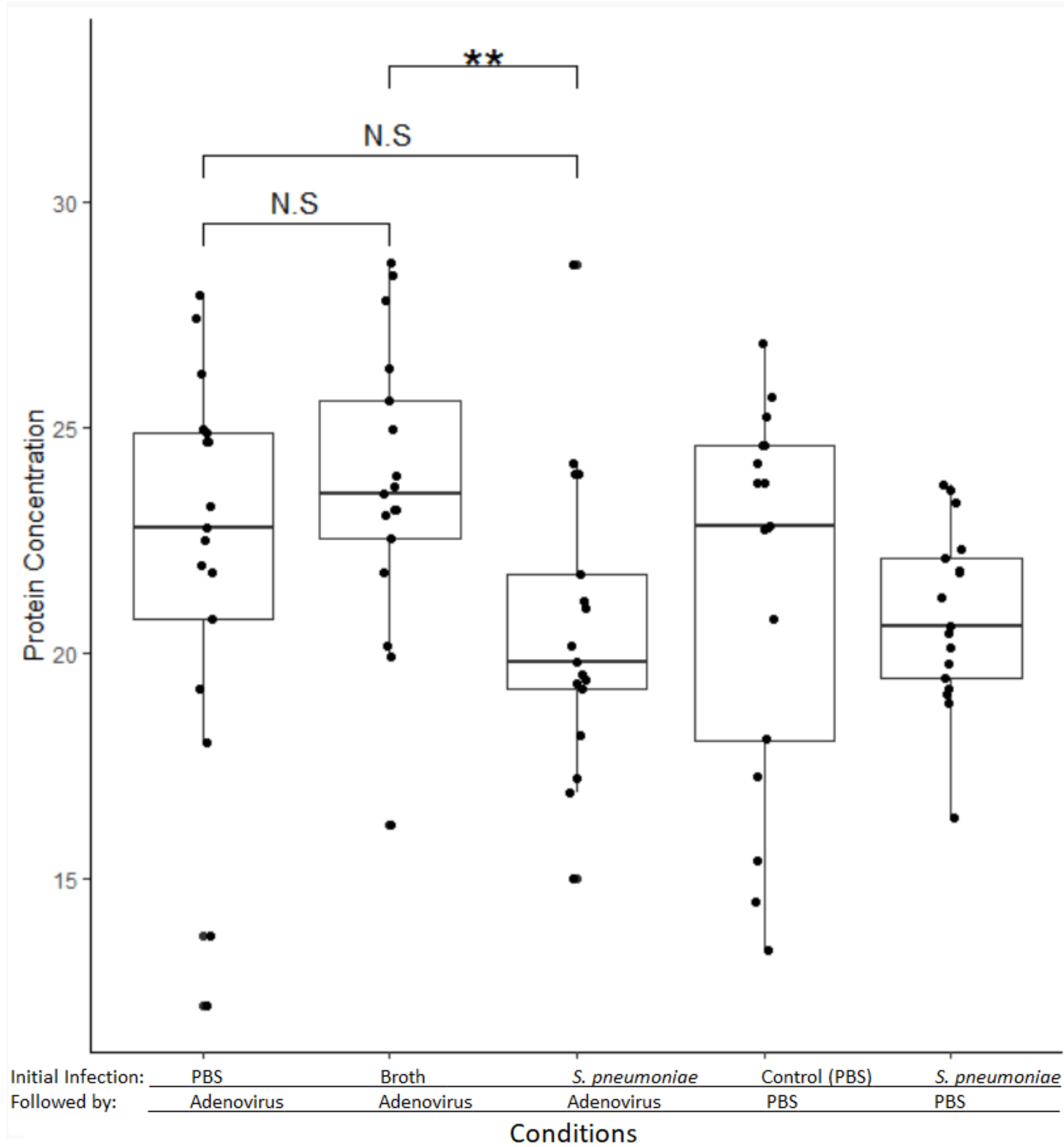

Figure 1: Boxplot for protein concentration ug/ul by condition. N.S. means no significant difference between conditions. ** There is strong evidence to suggest there is a significant mean difference between the protein concentration means for condition 2 and 3 (p value = 0.0033).

Luminescence

A luminescence assay was used to determine the β-galactosidase protein production from the LacZ reporter gene encoded in the recombinant adenovirus. β-galactosidase production indicates viral transduction of the Calu-3 cells. The results of the experiments provide strong evidence to suggest there is a significant mean difference between the natural log of the luminescence for conditions 1 and 2 (p value = 0.03) (Figure 2). There is also strong evidence to suggest there is a significant mean difference between the natural log of the luminescence for condition 1 and 3 (p- <0.0001). Lastly, there is strong evidence to suggest there is a significant mean difference between the natural log of the luminescence for condition 2 and 3 (p value <0.0001). There is no difference between conditions 3, 4, and 5.

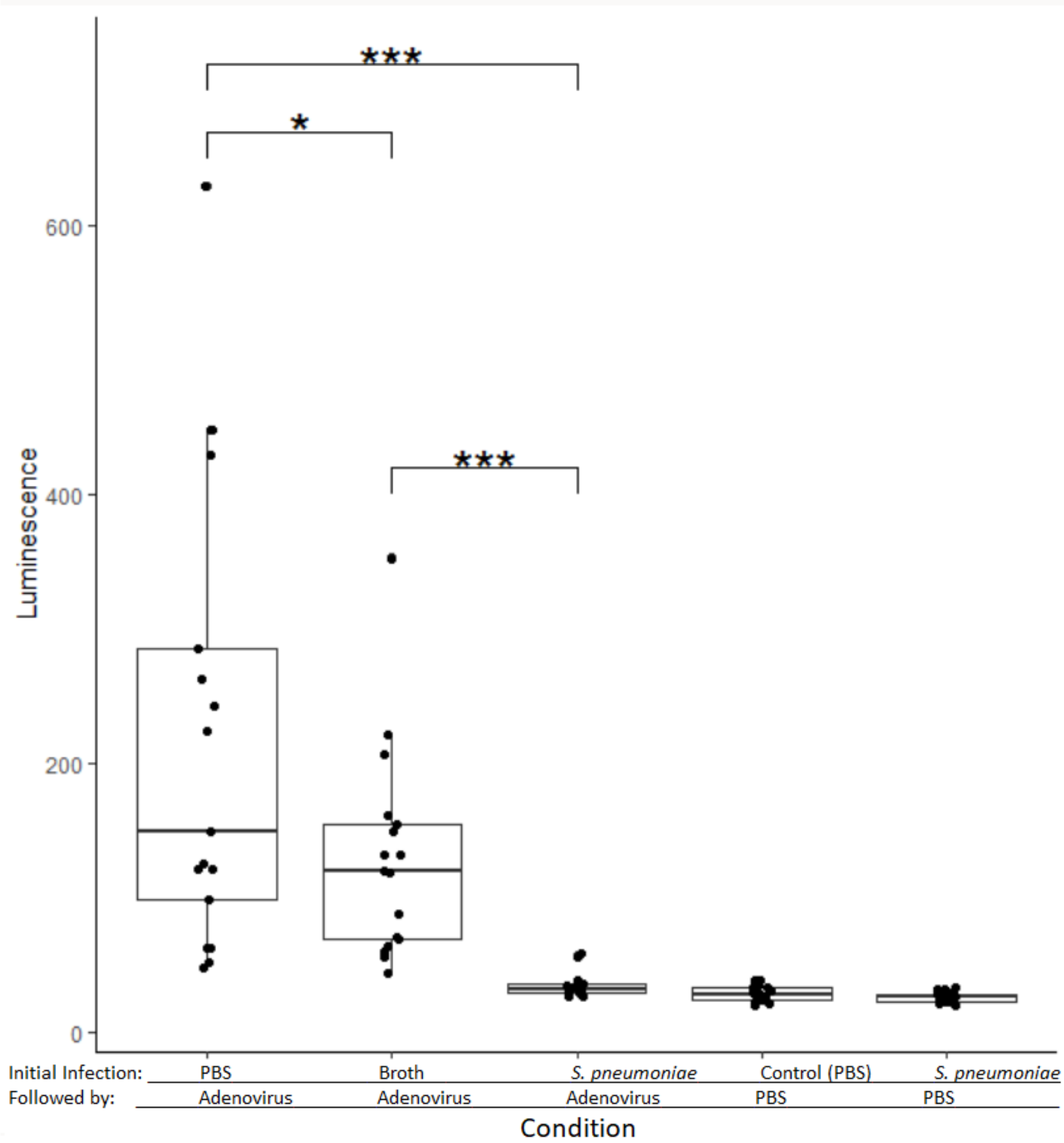

Figure 2. Boxplots for luminescence by condition. * Significant difference between condition 1 and 2, (p value = 0.03). *** Significant difference in the conditions of interest (3) compared to condition 1 and 2, where both p values were <0.0001.

Ratio of Luminescence/Protein Concentration

The ratio is determined by using the results of the luminescence assay and dividing by protein concentration. This allows the luminescence results to be normalized based on the amount protein, proportional to the number of cells in the sample, to determine if there is a significant difference between conditions. This analysis is used to determine if initial infection of lung epithelial cells with *S. pneumoniae* has an effect on subsequent infection with adenovirus. Not surprisingly, since the ratio is a function of luminescence, the constant variance assumption was again questionable, and another natural logarithm transformation was required. There is strong evidence to suggest there is a significant mean difference between the natural log of the ratio for 1 and 2 (p value 0.0044). There is also strong evidence to suggest there is a significant mean difference between the natural log of the ratio for 1 and 3 (p value <0.0001). Lastly, there is strong evidence to suggest there is a significant mean difference between the natural log of the luminescence for 2 and 3 (p value <0.0001).

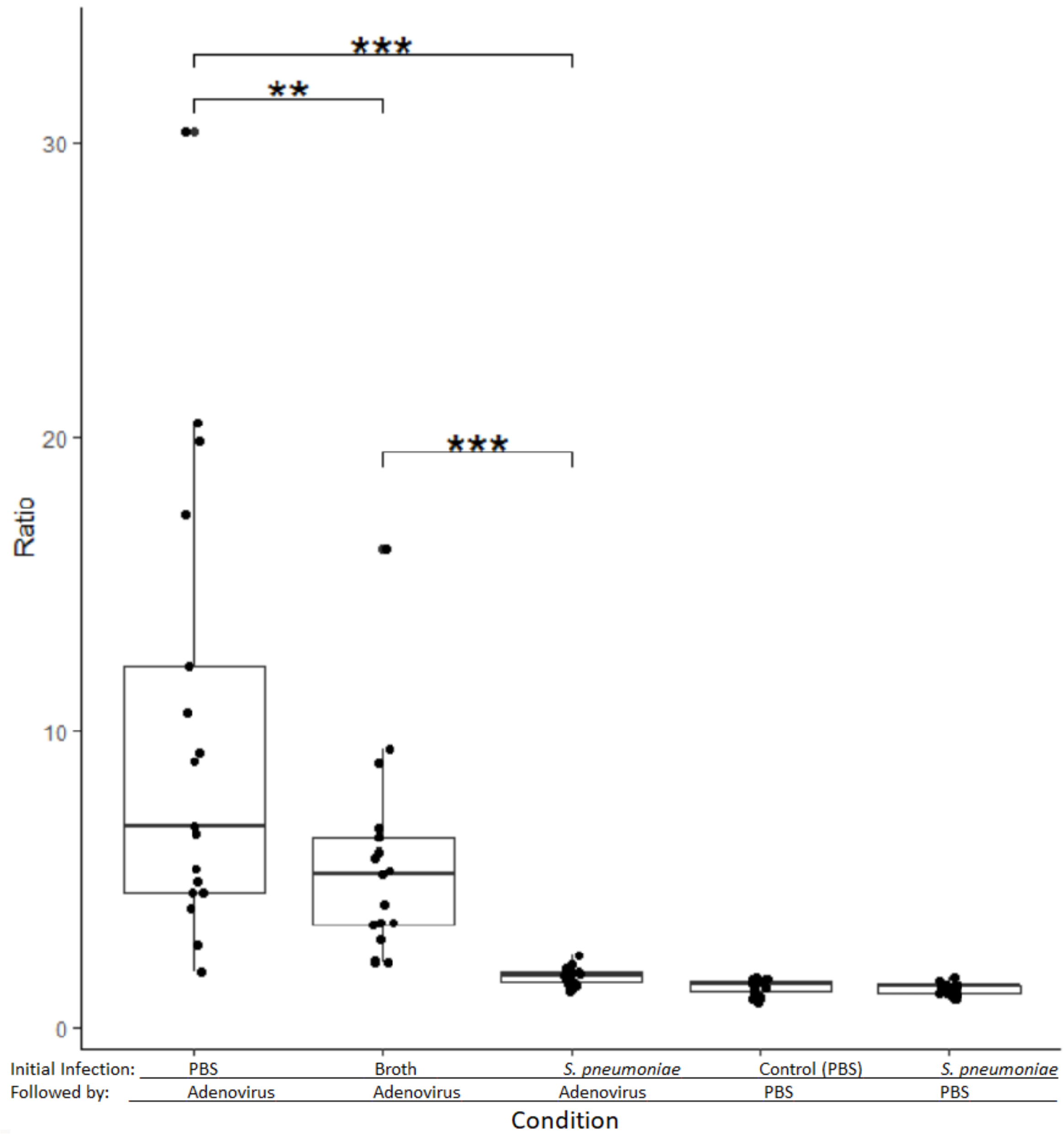

Figure 3: Boxplots for ratio by condition. **Significant difference between condition 1 and 2, (*p* value = 0.0044). *** Significant difference in the conditions of interest (3) compared to condition 1 and 2, where both *p* values were <0.0001.

Quantitative Polymerase Chain Reaction

Since Adenovirus has a DNA viral genome, DNA was isolated, and qPCR was used to determine the amount of cell-associated viral genomes. Determining whether initial infection of lung epithelial cells with *Streptococcus pneumoniae* has an effect on the amount of viral genome that is associated with the cells was determined in terms of relative quantification (RQ). Each sample was measured in duplicate to ensure validity of the RQ values. Since the duplicate measurements were identical, only one of them was used in the statistical analysis. Conditions 1, 2, and 3 are the focus of the results, so these were the only experimental conditions that were included in the statistical analysis. However, the analysis of variance resulted in a p value of 0.11, meaning there is not sufficient evidence to suggest there are any significant differences between any of the three conditions.

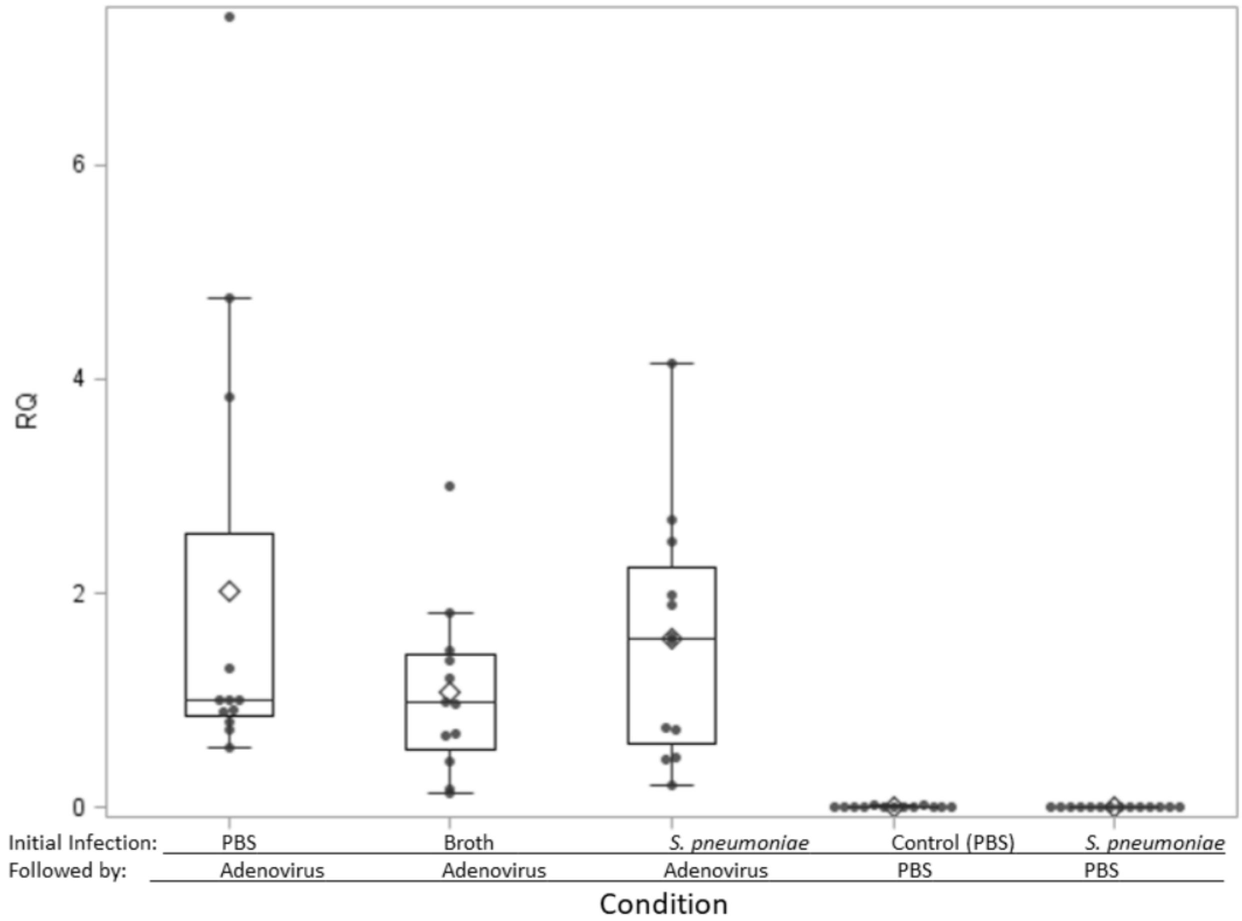

Figure 4: Boxplots for RQ by condition.

IV. DISCUSSION

The results of the experiments suggest that cell-associated AdLacZ viral genome was not affected by the presence of *S. pneumoniae,* but that the expression of the reporter gene in the AdV genome was hindered by initial infection of the Calu-3 cells with *Streptococcus pneumoniae*. The original hypothesis was that *S. pneumoniae* and AdV co-infections on polarized airway epithelial cells (CaLu-3 cells) will lead to decreased AdV transduction. An explanation for decreased AdV transduction is a decrease in CAR^{Ex8} receptors on the apical surface of the CaLu-3 cells. Another explanation could be the destruction of the CAR receptor by *S. pneumoniae.*

An explanation for decreased adenovirus transduction could be that initial infection of cells with *S. pneumoniae* activates an innate immune response in epithelial cells that served as a defense from the viral DNA expression. Lung epithelia produce surfactant proteins SP-A and SP-D that have opsonizing properties (33). Lung epithelial cells also secrete anti-microbial peptides. Beta-defensens are amphipathic antimicrobial peptides that form pores in microbial lipid bilayers (33 page 47). It has been shown that pneumococcal infection activates Type 1 IFN expression response in respiratory epithelial cells (27). Epithelial cells can also produce chemokines and cytokines including IL-1β, IL-8, IL-6. Other studies have shown that bacterial pathogen-associated molecular patterns (PAMPs) within the phagolysosome can produce a response that can mimic viral infection (28). Whether one or more of these pathways of the innate immune recognition of *S. pneumoniae* is activated still requires further identification. However, these responses may be shared between the *S. pneumoniae* and Adv.

The results of these experiments produced a model system to investigate co-infection of airway epithelia. The results also provide a greater understanding into how co-infections affect the innate immune response.

Future Studies

Future studies should identify which pathways are activated and whether there is any up/down regulation of the AdV receptor CAR on epithelial airway cells. Measurement of cytokines IL-1β, IL-8, IL-6 production by Calu-3 cells would help understand the host cell innate defense responses. Western blot to quantitate CAR protein levels should also be performed. Immunostaining for localization of CAR, Junctional Proteins like Zo-1, *S. pneumoniae*, and Adenovirus by confocal microscopy. This information could be utilized for prospective studies of AdV as vector for gene therapy and further understanding of AdV and *S. pneumoniae* pathogenesis on airway epithelia. A further analysis of the mechanism of this interplay is still to be elucidated. These data show that pathogen-pathogen interactions can be important modulators of lung infection.

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