Strategies for A Novel Anti-Influenza Therapy

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ABSTRACT:

This experiment compares the implications of two methods of measuring viral particles, specifically Influenza particles in human cell lines in vitro. These strategies include plaque assays and xCELLigence screening. Plaque assays, also known as reduction assays, are plates that are overlaid with semi-solid medium that limits the spread of the virus and shows where each particle is located based on the “plaque” or empty space on the plates where cells have died and been removed. xCELLigence screening is a newer program that checks for “impedance”, an artificial number that will measure the cells killed by virus as well as cell to cell interaction on a 96 well plate that utilizes gold microelectrodes. Both methods have variables that can make them useful in certain situations, however, the focus is on how reliable the xCELLigence program is in comparison to more traditional methods of quantifying viral particles.

Keywords:
Plaque assays
xCELLigence
Viral Particles

INTRODUCTION:

Virology: Influenza virus A (IAV) consists of 8 negative, single-stranded segments of viral RNA. These strands form a spherical shape that can be 100nm in diameter. Each of these vRNA strands contain noncoding regions which may include mRNA signals for polyadenylation as well as the signals for virus assembly (2). Hemagglutinin (HA) and neuraminidase (NA) are glycoprotein spikes that are located on the virion and can be used to characterize the type of Influenza virus. The Influenza virus attaches to host cells by recognizing N-acetylneruaminic, or sialic, acid on the cells’ surface which is found on many cell types of multiple animal species (2). In the human respiratory tract, there are more alpha-2,6 linkages compared to alpha-2,3 which are recognized by the HA glycoprotein spikes (2). HA is a trimer with 2 important regions, a “stem” of alpha-helices and a “head” of antiparallel beta-sheet. After gaining entry through endocytosis, the virus will uncoat in low pH due to HA structure change, resulting in the exposure of a peptide that helps facilitate pore opening between the viral envelope and endosomal membrane (2). The viral ribonucleoprotein (RNP) complex will then have access to the host cell’s cytoplasm. In addition to low pH, the M2 (matrix) transmembrane ion channel in the IAV envelope allow for hydrogen ions to be pumped into the virus particle from the endosome which is why most Influenza drugs are developed to block the M2 protein activity and stop viral RNPs from being released into the cytoplasm. When present in the cell’s cytoplasm the RNPs are then directed to the nucleus by the nuclear localization signals (NLSs) of viral proteins (2). It is in the nucleus that the vRNA will then be synthesized and translated into proteins.

Antigenic shift: The result of an Influenza strain incorporating HA or NA segments from a different Influenza subtype. This can occur when the virus is in different animals or humans which may create new antigenic proteins that are harmful to humans (2). Virus strains can have multiple amino acid changes when the cell’s antibodies try to neutralize the virus. It is most likely that Influenza A (H1N1) virus could have arisen
from an antigenic shift and had devastating effects because the human population did not have preexisting immunity (2).

**Antigenic drift:** The minor amino acid changes during antigenic shift can accumulate and lead to antigenic drift which makes it more difficult for the cell to neutralize the virus strain (2). Host antibodies can no longer recognize the mutated strains that circulate over the course of years. This is why there are continually emerging strains that have to be vaccinated against every year.

**Epidemiology:** Influenza is an infectious illness. Following the first Influenza pandemic that occurred in 1580, there have been about four pandemics in the 19th century and three in the 20th century. One of the most well-known pandemic is the “Spanish” Influenza which resulted in between 20 and 40 million deaths between 1918 and 1919 (6).

The “Spanish” Influenza appeared in North America, Europe, and Asia which makes it difficult to find the point of origin, which led to multiple outbreaks throughout the years (6). It was unusual to see these outbreaks within 1 or 2 years, since the Influenza virus is antigenically modified every 2 to 3 years. Sequences of the 1918 virus show that two of the surface proteins recovered from avian-like influenza virus (10). These proteins, in addition to novel Hemagglutinin (HA) and neuraminidase (NA) glycoproteins rendered swine and humans susceptible to the “Spanish” Influenza (10).

**CURRENT ANTI-VIRAL TREATMENTS:**

Vaccines have proved to be somewhat successful for seasonal Influenza, however researchers and companies cannot fully prevent the stains and treatment is only based on predictions of emerging strains. In addition, it takes time to develop and administer the vaccine. Currently, there are 2 classes of anti-viral treatments that are available: 1) channel blockers 2) inhibitors of viral proteins- for example, Tamiflu, which inhibits NA.

Anti-viral treatments such as amantadine and rimantadine can target viral proteins, specifically, the M2 proton channel. This channel is an excellent target because the M2 channel activity allows for protons to move away from the endosome which results in a lower pH, allowing viral genetic material to move into the cytoplasm to begin replication (1). The M2 proton channel will also maintain a pH gradient for the Golgi lumen and the cell’s cytoplasm to avoid Hemagglutinin from undergoing conformational changes while the virus is maturing (1). When M2 is disrupted then Hemagglutinin’s shape will be altered which will prevent future viruses from uncoating and there will be less pore openings between the viral envelope and endosomal membrane (1).

Certain neuraminidase inhibitors such as oseltamivir and zanamivir have been approved for the use against Influenza A and B, however, there have been emerging strains that seem to have more resistance to the inhibitors (11). This is due to the amino acid mutations that can be found on the active site of neuraminidase (11).

Tamiflu, an oseltamivir drug that inhibits NA works as an antiviral drug by inhibiting its enzymatic activity. Since NA is necessary to spread the virus to other cells, Tamiflu is successful in slowing down the virus. However, the NA gene segments—
specifically the H275Y—can change, resulting in a NA protein that is resistant to oseltamivir (5). This results in a mutation where the amino acid, Histidine, can replace Tyrosine (5). During the 2015-2016 influenza season there were only a small amount of H1N1 viruses that were resistant to oseltamivir (5). Because of this the CDC believes that there does not have to be any influenza antiviral drug use that has to be changed during the 2016-2017 flu season. Therefore, although Tamiflu seems to be a successful antiviral drug it cannot account for circulating strains that are resistant. This is why it is important to continue to study the mechanisms of the Influenza virus and to find new drugs that can target the non-mutating parts of the virus (5).

Recently, there are more antiviral drugs that are being tested that target the RNA polymerase that is necessary for the Influenza replication and transcription. Viral RNA or vRNA is important because the RNA genome act as templates for new copies of the RNA. In transcription, the viral RNA polymerase is responsible for creating RNA primers so that the vRNA can be synthesized (8). One particular RNA polymerase target is the trimeric RNA-dependent RNA polymerase or RdRp (8). This RNA polymerase is one of the largest vRNA polymerases known and it has been shown to be acquired from zoonotic transfer. RdRp can initiate transcription by cleaving the pre-mRNA near the 10-15 nucleotide long cap (9). Since it is positioned close to the 3’ vRNA terminus, this helps align the capped primer and the vRNA. During termination the poly-A tail is added when the RdRp stutters at the oligo-U tract (9).

Replication will produce complementary copies of the vRNA called cRNA (9). These cRNA can bind to new, viral NP and RdRp to form complexes that help synthesize more vRNAs. Previous studies have shown that cRNPs, the complementary ribonucleoprotein complexes can be taken from isolated cells and used to infect other cells in vitro which resulted in the production of new vRNA in the infected cells. This shows that by inhibiting viral RNA replication there is a chance to stop the cells from creating new vRNA.

It is necessary to conduct research on a new class of drugs. The main goal is to target the “human” parts of the virus, the host. IAV acts as a parasite, infecting multiple cells at the same time and reproducing genetic material and proteins rapidly. Since Influenza mutates quickly with its 8 chromosome-like strands it is possible to make drugs that incorporate hybrids which will make it more difficult for the virus to mutate. This is why new programs such as xCELLigence can be used to discover new data that could help create a new class of drugs against Influenza.

MATERIALS AND METHODS:

MDCK cells: The Madin-Darby Canine Kidney Epithelial cells originate from a female cocker spaniel in 1958. These cells are morphologically epithelial which make them an ideal model for IAV infection, since IAV affects cells of the respiratory system. Since epithelial cells do not access blood and nutrients directly, there are diffusion methods that are utilized to obtain oxygen as well as excrete metabolic waste. They grow well in the lab with DMEM 10% FBS media (Dulbecco’s modified Eagle’s medium with Fetal Bovine Serum) (7).

DMEM: Dulbecco’s modified Eagle’s medium is necessary for MDCK cells because it contains necessary amino acids, vitamins and supplementary components. It
is the best choice of media based on MDCK morphology (3).

**FBS:** Fetal Bovine Serum is used in conjunction with DMEM as a growth supplement because it contains metabolic factors that promote growth (4).

**Plaque Assay:**

**Day 1**- MDCK cells are grown in a 100mm dish with DMEM, 10% FBS media. To collect the cells for plating the media is discarded with via vacuum and glass pipet tip. Then, 1mL of Trypsin, an enzyme that digests protein is added to the dish and quickly discarded. Afterwards, 2mL of Trypsin is added to the plate and then incubated at 37°C for 7-8 minutes. This ensures that the cells will lift off the plate. The plate was taken out of incubation and the MDCK cells were collected in a clean conical tube. 5mL of DMEM and 0.5% BSA (Bovine Serum Albumin) was added to the dish and then moved to the conical tube with MDCK cells. The conical tube was then taken to a centrifuge for a 6 minute spin down at 0.4 rcf. After spin down the conical tube contained a pellet at the tip and supernatant. The supernatant was then discarded via vacuum and glass pipet and the pellet was loosened. 5mL of DMEM and 0.5% BSA media was added to re-suspend the cells for cell counting. Later, 10uL of the resuspension was injected between a cell counting grid and cover slip. The final confluency, cell volume, needed was $5 \times 10^5$ which was calculated by the following equation (Chen Lab):

$$X \text{ mL of cell } = \frac{(5 \times 10^5)(\# \text{ of wells})}{(\text{Avg. } \# \text{ of cells})(1 \times 10^4)}$$

The above equation was used to determine the mL of cells that were present in the conical tube. Then, the total amount of media needed to plate the cells was calculated by counting the number of wells needed and subtracting the mL of cells to reach the total volume with DMEM and 0.5% BSA. Finally, 1mL of cells and media were added to each well (24 wells in total). The plates were then left in the 37°C incubator for 24 hours.

**Day 2**- The 12-well plates were checked for confluency where the bottom was completely filled with MDCK cells. A serial dilution was prepared with 1.5mL Eppendorf tubes, a total of 24 tubes with 8 tubes for each treatment. For the first tube of each treatment 980uL of DMEM and 0.5% BSA was added to the Eppendorf tube with 20uL of IAV viral media. Tubes 2-8 of each treatment had 500uL of DMEM and 0.5% BSA added to them before dilution. From the first tube of treatment the viral media and DMEM/0.5% BSA were mixed and 500uL was transferred to tube 2. The mixing and 500uL transfer procedures were repeated until there was 1mL of mixture in the last tubes, tube 8, of each treatment. This resulted in a 1:50 dilution of viral media to DMEM and 0.5% BSA.

The plates were taken out of the incubator and the old media was discarded via vacuum and glass pipet. Afterwards, 400uL of each dilution was added to their respective wells and placed into the 4°C fridge for 1 hour. Then, the plates were moved to the 37°C incubator for 1 hour. During this final hour a mixture was made with 25mL of Avicel, 25mL of 2xDMEM and 25uL of NAT in a conical tube for a total volume of ~50mL. After the final hour the viral media was discarded via vacuum and glass pipet and 2mL of the Avicel, 2xDMEM and NAT mixture were added to each well and left, untouched, in the 37°C incubator for 48 hours.


Day 3- skipped

Day 4- After 48 hours the plates were then taken out of the incubator and the Avicel, 2xDMEM and NAT mixture was discarded via vacuum and glass pipet. 1mL of Crystal Violet dye was added to each well and then the plates were left at room temperature for about 25 minutes. After the 25 minute time period the plates were dunked in a Styrofoam container filled with running D.I water until the dye was washed off, leaving the plaque visible. To calculate the particles of infectious units the following calculation was applied (Chen Lab):

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pfu\ per\ mL = \frac{\text{# of plaques}}{(\text{dilution})(\text{amount of viral mixture added})}
\]

With this calculation the number of counted plaques could be used to estimate the final amount of plaque forming units per mL of media.

**Impedence:** An artificial number acquired by the use of electricity for cell-cell and cell death sensing measurement systems such as the xCELLigence program.

**xCELLigence RTCA 96-well E plates with gold electrodes:** These were utilized in conjunction with the xCELLigence program to help measure “impedence” with multiple gold electrodes that run on the bottom the plate.

**xCELLigence:**
Before the experiment a test run with the xCELLigence plate reader was conducted.

**RESULTS:**

For the plaque assay the assays look as predicted. As the virus was more diluted the cells were less affected. The plaques can be seen as the white spots where there is cell clearance because of the Influenza virus. The plaques were then manually counted starting with the second plate (Fig.
2) with dilutions 5 through 8. Si #1 was the control and it can be seen that cell clearance was higher with dilutions 1 through 4 as opposed to IAV SiCYPA and IAV SiCYPB (Fig. 1).

Figures:

Fig. 1: Dilutions 1-4 show high amounts of viral activity. This figure compares the control, IAV Si #1, to treatments IAV SiCYPA and IAV SiCYPB. The results seem to show that there are higher amounts of IAV particles in the control than in the treatments. IAV SiCYPA and IAV SiCYPB look relatively similar in the amount of cell clearance.

Fig 2: Dilutions 5-8 show less amounts of viral activity and can be used to determine the amounts of viral particles in the IAV media samples-
This figure also demonstrates the higher amounts of viral particles in the control, IAV Si #1, as compared to the treatments. Calculations to quantify the amount of viral particles can be made from this plate. Future testing would have to start from the 5th or 6th dilution of this experiment.

**Fig. 3:** IAV and IAV/PSC treatments show MDCK cell-plate interaction over 50 hours. This figure shows that the treatment curves are around baseline during hour 30. Around hour 40 the IAV treatments causes the curves to dip into negative numbers for the cell index. IAV/PSC post treatment show slight cell retention as opposed to IAV and pre IAV/PSC treatments.

**DISCUSSION:**

The plaque assays made it easy to see where the cells have infected the cells, causing cell death. By adding the 2xDMEM, Avicel and NAT mixture to the wells the Influenza virus spread was limited due to the mixture’s semi-solid quality. The virus can only infect neighboring cells which results in visible plaques in the wells. The xCELLigence program made it possible to monitor multiple treatments at the same time and had similar results to the plaque assay. It can be determined that the PSC treatment lessened the amount of cell dissociation, however, the actual amount of cell to plate interactions could not be quantified. The results for the xCELLigence program matched those of the plaque assay (Fig. 3). When the MDCK cells were treated with IAV the graph was close to baseline where there was no change in the adherence of cells to the plate around 30 hours and as time passed the graph dipped lower into negative numbers for the cell index, the interaction between cells and plates. This indicated cell death. The graph then flattens out as the cells either stop dying or the rate between cell division and cell death are the same.

Both methods of viral treatment on cells have proved to have their advantages and disadvantages. Through previous work, plaque assays have been useful in estimating and quantifying the amount of viral particles.
present in the media samples. However, plaque assays are labor-intensive and the data is only semi-quantitative. Plaque assays cannot be used to screen multiple wells at the same time, since each experiment is limited to the amount of wells on each plate.

The xCELLigence program makes it easier to test multiple Influenza treatments on the cells at the same time. It is compatible with 96-well plates which is ideal for conducting high-throughput experiments. The data acquired from this method is completely quantitative, however, “impedence” has no biological meaning and cannot be used to compare to more traditional methods. Another disadvantage is that the bottom of the plates are covered with gold electrodes which make it difficult to check on the plated cells through a microscope. Overall, the novel xCELLigence method is more efficient time-wise, but, the plates are more costly.

Both the traditional method of plaque assays as well as this cutting-edge technology with the xCELLigence program both lack in the fact that the data collected from them are based on in vitro experiments. An ideal strategy would be to quantify viral particles within live animals, in vivo. Another future direction would be to take some of the viral media collected to conduct a MTT assay. This is an assay that uses a stain to measure toxicity in cells by assessing the metabolic activity within cells. The results of an MTT assay would be useful additions to both plaque assay and xCELLigence data.
References:


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