12-1-2018

Evaluation of a natural challenge model of zebrafish with Influenza A virus

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By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for an Honors Thesis in the Shackouls Honors College

Mississippi State, Mississippi

December 2018

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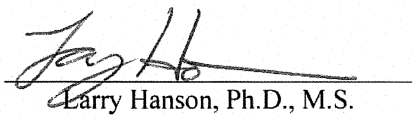
Approved:



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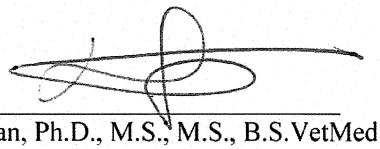

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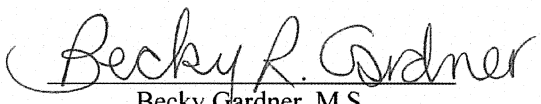

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Major Field: Microbiology Premed

Title of Study: Evaluation of a natural challenge model of Zebrafish with Influenza A virus

Pages in Study: 34

Honors Thesis

Influenza A virus (IAV) presents as a global public health concern, resulting in an extensive burden on morbidity and mortality rates in humans. IAV has high mutation rates, influencing emergence of new virus strains, which could lead to increased antiviral resistivity and altered virulence. Although prevention and treatment of influenza infections have been improved, animal models still play a major role in understanding of host responses and the pathogenesis of IAV infection. Zebrafish are useful host models for evaluating infectious diseases processes, especially with the ability to manipulate the genome of zebrafish. Research by others have shown that parenteral applied IAV could infect zebrafish embryos, supporting that zebrafish could be used as a host model of IAV infection. In this study, our goal was to determine if viral host adaptation or mucosal perturbation will provide a more useful model by allowing routes of infection through the respiratory epithelium. The prototype strain A/Puerto Rico/8/1934(H1N1) (abbreviated as APR8) was selected and used in this study. To host adapt the virus we attempted to propagate APR8 in SJD and ZF zebrafish cell lines, and to infect zebrafish embryos at 24-48 hours post-fertilization. In the process, we found that the zebrafish embryos and

cell lines were highly sensitive to the antibiotics and trypsin used in the medium to propagate the virus. We have therefore modified virus propagation procedures to optimize cell line production and embryo challenges. As a future study, once the adaption is successful, we will do comparative challenges of zebrafish embryos with parent strain and adapted virus, with and without mucosal perturbation. If successful, the zebrafish adapted virus can be used in pathogenesis, and innate defense research and comparative research will reveal the critical requirement for IAV adapting to a distant host.

ACKNOWLEDGMENTS

I would like to thank Dr. Hanson for mentoring and guiding me throughout the thesis process and for allowing me to conduct research in his lab. Your devotion to helping me become a better scientist is something that I will always be thankful for. Next, I would like to thank Lorelei Ford. Whenever I needed help with learning a new lab technique you were always there. Additionally, the endless amount of moral support is something I am extremely appreciative of. I would also like to thank the members of Dr. Wan's lab for helping me with all aspects of the training I received in the field of influenza. To my advisors Dr. Ashli Brown, Dr. Mary Reese, Ms. Becky Gardner, and core curriculum professors, I am very thankful for your guidance and mentorship throughout my time at Mississippi State. Finally, I thank my family for always loving me and encouraging me to strive towards my goals.

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CHAPTER I

INTRODUCTION

Host: Zebrafish

The zebrafish lifecycle after egg fertilization is divided into 4 major life stages: embryo, larvae, juvenile, and adult. The embryo is encased in a protective membrane called the chorion. In the chorion, there is a yolk sack that providing nutrients for embryonic development. The first 24 hours post fertilization (hpf) entails cleavage and permits cell division, but not cell growth. The blastula and gastrulation periods take place also. This is the stage at which they are most susceptible to genetic alteration. Gene expression can be seen as they develop into larvae because of their transparent body structures. After this 24-hour period, embryos will display activity and a beating heart. Embryos continue to develop and then hatch near 3 days post fertilization (dpf), where they form

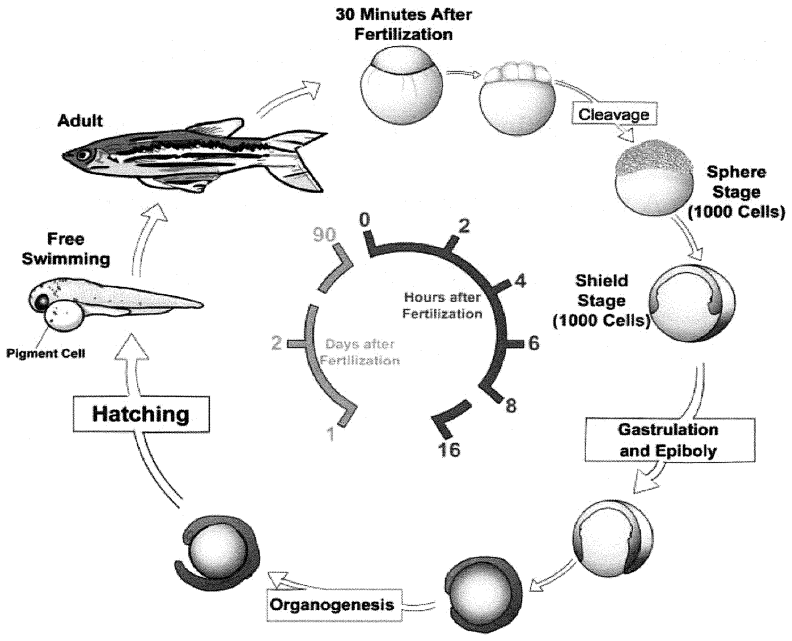

Figure 1. *Zebrafish life cycle*. D'Costa A and Shepherd IT, 2009.

specialized swimming structures, like the swim bladder, by 5 dpf where they rely solely on their innate immunity. From this point until 2-3 months of developing, they are in the juvenile life stage. After 3 months they reach adulthood which completes the lifecycle (**Figure 1**).

Pathogen: Influenza

Influenza viruses are enveloped negative-strand RNA viruses with a segmented genome holding seven to eight segments. Each of these segments encoding for at least one protein and are encapsulated by nucleoproteins (NP), which form ribonucleotide-nucleoprotein complexes (Palese and Shah, 2007). This virus is of the *Orthomyxoviridae* family and has four subtypes, influenza A, B, C, and D. Types B and C is mostly comprised of human hosts, with a few exceptions, influenza A (IAV) infects a wide variety of warm-blooded animals; influenza D virus is found in swine, cattle and small ruminants. Avian influenza viruses in aquatic birds serve as the natural reservoir for influenza A virus and probably are the ultimate source of human pandemic influenza strains (Webster et al., 1992). Influenza A viruses are subdivided by antigenic characterization of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins that project from the virion (**Figure 2**).

Due to influenza viruses RNA polymerase complex not containing proofreading activity, point mutations accumulate during replication and leads to the production of selective advantages for viral strains by allowing them to evade host immunity[2] (Taubenberger and Morens , 2008). The HA molecule initiates infection by binding to receptors on specific host cells. Antibodies against the HA protein can prevent receptor binding and are effective at preventing re-infection with the virus with similar antigenic properties. The HA and NA can evade previously acquired immunity by either (a) antigenic drift, in which mutations limit or prevent antibody binding, or (b) antigenic shift, in which the virus acquires HA or NA of a new subtype by reassortment between two influenza A viruses (Wright PF et al., 2007). These new strains from antigenic drift events can potentially cause pandemic outbreaks.

IAV has an extensive host range due to the crossing of different strains in various hosts. The mechanisms by which avian IAV cross species barriers to infect humans or other mammals, either causing dead-end infections or leading to subsequent transmission in the novel mammalian host, are not completely understood. Moreover, the zoonotic properties of IAVs that have the greatest medical and public health relevance, such as human infectivity, transmissibility, and pathogenicity, appear to be complex and polygenic and are not fully understood (Parrish et al., 2008; Taubenberger and Morens, 2009).

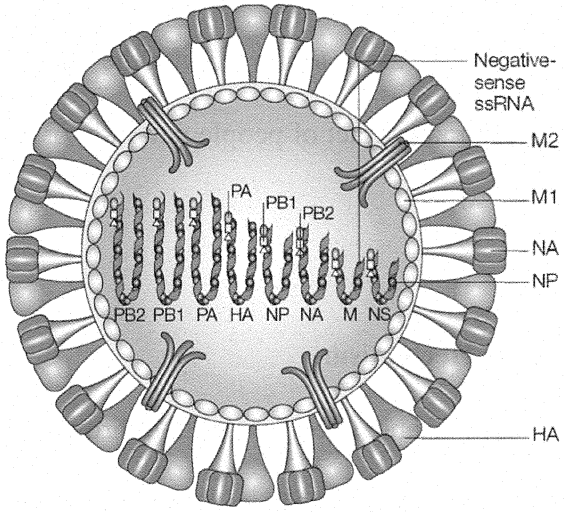

Figure 2. *Influenza virion structure.* Horimoto, T. *et al.*, 2005.

Avian IAV infections in humans: In the past decades, a large number of documented zoonotic avian IAV infections of humans have occurred, predominantly in association with epizootics of H5Nx highly pathogenic avian influenza (HPAI) in Eurasia and Africa (Peiris et al., 2007), and in epizootics of H7N9 low pathogenic avian influenza (LPAI) H7N9 in China (Xiang et. al., 2016) and a smaller epizootics with H7N7 HPAI in the Netherlands (Fouchier et al., 2004), and sporadic cases of H7N3 HPAI in Canada (Tweed et al., 2004) and H9N2 and H10N8 LPAI in China (Lin et al., 2000), all without evidence of stable adaptation or human-to-human transmission. Prior to this, there was limited evidence of direct avian-to-human IAV exposure, based on a small number of experimental human volunteer infections with LPAI (Beare and Webster, 1991), or by serological surveillance (Peiris, 2009). In humans, IAV replicates in the epithelial cells throughout the respiratory tree, with the virus being recoverable from both the upper and lower respiratory tract of people naturally or experimentally infected (Wright et al., 2007).

Infection Mechanism

IAV is able to be transmitted through aerosolized particles and enters the airways. It then attaches to the surfaces of respiratory epithelium. The viral membrane envelope contains HA proteins, responsible for the virus particle internalization through attaching to the host cell sialic acids, and NA proteins, responsible for releasing newly formed viruses. HA activation is dependent upon proteolytic cleavage of the HA precursor into HA1 and HA2. The cleaving of the HA molecule allows internalization of the host by endocytosis, forming an early endosome. In late endosomes, the pH drops which results

in a conformational change of the cleaved HA molecule. This conformational change permits the opening of HA1 which allows HA2 to extend to the endosomal membrane. Fusion peptide anchoring then permits the HA molecule to fold back on itself and fuses the viral membrane with the endosomal membrane. After fusion the 8 viral genomic segments are released into the cytosol of the cell, travel to the nucleus, and new virus particles are synthesized. The new virus particles are then released from the cell by budding from the host cell membrane and then infect neighboring cells (Samji Tasleem, 2009).

Zebrafish Challenge Models

Several members of the *Rhabdoviridae* family, having negative-sense ssRNA genomes, have been studied using zebrafish models. Spring Viremia of Carp virus (SVCV) was used to study IFN system and inflammation in zebrafish (Levraud et al., 2007). Snakehead Rhabdovirus (SHRV) was used to help understand the function of nonvirion genes (Alonso et al., 2004) and SHRV was the first viral infection reported in zebrafish embryos; however, this leads to an antiviral response with intraperitoneal injection of adult zebrafish (Phelan et al., 2005; Gabor et al., 2013; Gabor et al., 2015). Both viral Hemorrhagic Septicemia virus (VHSV), to study vaccine effectiveness (Novoa et al., 2006) and Infectious Hematopoietic Necrosis virus (IHNV), to study fish antiviral responses (Aggad et al., 2009) have been used in zebrafish models as well.

Nervous Necrosis virus (NNV) is a member of the *Nodaviridae* family and has a positive-sense ssRNA genome. Zebrafish infected with NNV displayed different interferon-1 (INF-1) responses in larvae than in adult zebrafish, which resulted in an induced acute and persistent infections respectively.

Two members of the *Iridoviridae* family, have also been shown to replicate in and infect zebrafish. Infectious spleen kidney necrosis virus (ISKNV) and European sheatfish virus (ESV), both containing dsDNA genomes, have been demonstrated to have the ability to successfully infect zebrafish (Lu et al., 2008).

Human pathogens chikungunya virus (CHIKV), herpes simplex virus (HSV-1), and influenza A virus (IAV) replicate optimally at 37°C. Palha et al. (2013) demonstrated that after recovery of challenged zebrafish, INF-1 responses determined the recoverability of the fish and CHIKV persisted in the brain. HSV-1 zebrafish models have served to demonstrate its persistence in adult zebrafish brains (Burgos et al., 2008), the presence of human homologs of viral entry receptors (Hubbard et al., 2010; Yakoub et al.), and the expression of IFN-1 (Ge et al., 2015). HSV-1 was the first successful zebrafish model for human viral infections demonstrated by Burgos et al. (2013). Caudal vein injection of IAV in zebrafish embryo was found to have an increase in viral burden and morbidity over time. The histopathology of these fish resembled the clinical symptoms of IAV infections in humans. This model, by Gabor et al. (2014), was the first live vertebrate model providing visualization of the interaction between the virus and host.

The growing temperature of zebrafish, 28°C, was considered a limitation for fish and human viral experimentation. However, these zebrafish models of fish and human viral diseases have contributed to a better understanding of viral diseases and host-pathogen interactions. Additionally, the ability to manipulate the genome of zebrafish makes it potentially useful as a host model and to study the process IAV uses to cause disease.

Benefit of Zebrafish IAV Models

Vaccines targeting specific viral antigens is challenging because of the high mutation rate of IAV. This pushes researchers to focus on host innate antiviral response as a better method for antiviral treatment. Animal models of IAV are essential to further understanding the pathogenesis of the virus and the responses of the host. Using zebrafish as an IAV model will be useful to study IAV transmission, adaptive immunity and improve vaccine development. Zebrafish are prime candidates for IAV studies because they have a substantial background as models for infectious diseases, are highly susceptible to genetic manipulation, and have a unique trait to rely only on their innate immunity in early stages of development.

CHAPTER II

OBJECTIVE & EXPERIMENTAL PLAN

Objective

Previous research has demonstrated that zebrafish embryos can be infected with influenza A virus (IAV) when the virus is injected into the bloodstream (Gabor et al. 2014). The objective of this study is to determine if adult zebrafish can be infected with influenza A, if the virus is allowed to host adapt.

Experimental Plan

This study will consist of 2 parts, virus propagation *in vitro* and titration viruses using different assays. *In vitro* virus production will be done in SJD and ZF cell lines with influenza A PR8 strain originated in MDCK cells. In the end, we want to determine the degree to which IAV can replicate in zebrafish, and whether host adapted IAV is more infectious to zebrafish.

CHAPTER III

MATERIALS AND METHODS

Cell Lines and Cell Culture

SJD (*Danio rerio* caudal fin fibroblast- ATCC) and ZF (*Danio rerio* fin cells-Nguyen, 2016) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)/(HAMS) media supplemented with 20% fetal bovine serum (FBS), 1% non-essential amino acids, 1% sodium pyruvate 1% penicillin-streptomycin. SJD and ZF Zebrafish cell lines were incubated at 28°C. MDCK (*Canis familiaris* kidney epithelial) cells were grown in enhanced Dulbecco's Modified Eagle's Medium (DMEM) complete media supplemented with 10% fetal bovine serum (FBS) and 1% PenStrep. MDCK cells were cells were incubated at 37°C, with 5% CO_2.

Viruses

The prototype strain A/Puerto Rico/8/1934(H1N1) (abbreviated as APR8) was selected and used in this study.

Virus Propagation

Method 1

For *in vitro* infections, MDCK cells were seeded into a T-75 flask and grown to confluence. The cells were then rinsed three times with 1X phosphate-buffered saline (PBS), and the APR8 stock virus was diluted to the desired MOI in 1X OptiMEM

supplemented with 1 μg/mL Trypsin and 100 U/mL PenStrep. The cells were inoculated and incubated for 1 hour at 37°C, with 5% CO2. The inoculum was removed and centrifuged at 4,000 x rcf. The suspension was frozen at -80°C in 1mL aliquots. *In vivo* virus propagation followed the same protocol; however, PenStrep and trypsin were not used in the process.

Method 2

The same steps as in *in vitro* infection method 1, were used in method two; however, TPS 1X OptiMEM was not used. Instead, trials of enhanced (DMEM) supplemented with 0%, 5%, and 20% FBS were used to infect zebrafish cells.

Hemagglutination Assay

In a V-bottomed 96 well plate, 50μL aliquots of PBS was added to all wells. 50μL of each sample was added to the first well of each column and diluted out to -8. A working solution of 0.5% turkey red blood cells (tRBC) was prepared and added to all wells. The plate incubated at room temperature for 30 minutes and the results were measured. A pellet of tRBC was formed at the bottom of the plate if negative. tRBC's suspended in the mixture indicated a positive sample.

$TCID_{50}$ Assay

A confluent monolayer of MDCK cells, in a flat-bottomed 96 well plate, were washed twice with PBS and infected with -1 to -8 dilution of a sample (triplicate or quadruplicate). After 72-120 hours of incubation (or until CPE is noticeable), 50μL of supernatant from each well was transferred to a V-bottomed 96 well plate. A working

solution of 0.5% tRBC was prepared and added to all wells. Following 30 minutes of incubating at room temperature, the results were measured. A pellet of tRBC was formed at the bottom of the plate if negative. tRBC's suspended in the mixture indicated a positive sample.

RNA Extraction

The cells were suspended in Tri Reagent® - MCRgene TR 118 in and the RNA extraction from supernatant and cells were done using the Zymo Research Group R2060 Direct-zol™ RNA MicroPrep kit. Using the bead beating method the samples were centrifuged at 16,000 x *g* for 1 minute. Then 400µL of the samples were transferred to a new microcentrifuge tube, 400µL of ethanol was added to each sample, and they were vortexed. This mixture was then transferred to Zymo-Spin™ columns and the vacuum method was used to collect the supernatant of the samples. For the DNase I treatment, 400µL of RNA Wash Buffer was added to the column and vacuumed. Next, a master mix with each sample receiving 5µL DNase I and DNA Digestion Buffer was made and distributed to each column. After 15 minutes of incubating at room temperature the supernatant from the samples were vacuumed. 400µL of Direct-zol™ RNA PreWash was added to the column and vacuumed. This step was repeated twice. Next, the columns were all transferred to collecting tubes and 700µL of RNA Wash Buffer was then added to the column. They spun for 4 minutes at 16,000 x g ensuring that all was buffer flowed through and then transferred to a RNase-free microcentrifuge tube. For the elution step, 50µL of DNase/RNase-Free Water was directly added to the column and centrifuged. The samples were then used for PCR.

qRT-PCR

RNA sample concentrations were then diluted to 50 ng/μL, determined with a NanoDrop™ spectrophotometer. Using AgPath-ID™ One-Step RT PCR 4387424 protocol, the PCR samples were prepared with a master mix containing 2X RT-PCR Buffer, Forward and reverse primers: (40μM) InfA Forward: 5'- GAC CRA TCC TGT CAC CTC TGA C -3' and InfA Reverse: 5'- AGG GCA TTY TGG ACA AAK CGT CTA -3', TaqMan® probe: InfA Probe: 5'- TGC AGT CCT CGC TCA CTG GGC ACG -3', and 25X RT-PCR Enzyme mix. 1μL of the RNA samples or positive or negative controls were added to PCR strip tubes containing 25μL of the master mix. These samples along with standards were done in replicates of 3. The system used to analyze the data was Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR Instrument. With a thermal cycling profile of: 1 cycle of Stage 1 – Reverse Transcriptase at 50°C for 10 minutes, 1 cycle of Stage 2 – RT inactivation/initial denaturation at 95°C for 10 minutes, and 40 cycle of Stage 3 – Amplification at 95°C for 15 seconds and 60°C for 45 seconds. The samples were then analyzed by the Applied Biosystems QuantStudio™ software to determine threshold cycles, established the standard curve, and calculated copy numbers in each sample.

CHAPTER IV

RESULTS & DISCUSSION

TPS 1X OptiMEM Infection Method

The method used to infect MDCK cells with APR8 (H1N1) influenza virus, was used to initiate the process of adapting the virus to zebrafish cells. These cells were incubated for 72 hours and the cytopathic effects (CPE) of the virus were observed at 24-hour intervals. At the 0hr time point, the wells displayed with a confluent monolayer of cells. However, at each time point, control samples exhibited signs of CPE similar to the low and high doses of infected cells (**Figure 3**).

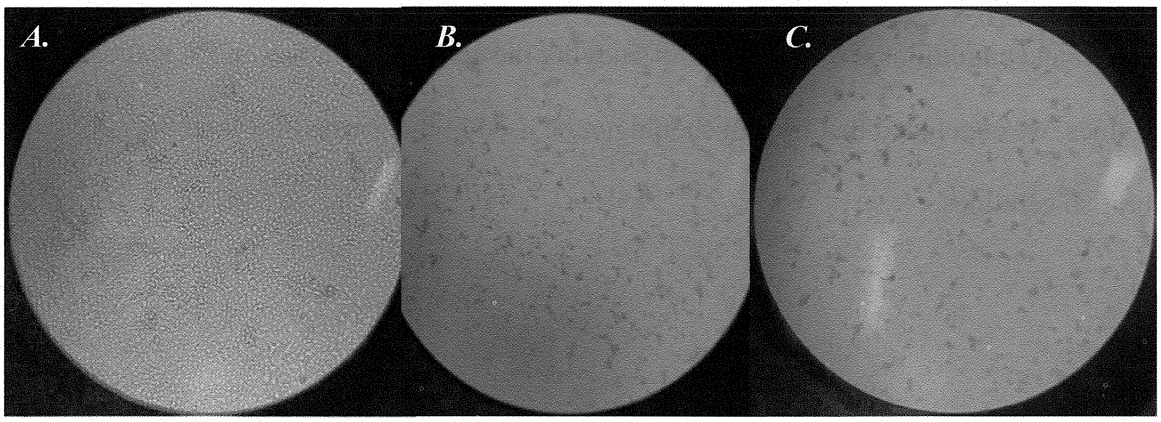

Figure 3. *Control zebrafish cells using TPS 1X OptiMEM protocol at A) 24 hours post-mock infection (HPMI), B) 48HPMI, and C) 72HPMI.*

Detecting Media Components Contributing to Cell Detachment

Trypsin is not a component of the cell culture media, and we wanted to see if trypsin was playing a role in false CPE readings in control samples. ZF and SJD cells were administered 1X OptiMEM with different compositions of trypsin. One sample of each cell line received media supplemented with trypsin and the other set receive media not supplemented with trypsin. These samples along with a control set of ZF and SJD cell lines with enhanced (DMEM) were incubated for 72 hours. The cells incubated using DMEM remained confluent over the 72-hour period. Less than 25% of the confluent cells were detached by the end of the 72-hour time period, upon observing cells with 1X OptiMEM not supplemented with trypsin (**Figure 4**). However, both cell lines receiving 1X OptiMEM supplemented with trypsin, displayed an increase in the amount of cell detachment at each 24-hour interval. At 72 hours, less than 15% of cells remained attached to the plate. These results were similar to infected cells held under the same duration and conditions. From the results studying the effect trypsin has on ZF and SJD cell lines, we concluded that trypsin should not be used in the method for infecting zebrafish cell lines.

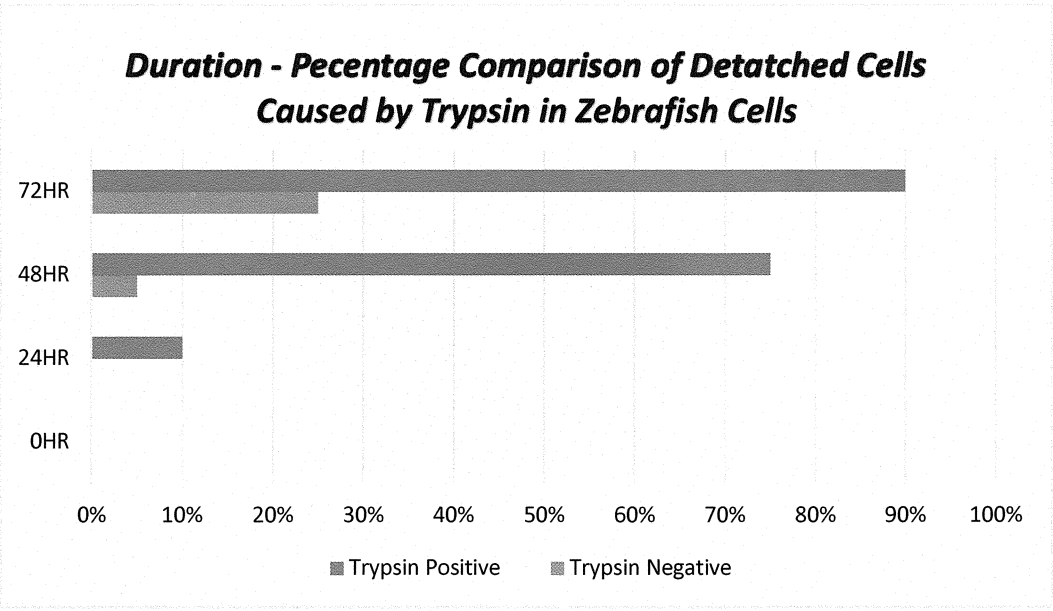

Figure 4. *The effect of the inclusion of 1 μg/mL trypsin in the medium on zebrafish cell lines.*

The Effect of Trypsin on Infecting Cells

During the viral replication cycle, cleavage of HA proteins embedded in the envelope of IAV is necessary to permit virus activation (Samji Tasleem, 2009). Trypsin has been shown to play an important role in the internalization process of influenza in MDCK cells (Zuhairi *et. al.*, 2012). Zuhairi *et. al.* demonstrated that trypsin enhances the infectivity of the virus by inducing endosomal membrane fusion in MDCK cells. To analyze how significant, the role of trypsin is in the viral titer produced during the infection of MDCK cells, we infected the cells with IAV and TPS OptiMEM with and without trypsin supplementation. We performed a $TCID_{50}$ assay and found the difference to be insignificant (**Figure 5**).

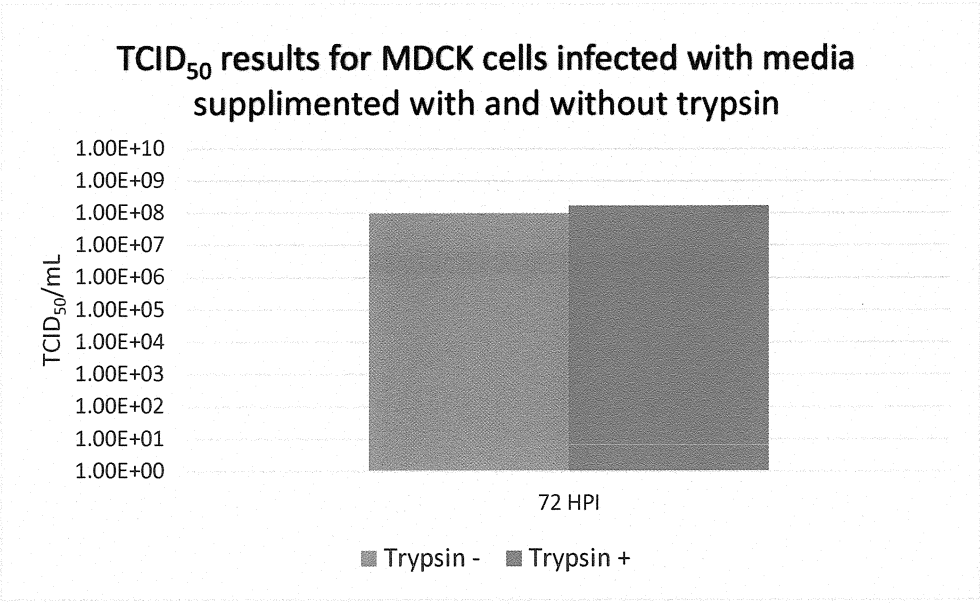

Figure 5. *The effect of 1 μg/mL trypsin on influenza A APR8 replication in MDCK cells.*

Results for Virus Detecting After Altering the Media

Therefore, we used of media lacking trypsin for zebrafish cell line infectivity assays. After these changes we did not detect virus when performing HA and $TCID_{50}$ assays on the zebrafish cell line extracts. We continued to see indications of cell detachment in sample not supplemented with trypsin. Given the gradual detachment of uninfected cells, 1X OptiMEM was concluded to not being optimal for zebrafish cells to be infected with and have the findings be conclusive. Subsequently, we switched the media to enhanced DMEM, because we know this media is optimal for zebrafish cell sustainability.

DMEM supplemented with Fetal Bovine Serum Infection Method

After switching the media, we incorporated FBS in the media for the infection process. Under normal infection methods with MDCK cell lines we would not use FBS because it inactivates trypsin. However, the zebrafish cells are not supplemented with trypsin and

this would not be an issue. With the addition of FBS and DMEM to our infection method, we saw consistent results in the cells remaining attached to the plates as we increased the FBS concentration and with infected cell samples we saw CPE. When using 20% FBS in the zebrafish cell lines we saw no indication of virus production in HA and $TCID_{50}$ assays. However, when analyzing PCR samples of the supernatant and cell samples of SJD and ZF cells, we were able to quantify viral RNA copies in the samples. In **Figure 6, 7** and **8**, we saw that there was not a significant indication of virus replication when comparing the 0 and 72 hours post infection samples. We also saw that there was not a substantial difference between the two cell lines.

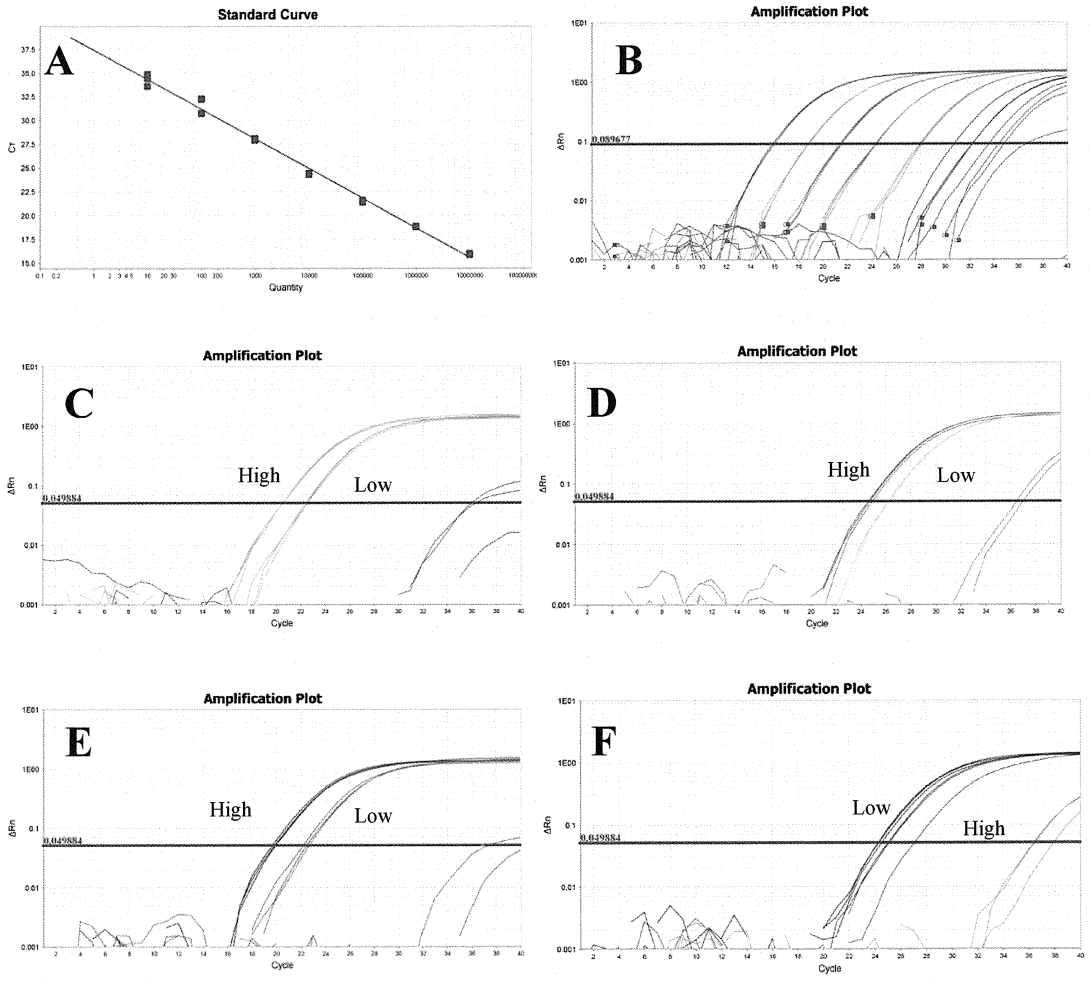

Figure 6. *0hpi PCR amplification plot. A) Standard curve for detecting M gene target, B) diluted standards (10-fold dilution from 10^7 copies to no copies), C) SJD cell samples (low and high dose), D) SJD supernatant samples (low and High) E) ZF cell samples (low and high dose), F) ZF supernatant samples (low and high).*

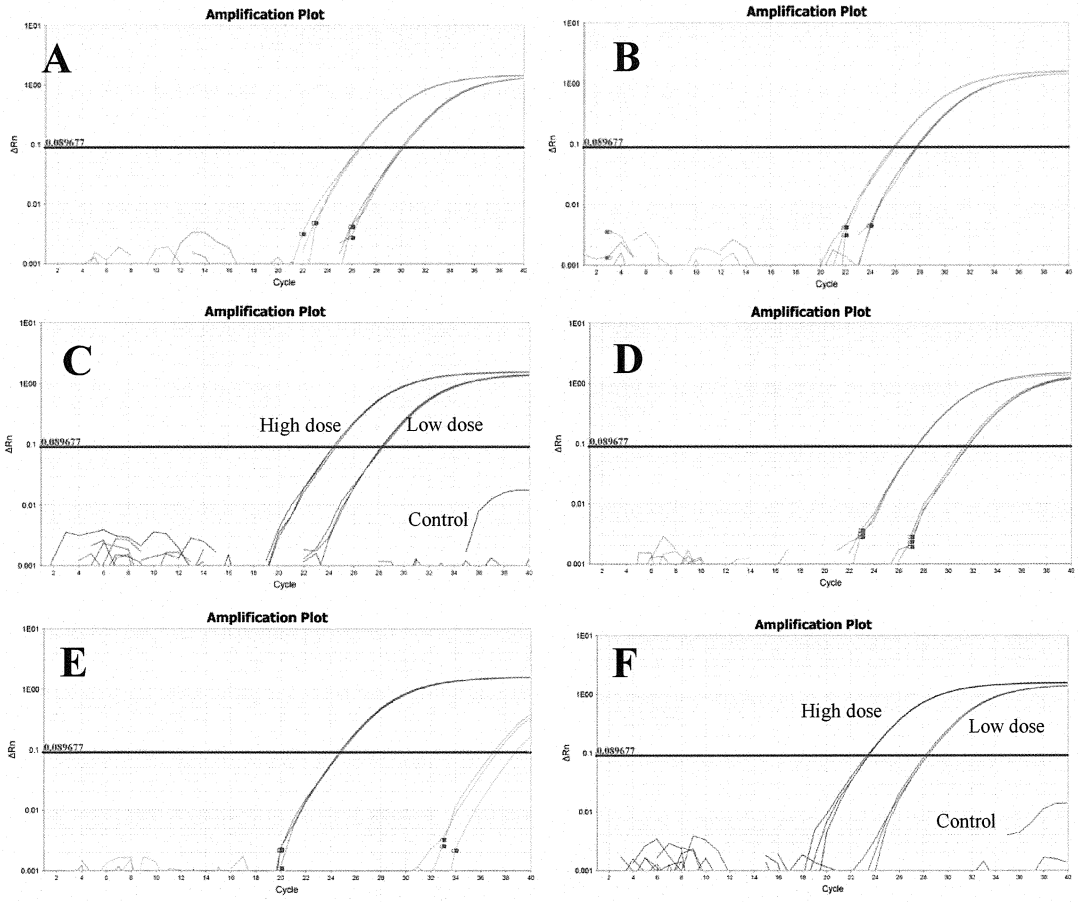

Figure 7. *72hpi PCR amplification plots. A) SJD low dose supernatant samples, B) SJD high dose supernatant samples, C) SJD cell samples (control, low dose and high dose), D) ZF low dose supernatant samples, E) ZF high dose supernatant samples, and F) ZF cell samples (control, low dose and high dose). Each amplification plot represents a replicate of the indicated sample, with the exception of cell samples (C and F). (The same standards and standard curve values were used from* ***Figure 6.****)*

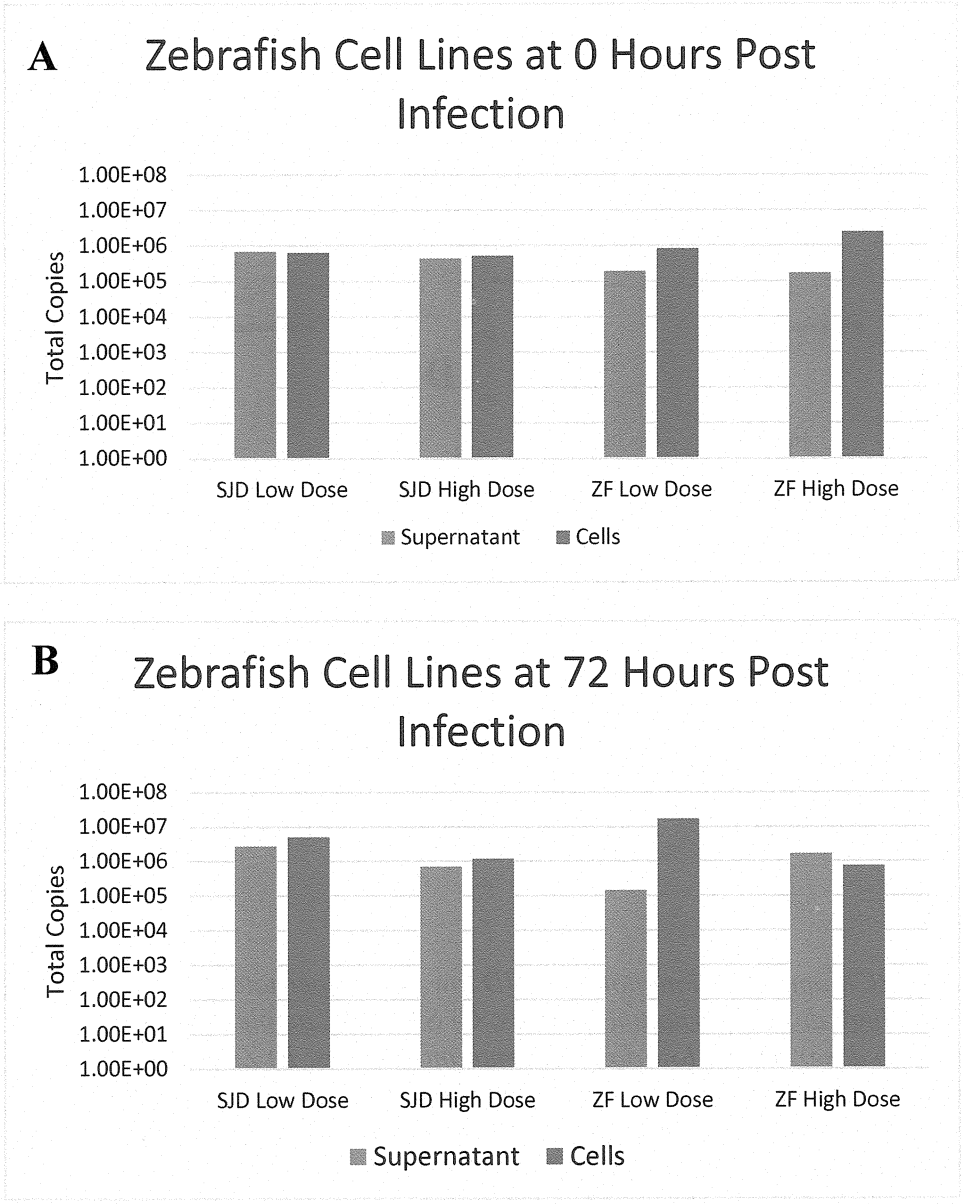

Figure 8. *Production of APR8 strain of influenza A (total copy number) in SJD and ZF cell lines at 0hpi (**A**) and 72hpi (**B**) with $1.78x10^8$ $TCID_{50}$ (high dose) or $8.9x10^7$ $TCID_{50}$ (low dose).*

Analyzing Temperature Differences and Their Relation to Infectivity in MDCK Cells

There is a distinct incubation temperature difference of 9°C between MDCK cells and zebrafish. Because of this we wanted to assess if this temperature difference had a significant role in the infectivity of the virus. Samples of MDCK cells were infected and incubated at 28°C. Another set of samples were infected and incubated at the normal temperature of the MDCK cells (37°C). $TCID_{50}$ values indicate that lowering the temperature to 28°C, decreases the virus titer at 72 hours post infection (hpi) (**Figure 9**).

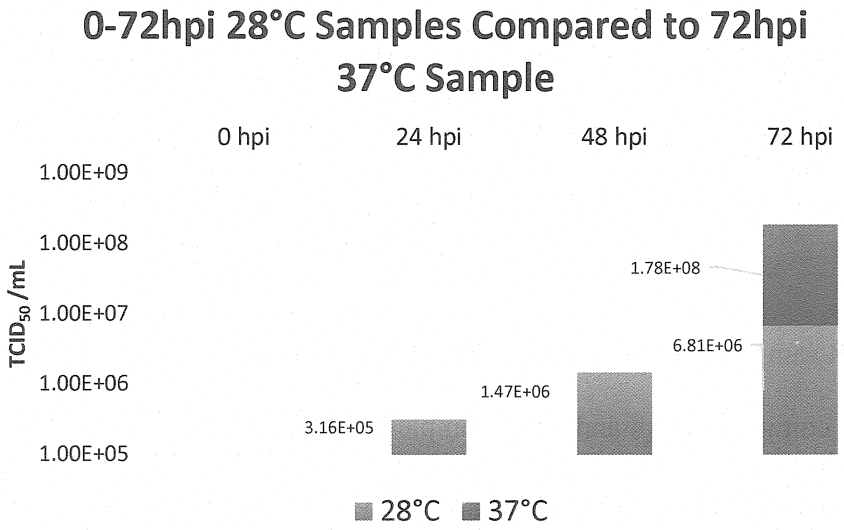

Figure 9. *The production of Influenza A virus in MDCK Cells at 28 ℃ and 37 ℃. Samples of supernatant from infected MDCK cell were taken at 0, 24, 48, and 72 hours post infection (hpi) when incubated at 28 C and compared to the 72 hpi MDCK cell titer when grown at 37 C.*

CHAPTER V

CONCLUSION & FUTURE DIRECTIONS

We conclude that using trypsin does not significantly alter the virus titer in MDCK cell, the original host. We also found that trypsin has a negative effect on zebrafish cell, causing them to express CPE. Therefore, we used media lacking trypsin. The 9°C temperature difference between MDCK cells and zebrafish cell lines, has been found to have a significant impact on the virus titer in MDCK cells. These findings provide a step in the right direction for establishing the adapted virus for this *in vitro* model.

Our PCR samples allowed us to quantify viral RNA in our infection samples. However, when comparing 0hpi and 72hpi samples we concluded that there was not a clear indication that the virus was replicating over the 72-hour time frame. An aspect of the infection method we noticed when analyzing the PCR, was that the control supernatant samples were positive for having amplification. This could have been due to the control and infected samples being cultured on the same plate. This will be noted for future infections.

After altering the methods for infecting the cell lines and getting potential amplification in PCR samples, completing the adaption is the next step for this project.

This will be done by second and third rounds of blind passages with supernatant collected from the first passage of each cell line.

The next project we are working on is a trial having 20 zebrafish/group injected with 10^4 $TCID_{50}$ IAV. In Group 1, 20 wild-type zebrafish will be injected with the cell culture virus, group 2 will have 20 wild-type zebrafish were injected with the fish passaged virus (or zebrafish cell passaged virus). Group 3, - 20 rag-1 mutant zebrafish (this mutant strain has no lymphocyte-based components of the immune system) will be injected with the fish passaged virus. A group of 10 wild-type zebrafish will be injected with HBSS as an injection control. This infection control group will be run during each trial to evaluate any negative effect of handling and injecting the zebrafish. In each of the virus infected groups 5 fish will be sampled at 1 hour, 24 hours, 48 hours, and 96 hours post injection. Kidney, spleen and gill samples will be taken, the IAV RNA will be extracted, and analyzed with qRT-PCR. This study will indicate if host adaptation provides a better infection then the parent virus.

In conclusion, the zebrafish cell lines used were relatively refractive to infection with the APR8 strain of IAV, but qRT-PCR data suggests some infection and replication had occurred. This indicates that repeated passage in these cell lines may resulted in a host adapted strain of this virus. In the process we found the fish cells were adversely affected by trypsin and the use of serum free medium that are commonly used in when infecting mammalian cell lines. These procedural modifications will be used in future studies with IAV in zebrafish cells.

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