Urban Rodents and Influenza A

Matthew Araujo & Joseph Evans

April 22, 2019

Abstract

Influenza A is a virus that can infect a wide range of hosts and thus has the potential to cause widespread infection. Due to urban interactions between avian and rodent species it is possible that the common brown rat, Rattus norvegicus could become a transmission vector, triggering an influenza epidemic or pandemic. To test the potential for viral transmission, samples taken post mortem as swabs and tissue samples from urban rats collected in the city of Boston were tested for the virus using RNA extraction and PCR. Preliminarily positive results were obtained from lung tissue and oronasal swab samples and used to inoculate chicken eggs for viral amplification. Using current methodologies, no virus was detected. Future studies may focus on other model systems for viral amplification.

Introduction

Rattus norvegicus, common names include the brown rat or Norway rat, were believed to have migrated to North America on ships traveling from Europe in 1775. Norway rats, who are known to live in close association with human populations, are often found in present day urban and suburban areas. Their close interaction with humans have made them conduits of viral disease to human populations in the recent past. For example, the Norway rat is believed to have been the source of an outbreak of the Seoul Hantavirus in 2017 [Kerins, 2018]. The outbreak was found to have started in an in-home rattery that bred Norway rats primarily as pets. The virus was confirmed to have spread to 31 facilities, located throughout 11 different states in the United States, as well as 6 ratteries in Canada. Although no deaths occurred and only three persons were hospitalized, this 2017 outbreak is an example of how Norway rats are able to act as a vector spreading viral infection among the human population in which they cohabitate and the real possibility of a more serious viral outbreak occurring.

2009 H1N1 Pandemic

In 2009, a new strain of influenza appeared in the United States and Mexico. It was created from the genetic material from influenza strains of three different species being exchanged to create what became the H1N1 Influenza A virus [Al-Muharrmi, 2010]. This virus was unlike most other influenza viruses that came before it. Older individuals appeared to have a higher immunity to the virus than younger individuals. Deaths and other complications associated with the virus also occurred mostly in younger people. In the span of about 2 months, the newly created virus was announced by the WHO as a pandemic event, the first in 40 years [WHO, 2018].

Influenza Virus

Influenza viruses are a subset of the family Orthomycoviridae, which cause the flu in vertebrates. Of the viruses in this family, the genus Influenza A most commonly infects humans. The genus is further classified by their surface glycoproteins, hemagglutinin [HA] and neuraminidase [NA], into subtypes. There are 18 unique hemagglutinin types, 8 of which are able to infects humans, and 11 unique neuraminidase types, 6 of which can infect humans (CDC, 2017). Hemagluttanin binds to sialic acid receptors on cells to infect them and neuraminidase cleaves the sialic acid receptors off the host cell, preventing agglutination of newly created virions, allowing them to spread [Shtyrya et al., 2009].

Of the known influenza A viruses, all but two [H17N10 & H18N11] have been found to also infect birds. Avian influenza A viruses are designated as highly pathogenic avian influenza (HPAI), which include H5 and H7 subtypes, or low pathogenicity avian influenza (LPAI), which can include H1-H16 subtypes, based on molecular characteristics

of the virus and the ability of the virus to cause disease and mortality in chickens in a laboratory setting [Barnard, 2009]. Influenza binds to the sialic acid [SA] receptors in cells, which differ among species. The difference between binding to an avian host or human host comes from having an alpha 2,3 linked SA glycoprotein versus an alpha 2,6 linked SA glycoprotein [E. Driskell, 2012]. Mutations affecting a virus's ability to bind to these receptors may allow infection of new hosts or prevent infection of traditional hosts.

Reassortment of genes for influenza virus has the possibility to occur when two viruses co-infect a cell. In cases such as these, genomic segments can be exchanged, and novel strains of virus not seen in nature are generated, as was the case in the 2009 H1N1 pandemic [Al-Muharrmi, 2010]. The influenza genome is divided into eight negative RNA segments, which are attached to each other through a polymerase protein complex. Reassortment events can allow hosts previously immune to strains of a virus to be infected. Influenza A viruses can infect a range of different hosts other than humans. Possible hosts include birds, pigs, horses, seals, and whales. Different subtypes of Influenza A infect a different range of hosts, with the exception of birds, who as previously stated are hosts to all known Influenza A subtypes.

Norway Rats and Influenza

It has been shown that Norway rats are able to become infected with a type A Influenza virus (H3N2) in an experimental setting [Daniels, 2003]. While testing the viral influenza infection among three rat strains (Norway, Sprague-Dawley, and Fischer-344), Norway rats were shown to have a unique response to the viral infection. The Sprague-Dawley and Fischer-344 rats showed a similar infection profile and immune response that resulted in an approximate 100-fold increase of virus titer when compared to the Norway Rat. When confronted with this strain of influenza virus the Norway rat was able to inhibit viral replication in the lung at a much more efficient rate than that of the other two rat strains. This difference was attributed to a difference in the utilization of host defenses. Sprague-Dawley and Fischer-344 rats were shown to have an immediate recruitment of neutrophils and greater interleukin response, whereas Norway Rat's immune response tended to heavily favor the utilization of macrophages and natural killer cells. This unique, innate immune response could be the reason to why Norway rats have not experienced widespread infection with the influenza virus. If a crossover event were to occur in the ever-changing influenza viruses that made it to be able to better bypass this host immune strategy, the close proximity of Norway rats to human populations could cause an influenza outbreak similar to that of the 2009 H1N1 pandemic.

Materials and Methods

Rat Necropsy

Rats were received from Boston Department of Public Health, from various locations within Boston (see Figure 1), and brought to the Cummings School of Veterinary Medicine. Necropsies were performed on a total of 55 rats, 32 of which were males and 23 were females. When rats were received, each was assigned a specimen identification number (i.e. R-300) in sequential order. Latitude and longitude of where the rats were trapped was listed. The rats were weighed and species, sex, and external exam notes were recorded. Any ectoparasites were collected and transferred into 2 mL cryovials containing 70% ethanol. Two swab samples were then taken and stored separately in 2 mL cryovials containing ThermoFisher Remel Micro Test M4RT (VTM). The first sample included swabbing each of the rat's paws, a sweep of the hair, and base of tail. The second sample was a swab of the oronasal cavity. Rats were placed dorsally and secured. Scissors were used to open the thoracic cavity. The lungs were removed and two samples were retrieved. A sample of each lobe of lung was taken and stored together in two separate 2 mL cryovials containing VTM. The incision was extended caudally and the abdominal cavity was then opened. All abdominal organ samples were stored in empty 2 mL cryovials, one vial for each different sample taken. The liver was removed and a sample of each liver lobe was taken. The spleen was then removed and cut into sections small enough to insert into the cryovials. Each kidney was located and removed. Kidneys were stored in two individual cryovials. Fecal samples were taken from the descending colon. One sample was placed into a cryovial containing VTM and another placed into an empty cryovial.

In the event that only enough fecal material for a single sample was present, the VTM sample was prioritized. Internal examination notes were recorded as necessary. All samples were stored at -80 degrees Celsius until RNA extraction was to be performed.

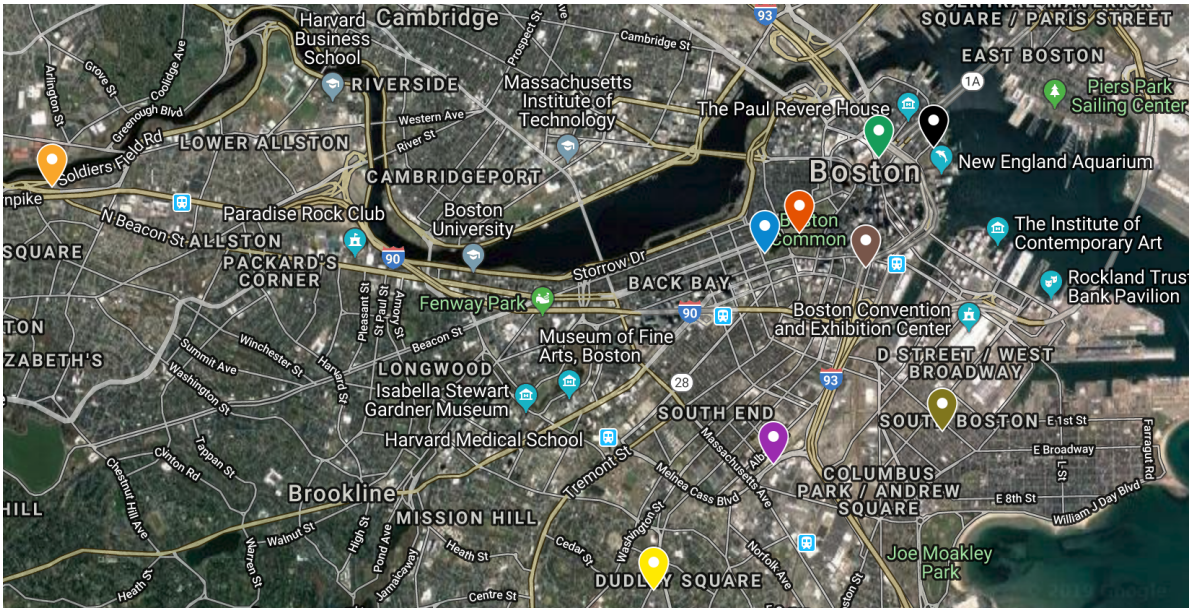

Figure 1: An area map of locations that rat specimens were retrieved from. The color of the marker represents a different location in the Boston area. Red: Boston Commons/Public Garden (BC/PG), Brown: Chinatown, Orange: N. Beacon St., Beige: South Boston, Purple: Suffolk County House of Corrections, Green: Holocaust Museum, Black: Boston Waterfront, Blue: Garden of Remembrance Memorial, Yellow: Roxbury

Swab Sample RNA Extraction

In order to extract viral RNA from the swab samples and fecal samples the following extraction protocol was performed. Samples to be extracted were first identified and removed from the -80 degrees Celsius freezer and transferred to the 4 degrees Celsius refridgerator to thaw. For a full extraction run, 86 samples were processed across 8 ThermoFisher KingFisher extraction plates (labeled A-H). The biosafety cabinet, as well as all material entering the cabinet (pipettes, tip boxes, vortex, etc.), were treated with RNase Zap prior to extraction. This promotes the inactivation of the RNase enzyme that degrades RNA. Lysis mix is prepared using the Omega Bio-tek Mag- Bind Viral DNA/RNA 96 kit (Mag-Bind® Particles CNR, TNA Lysis Buffer, VHB Buffer, Carrier RNA, Proteinase K Solution (40mg/mL), SPR Wash Buffer & Nuclease Free water). In a 15mL conical tube, 6.72 mL TNA lysis buffer, 44.8 μL linear polyacrylamide, and 7.84 mL of 100% isopropanol are mixed. This mix was vortexed briefly and 130 μL were transferred into every well in Row A of each extraction plate. 200 μL of VHB buffer are transferred into Row B of every well of each extraction plate. In every well of both Row C and Row D, 200 μL of SPR wash buffer are added. In row E 50 μL of nuclease free water are added. Each Kingfisher plate is sealed with a foil cover until samples are ready to be added.

For extraction plates A-G 50 μL of sample are micropipetted into an individual well of Row A. In the last column of Row A 50 μL of VTM is added as a negative control. In every well of Row A 10 μL of magnetic bind-bead solution is added (contains 530 μL of Mag-Bind Beads CNR and 530 μL of proteinase K solution). For Plate H, samples and bead solution are added into wells A 1-9. In well 10, 50 μL of the PR8 (Puerto Rico-8 Influenza A virus strain) positive control is added with the magnetic bead solution. Row 11 is left empty as a placeholder for the rtPCR positive control. Row 12 is an identical negative control to all the other plates containing VTM. Plates are then transferred to the Kingfisher machine for the extraction and wash cycles. When finished, 50 μL of the contents of Row E (where the extracted RNA is located) were transferred into the PCR plate. The samples are transferred into rows in the same manner as they were transferred to the extraction plate, wit When all of the samples have been transferred into the PCR plate, the plate is then covered with a plastic plate seal cover.

Tissue Sample RNA Extractions

RNA extractions were performed on rat lung tissue preserved in VTM at -80 degrees Celsius.

Samples to be run were identified and removed from the freezer to thaw. Once thawed, pieces of tissue were added to a bead beater vial (2 mL), with enough tissue to fill the tube approximately 1/3 of the way full. To this vial, 600 μL of VTM was added. The vials were then agitated using a Scientific Industries, Inc Cell disruptor Genie at 3000 rpm for 5 minutes. Samples and tissue were then moved to RNase/DNase-free 2mL microcentrifuge tubes. This tube was centrifuged at a G-force of 250 for 7 minutes and a small amount of supernatant removed. 750 μL of chilled Sigma RNAzol RT was transferred into this tube and mixed via pipetting up and down ten times and then 300 μL of RNase/DNase free water was added. The tube was then repeatedly inverted for 15 seconds. Tubes were then allowed to incubate at room temperature for 15 minutes. Following this incubation period, samples were centrifuged at 12,000g (14,000 rpm) for 15 minutes at 4 degrees Celsius. 750- 1000 μL of the resulting supernatant was then transferred into a separate 2 mL RNase free tube. Isopropanol was added in an equal volume of the extracted supernatant to the RNAse free tube. The tube was then inverted 5-7 times and again allowed to incubate at room temperature. After 10 minutes of incubation the tubes were then centrifuged at 12,000g for 10 minutes at 4 degrees Celsius. If a pellet was visible at this point, all of the supernatant was removed from the tube. In cases where a pellet was not visible, 50 μL of supernatant was left in the tube. The pellets were then washed with 600 μL of 75% ethanol and inverted 5 times. Samples were then centrifuged at 8,000g (11,400 rpm) for 3 minutes at 4 degrees Celsius. The supernatant was removed, making sure not to disturb the pellet. The ethanol wash and centrifugation process was then repeated. The supernatant was again removed. The pellet was resuspended in 30-100 μL of RNase free water and vortexed for 3 minutes. If RT-PCR was not being immediately performed, then the centrifuge tubes were placed in a -80 degrees Celsius freezer until needed.

Real Time PCR

All surfaces were first wiped down with Pharmacal Quatricide (2.25% alkyl (60% C14, 30% C16, 5% C12, 5% C18), Dimethyl benzyl ammonium chlorides, 2.25% dimethyl ethylbenzyl ammonium chlorides, 95.5% inert ingredients). Surfaces, as well as materials (pipettes, tip boxes, vortex, etc.), were wiped with Molecular BioProducts DNA AWAY. The RNA plate, created from RNA extraction samples, was removed from the freezer and allowed to thaw over ice. If samples were in centrifuge tubes from a tissue sample extraction, they were used in the place of an extraction plate. Aliquots of qSCRIPT Quanta Toughmix (rtPCR master mix), forward and reverse primers, and probes were also removed from the freezer at this point. In each well of a 96-well optical rtPCR plate the following reagents are added: 10 μL qSCRIPT Quanta Toughmix, 3.6 μL Nuclease free water, 0.4 μL of 20 μM Avian Influenza Matrix probe forward primer(Avian Influenza 5' ARA TGA GTC TTC TRA CCG AGG TCG 3'), 0.4 μL of 20 μM Avian Influenza Matrix probe reverse primer (Avian Influenza 5' TGA AAA GAC ATC YTC AAG YYT CTG 3'), and 0.6 μL of 5 μM Avian Influenza Matrix probe (Avian Influenza H5 5' FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA 3').

The RNA plate was spun down at 315g for approximately 8 seconds and the bottom was wiped down with Quatricide and DNA away. The cover of the RNA plate was punctured and 5 μL of sample transferred to the corresponding well on the rtPCR plate. In column 12 of the rtPCR plate 5 μL of water was added as a negative control. In well H11 5 μL of the PR8 RNA positive control (A/Puerto Rico/8/1934) was added. The plate was then sealed with a transparent optical film cover. The PCR plate was run for one cycle at 50 degrees Celsius for 10 minutes for cDNA synthesis and then 95 degrees Celsius for 1 minute to initiate denaturation. Following this, 45 cycles of 95 degrees Celsius for three seconds (allowing for denaturation) and 30 seconds at 60 degrees Celsius (allowing for annealing and elongation) occurred. Samples were considered positive when they produced cycle threshold values ¡45 with the primer and probe sets.

Egg Inoculation and Harvest

Chicken eggs were secured through Charles River Laboratories and incubated at 37 degrees Celsius and 45-50% humidity. Inoculation of eggs took place after 10 days of incubation. All samples were removed from the freezer and allowed to thaw. All surfaces of the biosafety cabinet were wiped down with Quatricide, as well as all materials being placed into the hood. 200 μL of each sample was then pipetted into 0.5 ml Eppendorf tubes containing 100 μL of an antibiotic cocktail Tubes were briefly vortexed and allowed to incubate on ice for 1-2 hours. During this time, eggs were removed from the incubator and placed into a separate biosafety cabinet. Lights were turned off and eggs were candled to check survival rates and location of air sac and blood vessels. A small "x" was penciled onto the outer shell of the eggs, slightly above the

intersection of the air sac and embryo, being careful to avoid blood vessels.

Upon completion of candling, eggs were wiped down with 70% ethanol and placed into the biosafety cabinet containing sample. Eggs were paired with each of the two eggs set up to be inoculated with the same sample. A 23G lancet was then used to penetrate the eggs at the center of the "x" created during the candling process. A 25G x 5/8" luer-lock needle, attached to a 1ml syringe, was used to draw up the 300 μL of sample (200μL sample + 100μL antibiotic cocktail). 150 μL was then injected into the allantoic fluid of each of the two eggs that were paired. The holes created by the lancet were then sealed with a drop of Elmer's glue. The glue cap was wiped wiped down with 70% ethanol after each egg pair. After the inoculation of all eggs, again, they were lightly sprayed with 70% ethanol and then transferred from the biosafety cabinet to the incubator under the same temperature and humidity conditions. All materials removed from the hood, as well as all surfaces, were wiped down with Quatricide. Eggs were then monitored for viability via candling until it was time to harvest. Any unviable eggs were removed from the incubator and stored in a secondary containment unit, to be disposed of upon the completion of the harvest of the other eggs.

After approximately 72 hours of incubation following inoculation, eggs were placed into a secondary containment unit at -20 degrees Celsius for 1 hour to euthanize embryo. Eggs were sprayed with 70% ethanol and transferred into the biosafety cabinet. The top of each egg was then gently cracked open with a pair of tweezers and shell pieces removed. A disposable spatula was then utilized to pull back the allantoic membrane from the inside of the shell and an 18G luer-lock needle, attached to a 10ml syringe, was utilized to puncture the membrane. Approximately 5 ml of allantoic fluid from each egg of a pair was then transferred into a pre-labeled 15 ml Falcon conical tube. All tubes were stored on ice until the completion of the harvest. Between eggs, tweezers were wiped down with 70% ethanol and inserted inserted into a heated glass bead sterilizer for 10-15 seconds. All Falcon conical tubes were then centrifuged, utilizing aerosol containment rotors, at 454g and 4 degrees Celsius for 7 minutes. The containment rotors were then sprayed with 70% ethanol and transferred into the biosafety cabinet before being opened. All tubes were placed on ice and then 1.5-2ml of each allantoic fluid sample were aliquoted into five separate 2 ml cryovials. The cryovials were then stored in a -80 degrees Celsius freezer. All materials removed from the hood, as well as all surfaces, were wiped down with Quatricide.

Results and Discussion

Cycle threshold (CT) was used to measure amplification of extracted RNA samples. The value assigned to the cycle threshold was determined if or when measured fluorescence surpasses the threshold by assigning the cycle number to that sample. Fluorescence was measured using ΔRn or the normalized reporter value. ΔRn is measured by the amount of reporter fluorescence divided by a passive reference dye. The limit of cycle threshold values was set to 45, meaning any sample that does not reach the threshold by the 45th cycle will be considered negative or undefined.

The first round of real time PCR was composed of a mix 86 feces and various oronasal, paw, and hair swab samples (Figure 2). No samples within this run reached a CT value below 45.

From the second round of real time PCR, which was performed on ten preliminary lung tissue samples from ten rats (Figure 3.) There were four samples with a CT value lower than 45 which were 17MR00206, 17MR00254, 17MR00266, & 17MR00270 (Table 1). This CT value made sample 17MR00270 an outlier from the other nine samples. The lung sample from the specimen was used up during the extraction process so a second lung sample (17MR00271) from the same rat was used as a proxy for RNA to be inoculated into eggs for viral culture later on.

The third round of real time PCR was performed on a full extraction plate (Figure 4), or 86 samples, from swabs of the oronasal cavity and the paws of Rat #201 through Rat #318. Swab samples took much less time to extract RNA and therefore, more samples could be tested in a shorter period of time. Samples with the top ten lowest ct values, including the two samples above the threshold, whose swab samples produced low ct values. One lung sample from each rat specimens where high swab samples were found was put through the RNA extraction and PCR process in an attempt to determine whether there was a correlation between low ct values in lung tissue and low ct values from swab samples. Results from the PCR of these samples (Figure 5) show sample 18MR00539, coming from Rat # 316 to approach the threshold but not pass it. Two of the ten preliminary swab samples were found in this specimen, meaning only eight lung tissue samples were tested.

The fourth round of real time PCR was performed on the extracted RNA of eight lung tissue samples: 17MR00366, 17MR00371, 17MR00406, 17MR00412,

Table 1: In this table are the vial sample numbers and the corresponding rat the sample came from. A measurement of '-' for the CT value indicates in 45 cycles of real time PCR, no significant replication of Influenza A could be measured. Although not true positives in terms of their ct values, these stood out on amplification plots when compared to other samples during the same round of real time PCR.

ID	Location	Source	Pre-inoculation CT	Post-inoculation CT
R201 - 17MR00206	Roxbury	Lung	42.66	-
R213 - 17MR00254	BC/PG	Lung	42.89	-
R216 - 17MR00266	9/11 Memorial	Lung	40.61	-
R217 - 17MR00270	9/11 Memorial	Lung	37.44	-
R247 - 17MR00364	BC/PG	Oronasal Swab	-	-
R248 - 17MR00368	BC/PG	Paw Swab	-	-
R250 - 17MR00409	9/11 Memorial	Paw Swab	-	-
R255 - 17MR00404	South Boston	Oronasal Swab	-	-
R305 - 18MR00429	Bradston St.	Oronasal Swab	-	-
R307 - 18MR00448	BC/PG	Paw Swab	-	-
R316 - 18MR00537	Bradston St.	Paw Swab	-	-
R316 - 18MR00539	Bradston St.	Lung	-	-
R325 - 18MR00620	Holocaust Museum	Oronasal Swab	42.63	-
R328 - 18MR00645	BC/PG	Oronasal Swab	44.05	-
R333 - 18MR00693	Holocaust Museum	Oronasal Swab	-	-

18MR00430, 18MR00450, 18MR00539 (Figure 5). The extracted lung tissue samples correlated to extracted oronasal and paw swab samples from the third round of real time PCR that had the lowest ct values. Of these eight samples, 18MR00539 stood out from the rest based on its high amplification. From the previous real time PCR, an extracted swab sample from both the oronasal cavity and paws came back with higher amplification, making this the third sample tested from Rat #316 to come back with evidence of influenza A exposures. Again, the sample of lung tissue used in the extraction was used up, so a second sample of lung tissue (18MR00540) from the Rat #316 was used as a proxy.

On the fifth round of real time PCR, the 31 remaining swab samples to that date were tested. (Figure 6) Three samples of the group came back with ct values lower than 45. These samples were: 18MR00620, 18MR00645, and 18MR00693 (Table 1).

Necropsy samples were selected for inoculation into eggs based on their ct values with consideration of the other ct values in their respective rounds of PCR (Table 1). A total of eight samples were selected to be inoculated into chicken eggs: 17MR00206, 17MR00266, 17MR00270, 18MR00134, 18MR00620, 18MR00645, 18MR00693, and 18MR00539. The process of inoculating potential virus into eggs is done to allow virus to proliferate. After a 72 hour period of incubation, the alantoic fluid of the eggs was harvested, spun down using a centrifuge, and RNA extraction and real time PCR were performed again. No samples from the post-inoculation real time PCR had any significant amplification of influenza virus (Figure 7).

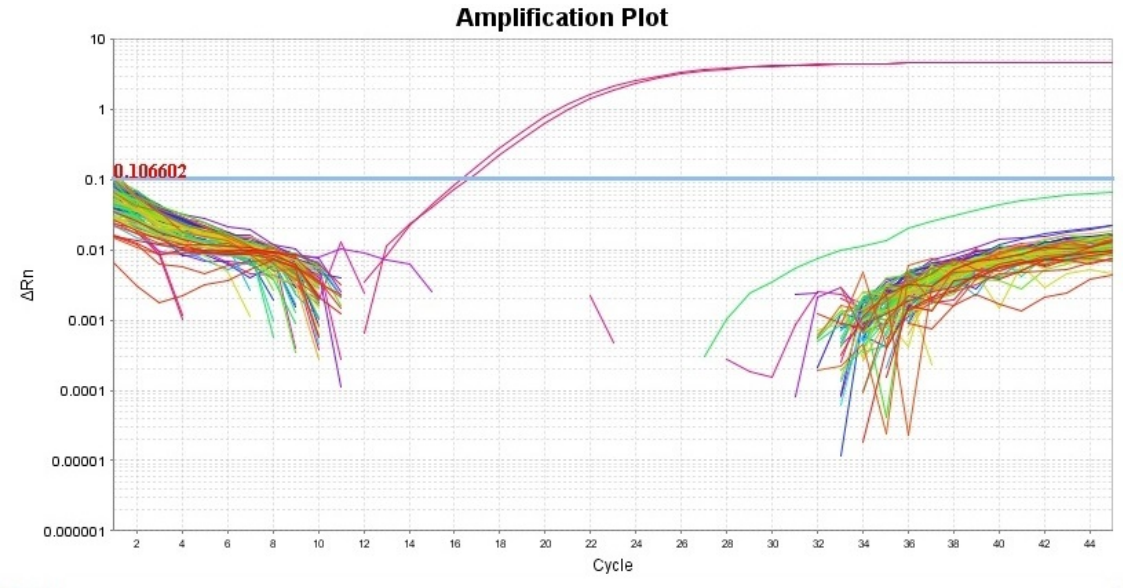

Figure 2: A real time PCR amplification plot from 86 samples of extracted RNA. Oronasal swab samples, swab samples of the paw, and fecal samples were used for extraction. Each line represents one sample. ΔRn values on the y-axis are equivalent to the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Ct values were taken when the ΔRn passed the threshold. Thresholds were placed at the highest point of the sample noise on the left of the graph. From this round of PCR, no samples crossed the threshold and were therefore all negative.

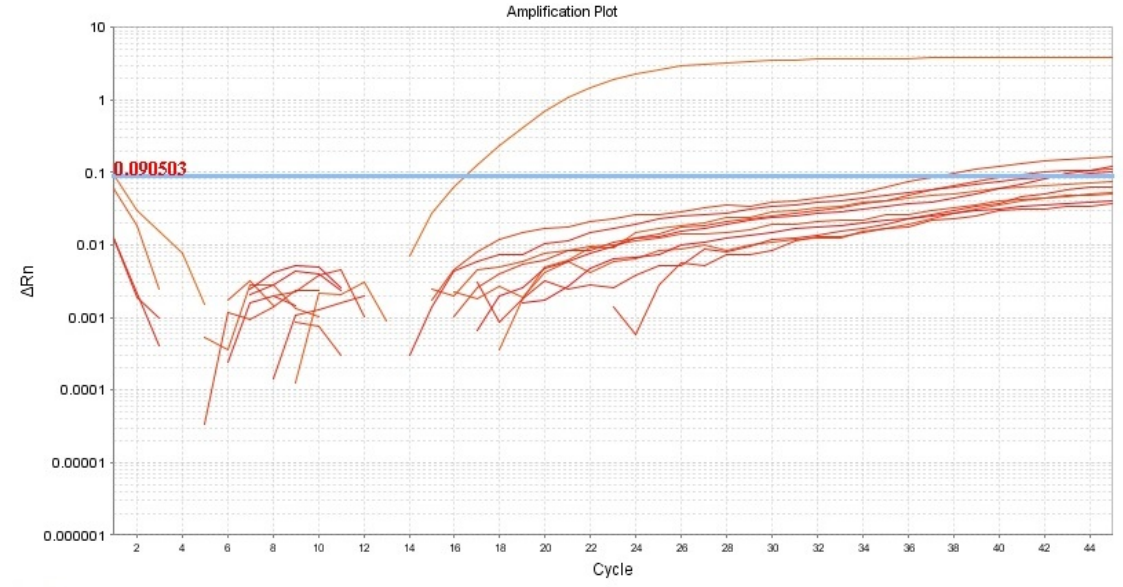

Figure 3: A real time PCR amplification plot from 10 samples of extracted RNA. Lung tissue samples were used for RNA extraction. Each line represents one sample. ΔRn values on the y-axis are equivalent to the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Ct values were taken when the ΔRn passed the threshold. Thresholds were placed at the highest point of the sample noise on the left of the graph. Samples 17MR00206, 17MR00254, 17MR00266, & 17MR00270 passed the threshold below 45 cycles.

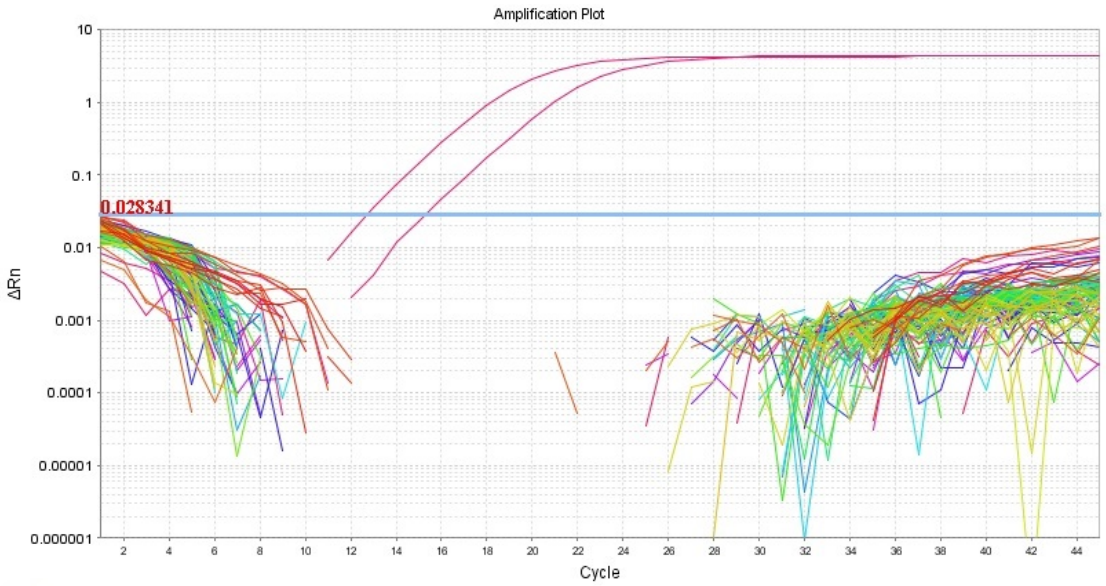

Figure 4: A real time PCR amplification plot from 86 samples of extracted RNA. Oronasal swab samples and swab samples of the paw were used for extraction. Each line represents one sample. ΔRn values on the y-axis are equivalent to the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Ct values were taken when the ΔRn passed the threshold. Thresholds were placed at the highest point of the sample noise on the left of the graph. From this round of PCR, no samples crossed the threshold and were all negative.

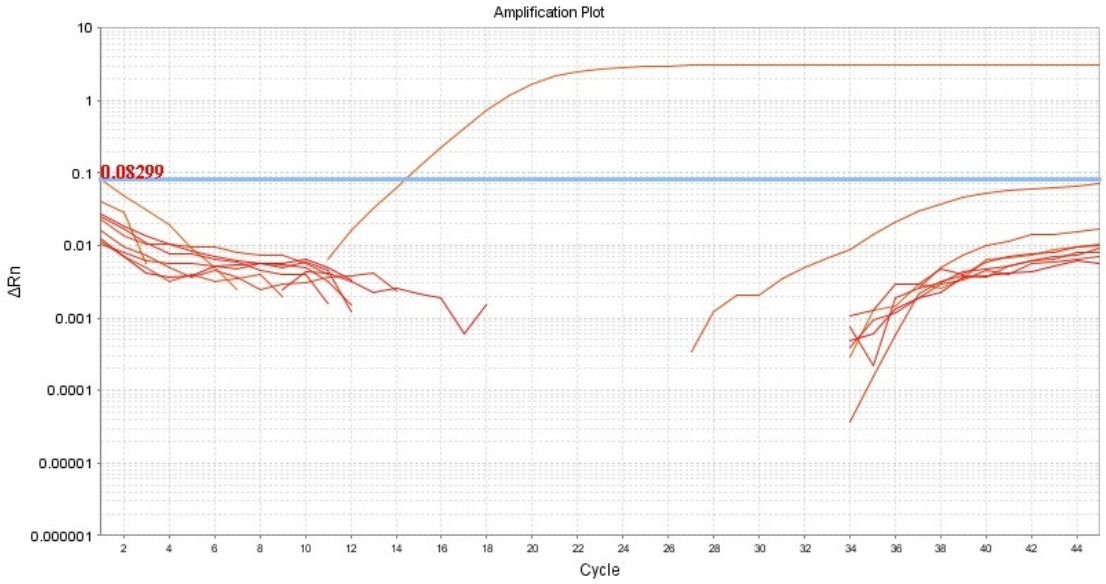

Figure 5: A real time PCR amplification plot from 7 samples of extracted RNA. Lung tissue samples were used for extraction. Each line represents one sample. ΔRn values on the y-axis are equivalent to the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Ct values were taken when the ΔRn passed the threshold. Thresholds were placed at the highest point of the sample noise on the left of the graph. From this round of PCR, no samples crossed the threshold and were all negative.

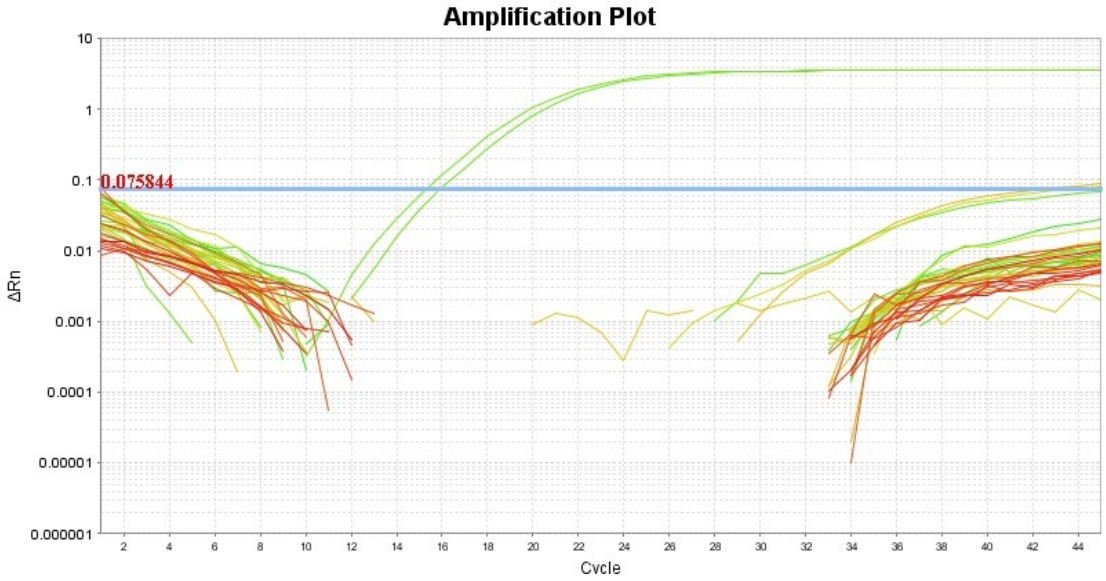

Figure 6: A real time PCR amplification plot from extracted RNA. Oronasal swab samples and swab samples of the paw were used for extraction. Each line represents one sample. ΔRn values on the y-axis are equivalent to the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Ct values were taken when the ΔRn passed the threshold. Thresholds were placed at the highest point of the sample noise on the left of the graph.

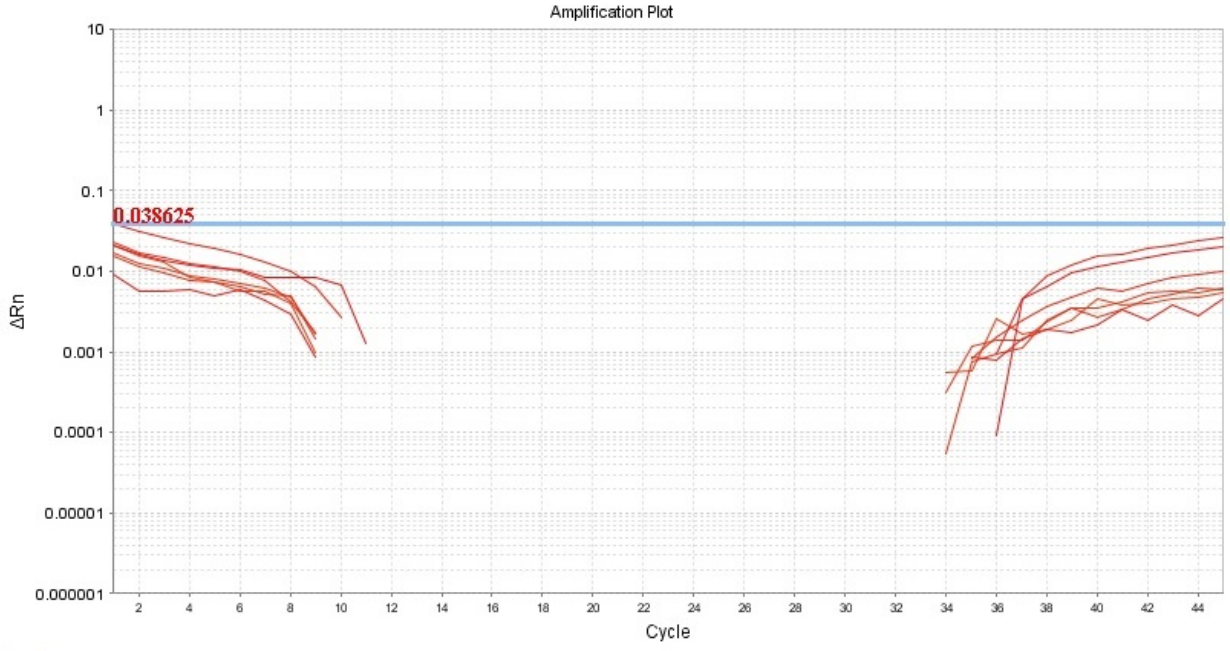

Figure 7: A real time PCR amplification plot of eight viral RNA samples that were inoculated into chicken eggs to amplify virus and then extracted a second time. Of the eight samples, no samples had a ct value lower than 45. Ct values were taken when the ΔRn passed the threshold. Thresholds were placed at the highest point of noise created by the samples to the left. ΔRn values are equivalent to the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. From this round of PCR, no samples crossed the threshold and were all negative.

Though the data collected appears negative, there may be variables causing false negative results. Low ct values from PCR are inversely proportional to the amount of virus found in the sample. Even if a ct value is high, or even undetectable, virus can still be isolated from the samples, creating false negatives [Lindsey et al., 2013]. While this does not account for every sample, which most likely does contain some true negatives, keeping this in mind, it is also possible that rats could be infected with Influenza A virus but not shed virus in their feces or the oronasal cavity. A low viral load to begin with may result in an inability to detect levels of viral RNA that were in the samples.

The RNA extraction process for tissue samples varied from the process of extracting RNA from swab samples of both paws and the oronasal cavity. It was assumed that viral particles would be released from the swab and into the Viral Transport Medium (VTM). Pairing a low viral load with failure of virus to release into VTM could cause PCR results to show a false negative. The RNA extraction process for lung tissue may also decrease the viral load available for PCR. To release the RNA from tissue, samples were agitated using metal beads. Two concerns with tissue agitation arose. Agitating tissue too hard may destroy the viral capsid and RNA, making it unable to be detected with PCR. Too little agitation, and viral particles may not be released into the VTM and therefore undetectable to PCR. The line between being too rough with the sample but not forceful enough may need to be researched more in future experiment.

With low viral loads, extending the number of cycles that PCR runs through may increase ct values for samples that don't have high levels of virus. From choice samples seen in Figure 1 a, d & e, there are outliers that may be able to break the threshold. Given enough time, all samples can theoretically break the threshold but these outlier samples may have levels of virus that are too low to be detected in 45 cycles of PCR but still contain virus. As previously mentioned, Norway rats display a unique immune response when exposed to and infected with influenza. It is a possibility that this alternative immune response plays a part in keeping the viral load at suppressed enough level as to hinder the the amplification process. This may explain why most of the samples displayed "weakly positive" results.

All controls for real time PCR were successful and behaved as expected. It should be noted that these controls were pipetted directly into the wells for PCR and did not undergo the same extraction process as the samples retrieved from the rats. This would account for the strong difference between the resulting PCR values of the positive controls compared to those of the rat samples. If the PR8 positive control had a barrier of extraction for the RNA similar to that of the tissue/swab samples barrier to extraction of RNA, perhaps these values would have been more similar.

Once all the samples that were marked as positive, or having a ct value lower than 45, were inoculated into eggs for virus amplification, no virus was detected. Future experiments may seek to explore different methods of culturing influenza A virus from rats [J. Katz, 1989]. Some research teams in the past have had difficulty amplifying non-avian influenza in chicken eggs [C. McWhite, 2016]. One reason non-avian influenza may not efficiently amplified in the eggs is that non-avian influenza may be more effective against receptors in their hosts compared to birds. This would cause lower rates of viral amplification in non-host species.

Acknowledgements

We would like to thank our advisors, Dr. Jill Rulfs, Dr. Jonathan Runstadler, and Dr. Marieke Rosenbaum for guiding us and helping plan out the project. We would also like to thank Dr. Wendy Puryear, Dr. Nichola Hill, Alexa Foss, Dr. Kate Sawatzki, and Katherine Zhou for all of their guidance and assistance with lab protocols, research, and data interpretation. We would also like to thank the City of Boston's Public Works Department for donating the specimens to the lab.

References

[WHO, 2018] (2018). Influenza (flu).

[Al-Muharrmi, 2010] Al-Muharrmi, Z. (2010). Understanding the influenza a h1n1 2009 pandemic. *Sultan Qaboos University Medical Journal*, (10):187–195.

[Barnard, 2009] Barnard, D. (2009). Animal models for the study of influenza pathogenesis and therapy. (82):A112.

[C. McWhite, 2016] C. McWhite, e. a. (2016). Sequence amplification via cell passaging creates spurious signals of positive adaptation in influenza virus h3n2 hemagglutinin. *Virus evolution*, (2).

[Daniels, 2003] Daniels, M. e. a. (2003). Kinetic profile of influenza virus infection in 3 rat strains. *American Association for Laboratory Animal Science*, (53):293–298.

[E. Driskell, 2012] E. Driskell, e. a. (2012). Low pathogenic avian influenza isolates from wild birds replicate and transmit via contact in ferrets without prior adaptation. *PLOS*, (7).

[J. Katz, 1989] J. Katz, R. W. (1989). Efficacy of inactivated influenza a virus (h3n2) vaccines grown in mammalian cells or embryonated eggs. *Journal of Infectious Disease*, (160):191–198.

[Kerins, 2018] Kerins, J. (2018). Outbreak of seoul virus among rats and rat owners- united states and canada, 2017. *Center for Disease Control*, (67):131–134.

[Lindsey et al., 2013] Lindsey, L., Kelly, T., Plancarte, M., Schobel, S., Lin, X., Dugan, V., Wentworth, D., and Boyce, W. (2013). Avian influenza: Mixed infections and missing viruses. *Virology*, 5(8):1964–1977.

[Shtyrya et al., 2009] Shtyrya, Y., Mochalova, L., and Bovin, N. (2009). Influenza virus neruaminidase: Structure and funtions. *Acta Naturae*, (1):26–32.