Investigating Hemagglutinin in Influenza A using pseudoviruses

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Abstract

The rise of Influenza A virus (IAV) infections continues to fuel a global epidemic due to rapid mutations within the virus and its ability to cross between species. In northeastern seal populations, this antigenic shift is observed in maternal antibodies found in sera samples of seal pups. However, the chances of identifying and propagating live viruses from these samples are extremely low. An alternative approach is to use a pseudovirus (PV), which is a non-infectious viral particle that can be built to express select surface proteins. Our aim is to develop a panel of PVs, each of which carries a single sub-type of hemagglutinin (HA). We hypothesize that a PV will be able to identify the HA-subtype of an IAV from these seal samples and observe emerging IAV trends.

Introduction

Avian Influenza A virus (IAV) is a highly contagious respiratory virus that has caused over 79,000 deaths between 2016-2017 in the United States alone (CDC, 2019). Due to continual antigenic drift, IAV can rapidly mutate and cross between species rather than remain in a species-specific cycle such as Influenza B or C (See Figure 1). What contributes to its deadly nature is that IAV strains have the ability to cross into the human species, causing global epidemics when novel IAV strains arise (e.g. H1N1, the 'swine flu'). While measures have been taken to prevent these strains of IAV through seasonal vaccinations, this high rate of reassortment raises the virion's resistance against antibodies and allows the creation of novel strains (Shao et al., 2017).

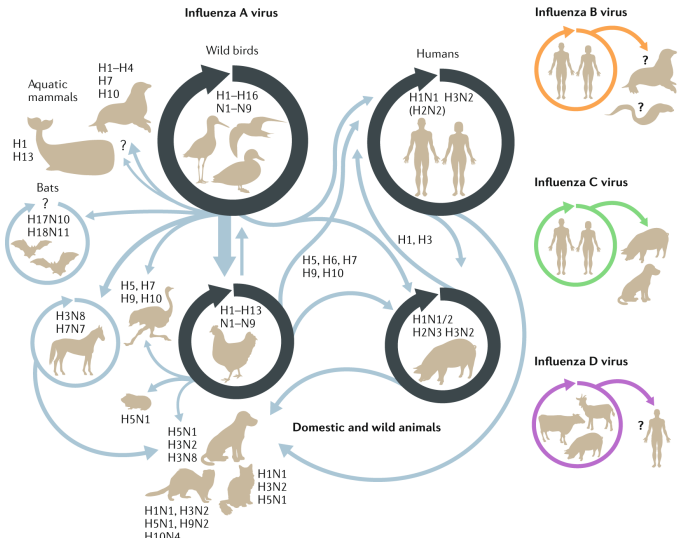

Figure 1: Influenza virus cross-species cycle.

The figure above depicts the host range that has been recorded for different Influenza viruses. Note the high rate of cross-species interactions concerning Influenza A in comparison to a lower rate as in the case of Influenza B. Image retrieved from Long JS. Nat Rev Microbiol 2019.

This antigenic shift is caused by the reassortment of two major glycoproteins that rest on the surface membrane of the IAV virion, hemagglutinin (HA) and neuraminidase (NA). These glycoproteins

work together to permit the infection of a host cell to occur and there are 18 known subtypes of HA and 11 known subtypes of NA (Qiu, C., et al 2013). Once the virion has entered the host body, the HA protein allows the virion to bind to the sialic acid receptor on the host cell membrane (Shao et al., 2017). However, if the host body contains antibodies against the specific HA subtype, then binding cannot occur between the receptor and HA protein as the virus is bound by the antibodies. As the virus can no longer bind to the receptor resulting in infection, this binding specificity is a common antigenic target in seasonal vaccines. Therefore, if a novel strain arises with mutations that alter gene segments of the HA glycoprotein, the efficiency of vaccines decreases (Shao et al., 2017 & Ning et al., 2018).

To test IAV strains within a mammalian serum sample, previous studies used conventional methods such as hemagglutination inhibition (HI) titers, micro-neutralization assays, and hemagglutination assays (HA) to identify which neutralizing antibodies prevent the infection. However, these procedures require the live propagation of viruses and a BSL level 3 laboratory setting (Qiu, C., et al 2013). Even if the risks are disregarded, if the virus is to be propagated from a serum sample, the process of growing viruses is labor-intensive (roughly 2 weeks) and relatively unsuccessful (Evans, J. R., & Araujo, M. J. 2019). Live viral amplification is commonly done through chicken eggs, which are vastly different from the mammalian host serum samples (Qiu, C., et al 2013). Additionally, due to IAV's antigenic drift, it is difficult to discern which glycoprotein is being expressed as the glycoproteins can shift between subtypes as cross-species infection occurs.

Recently, there has been an increase of fatal infections of IAV in *Phoca vitulina* (Harbor seals) located along the northeast region in the United States. While some of these cases have been linked to IAV (H10N7), previous research suggests that this is not the case for all serum samples and it is unknown which glycoprotein is mutated (e.g. HA or NA) (van den Brand, J. M A., et al 2016). Neutralizing antibodies have been found in a majority of serum samples confirmed to be infected with H10N7 strain, but not all samples, suggesting the rise of an antigenic shift. Currently, it is a common practice to collect serum samples from seal pups during "pupping season" rather than mature subjects due to safety concerns. This can be used to observe an antigenic shift within a population, as the maternal antibodies are present in sera samples of seal pups. Due to the BSL 3 laboratory restriction and the inability to successfully propagate live virus from sera collected in these mammalian populations, another way to test these serum samples is by creating a "pseudovirus".

A pseudovirus is a non-infectious recombinant viral particle that can derive its backbone and membrane envelope from a variety of viruses, such as human immunodeficiency virus (HIV) (Qianquian, 2017). A pseudovirus is incapable of expressing its own surface protein, instead, the pseudovirus is assembled from a number of select plasmids transfected into a host cell. After transfection, a pseudovirus will bud off the host cell and be released outside of the cell into the media. These pseudoviruses will then express the desired proteins on its surface (Qianquian, 2017).

Our aim was to develop a panel of pseudoviruses that carried a single sub-type of hemagglutinin (HA) glycoprotein of IAV. While both HA and NA can be expressed on the pseudovirus, HA has shown a stronger relationship to viral infectivity and thus will be used for the project. A panel of H1 through H16 plasmids would individually be expressed on a pseudovirus (using 293T cells as the host cell) along with enhanced green fluorescent protein (GFP). Using sera from infected seal samples, all 16 HA proteins would be individually tested via different neutralizing antibody assays to identify which antibodies are found in the sera. We hypothesize that by using a pseudovirus, we will be able to identify the HA-subtype of a virus from serum samples of seals subjects and potentially derive which IAV strain it may be from. Identification of the HA proteins would confirm if an emergence of a new avian influenza virus is observed in the mammalian northeastern seal population in the United States.

Materials & Methods

Cell Line

HEK 293T/17 cells were obtained from the Rockefeller University via the American Type Culture Collection (ATCC). The 293T/17 cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Pen Strep (5000 Units penicillin, 5mg/ml streptomycin). The cells were then incubated at 37 °C and 5% CO_2, and were split every three days to maintain a 2.6 x 10^6 cells/mL concentration.

Plasmids & Antibodies

All 16 HA plasmids and their related antisera were provided by BEI Resources. A full list of reagents provided by BEI Resources can be found in the Supplementary Information: Appendix A. Each HA plasmid contains an ampicillin-resistance gene and a specific SacI restriction enzyme site. In addition to these, a plasmid containing a GFP reporter gene (pMET7-gag-eGFP, Addgene, 2019) was used for qualitative observations.

Preparation of Plasmids for Transfection:

In order for the plasmids to be expressed in the HEK-293T/17 cells, each plasmid type was initially transformed into a separate culture of NEB® 10-beta Competent E. coli (High Efficiency) (New England BioLabs Inc., 2019). Transformed *E.coli* were then grown on an ampicillin-treated plate for 24-36 hrs and isolated colonies were then grown in 2mL of Lysogeny broth (LB) with 50 mg/mL ampicillin. Plasmid preparations from the remaining bacteria were done using a QIAprep Spin Miniprep Kit (Qiagen, 2019). HA plasmids and the GFP plasmid were then confirmed through a restriction enzyme digest using SacI/EcoRI, respectively, before plasmid transfection into HEK 293T/17 cells.

Hemagglutinin-Plasmid Cocktails

To optimize co-transfection, the HA plasmid to pMET-eGFP-gag (GAG) plasmid ratios were varied. To test the variations of HA to GAG ratios, multiple plasmid cocktails for each of the different ratios

were made. Each ratio had a total of 250 nanograms of DNA. The concentration (nanograms per microliter) varied from plasmid to plasmid. Therefore, the amount (in microliters) of GAG and HA depended on the desired GAG:HA ratio.

Transfection of Hemagglutinins (HA)

To introduce a single type of hemagglutinin into 293T cells, two dilutions were made: lipofectamine was diluted with 250 µl of Opti-MEM (Mix A) and p300reagent was diluted with 250 µl Opti-MEM (Mix B) (ThermoFisher Scientific). Afterwards, a 1:1 µl ratio of Mix A and Mix B was added to each plasmid cocktail. The diluted DNA mixes were then incubated for approximately 10 to 15 minutes at room temperature. 70 µl of each DNA mixture was directly placed into a designated well containing 1 x 10^5 cells and 3 mL culture media. 1µl of enzyme solution (~20U/mg) Neuraminidase (Sialidase/*vibrio cholerae*, SigmaAldrich 2019) was added to each well to aid in the release of pseudoviruses by separating HA protein from the host cell (Su, B. et al, 2009). The 12-well plate was incubated at 37 °C for approximately 24 to 48 hours.

Harvesting the Pseudovirus Supernatant

For the harvest, the supernatant (which contained the pseudoviruses) was pulled off from each treated well after 24-48 hours and frozen at -80 °C for later use in the hemagglutinin assay.

Red Blood Cell Washing

Before beginning the hemagglutinin assay, rooster red blood cells (RBCs) were washed with Dulbecco's Phosphate Buffered Saline (DPBS) at a ratio of 1:9, RBC : DPBS (v/v). From this, a final concentration of 0.5% RBC in DPBS (v/v) was utilized in the hemagglutinin assay.

Hemagglutinin (HA) Assay

To test whether the pseudovirus can adhere to the RBCs (indicating the presence of a virus), a hemagglutinin assay was performed. The HA assay is plated onto a 96 well plate with a V bottom. 1:2 serial dilutions in PBS were made along the row, with the exception of the final row to maintain the control with only PBS and RBCs. Finally, 50 µl of the 1:20 RBC dilution was added to all wells. The 96 well plate was left on the lab bench for approximately 20 minutes to allow RBCs to precipitate. If RBCs show evidence of precipitation, then there is no virus present; whereas if the RBCs in the well have not precipitated, then there is indication of a virus present.

Results

The first trial of the Hemagglutinin (HA) Assay consisted of the pseudovirus with hemagglutinins H1, H2, and H3. After the 96-well plates sat for 20 minutes at room temperature, the results showed there was hemagglutination in all the 1:5 HA plasmid to GAG plasmid ratio wells (Figures 2, 3 & 4). For H1 1:5 and H2 1:5, the pseudovirus agglutinated the red blood cells up until the 1:16 dilution well. There were two exceptions: one H1 column that showed hemagglutination until the 1:8 dilution

well and one H2 column that showed hemagglutination at the 1:32 dilution well. On the other hand, the red blood cells of all the 1:5 H3 wells had agglutinated up until the 1:32 dilution. The 1:10 and 1:20 wells for all hemagglutinins had the red blood cells settled down. No agglutination was evident for any of the HA samples at other HA:GAG ratios.

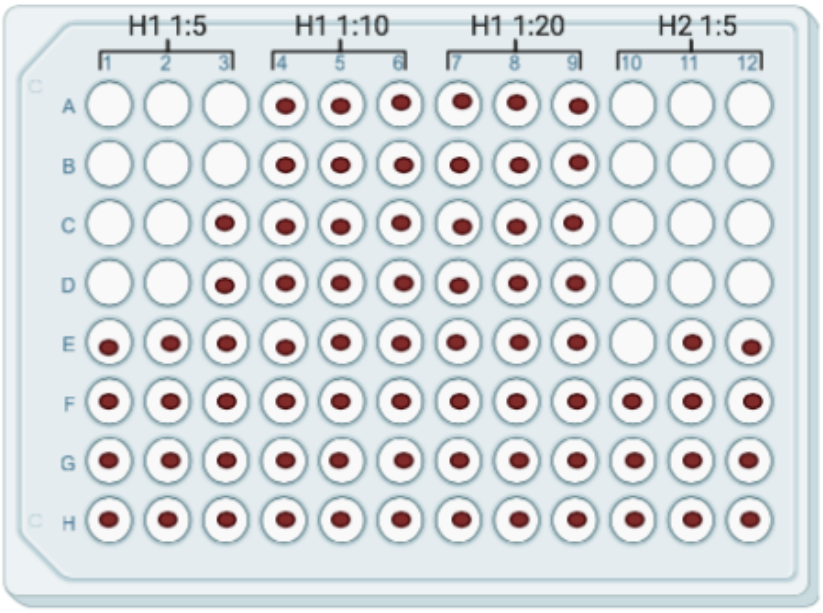

Figure 2. A Hemagglutinin (HA) Assay of Hemagglutinins 1 (H1) & 2 (H2)
The diagram above of a 96-well plate shows the HA Assay results of the HA:GAG ratios: 1:5, 1:10, and 1:20 for Hemagglutinin 1 (H1) and a 1:5 ratio for Hemagglutinin 2 (H2). 1:2 serial dilutions were made in each successive well in a column. Plates were read after a 20 minute incubation at room temperature. The blank wells represent hemagglutination, and the red wells represent no reaction.

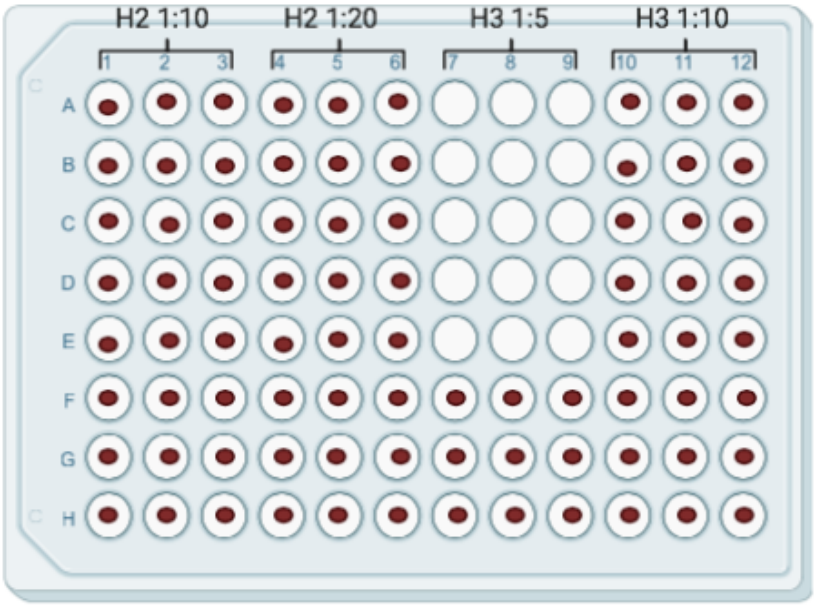

Figure 3. A Hemagglutinin (HA) Assay of Hemagglutinins 2 (H2) & 3 (H3)

The diagram above of a 96-well plate shows the HA Assay results of the remaining ratios: 1:10, and 1:20 for Hemagglutinin 2 (H2) and 1:5 and 1:10 ratios for Hemagglutinin 3 (H3). 1:2 serial dilutions were made in each successive well in a column. Plates were read after a 20 minute incubation at room temperature. The blank wells represent hemagglutination, and the red well represents no reaction.

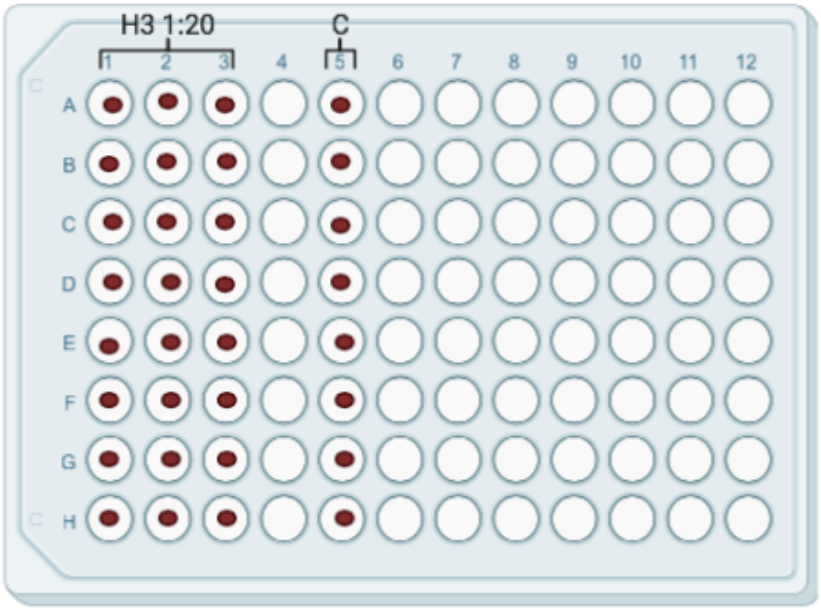

Figure 4. A Hemagglutinin (HA) Assay of Hemagglutinin 3 (H3) & the HA Control

The diagram above of a 96-well plate shows the HA Assay results of the 1:20 ratio for Hemagglutinin 3 (H3) and the control. 1:2 serial dilutions were made in each successive well in for the H3 column. Plates were read after a 20 minute incubation at room temperature. The control column consisted of only PBS & RBCs. The blank wells represent hemagglutination, and the red well represents no reaction.

Discussion

Our results suggest successful construction of three hemagglutinin (H1, H2, and H3) expressing pseudoviruses and demonstrates capability of infection as all three were able to agglutinate the red blood cells for multiple dilutions of the 1:5 ratio. Thereafter, the H1, H2, and H3 pseudoviruses were prepared using the measurements for the 1:5 HA and GAG plasmid ratio. Since the presence of the pseudoviruses was determined, the next step would be to test if the antibodies recognize and bind each pseudovirus through an hemagglutination inhibition assay (HI Assay).

Part II

Methodology

Hemagglutination Inhibition (HI) Assay

The hemagglutination inhibition assay (HI Assay) would be used to observe the antibody response to a specific HA-subtype pseudovirus. If the antibody recognizes the HA pseudovirus, then hemagglutination inhibition will take place. Before starting the HA assay, the antiserum (provided by BEI Resources) was prepared with an addition of 1 mL of distilled water, and was allowed to dissolve at room temperature. Afterwards, the prepared antiserum was stored at 20°C until it was needed. The red blood cells (RBCs) are washed with PBS to make a final 1:20 dilution of 1 mL of washed RBCs with 19mL of PBS. Finally, the HA unit (from a previous HA assay) is calculated to determine the minimum amount of the HA pseudovirus needed to cause agglutination of the RBCs. Typically there are two different HA pseudoviruses tested in a 96-well plate. The plate consists of three columns of controls: one with only RBCs and PBS, and the other two are the HA pseudoviruses, RBC, and PBS. Six columns contain the antibody, HA virus, RBC, and PBS. First the 25 µl of PBS is added to all wells in rows 2-12, followed by 50 µl of antibody into its designated group of wells. Based on the HA unit, 25 µl of the HA pseudovirus is added into their designated wells. Afterwards, a 2-fold dilution is done by transferring 25 µl from the first row to the successive well, the last 25 µl is discarded. The plate is incubated for 5-10 minutes at room temperature before adding 50 µl of RBCs to all wells. After adding 50 µl of RBCs, the plate is covered by a film sealing sheet and incubated at room temperature for an hour before interpreting the results.

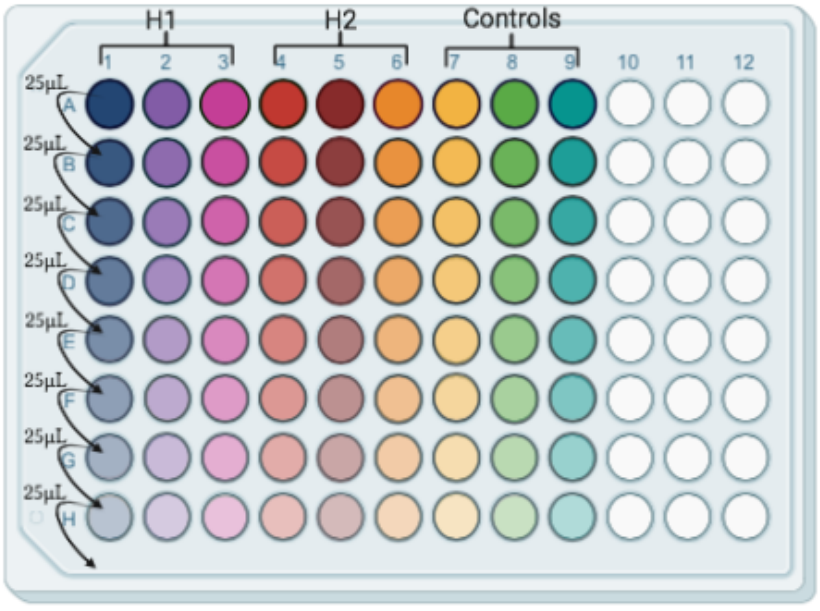

Figure 5. A 96-well plate setup for the Hemagglutination Inhibition Assay.

In the above 96-well plate setup, columns 1-3 contain hemagglutinin 1 (H1), columns 4-6 contain hemagglutinin 2 (H2), and columns 7-9 are the controls. For the H1 columns: 1 has the H1 antiserum, 2 has the H2 antiserum, and 3

has H3 antiserum. Similarly to H1, the H2 columns: 4 has the H1 antiserum, 5 has the H2 antiserum, and 6 has H3 antiserum. It is expected that the antiserum should only recognize and bind to their corresponding hemagglutinin (i.e. anti-H1 should bind H1 and anti-H2 should bind H2). The control columns: 7 contains only PBS and RBCs, 8 contains H1, PBS, and RBCs, and 9 contains H2, PBS, and RBCs. The vertical 2-fold dilution was done by transferring 25 μL from the first row to the successive wells (except column 7) where the last 25 μL are discarded.

Enzyme-linked immunosorbent assay (ELISA)

To measure the presence of the glycoprotein HA, an ELISA would be performed on each pseudovirus sample as adapted from F. Bishai (1978) as follows. An immunoassay 96 well plate would be coated with an HA-specific antibody (provided by BEI Resources), followed by a DPBS wash. Dilutions of the corresponding HA samples are added to triplicate wells with a total volume in each well of 200μL. An additional well would be prepared for a negative control with 1% Bovine Serum Albumin in DPBS (w/v). After 1hr of room temperature incubation, the samples are washed 10 times in DPBS. 200μL of horseradish peroxidase-conjugated anti-human immunoglobulin G (IgG) enzyme is added, followed by 15 washes and incubation for another hour. 2mL of o-phenylenediamine dihydrochloride (OPD) enzyme substrate is then added to these wells and after a 20 minute incubation a yellow color should appear. 100μL of 1N NaOH solution is added to halt the reaction and the results (OD) read at 492 nm using a plate reader.

HI Assay with mammalian seal sera samples

After the pseudovirus has been confirmed to carry the HA-specific glycoprotein, the HI titer assay would be repeated with mammalian seal sera samples instead of the provided antisera panel (BEI Resources, 2019) to determine if the serum samples have antibodies that neutralize a specific HA protein. After returning to room temperature, serum samples would follow a vertical 2-fold dilution as directed in 'Hemagglutination Inhibition Assay' above. After the assay has been performed, if the well has precipitation of RBCs, then it indicates the serum contains antibodies to a specific HA antigen. Therefore, it can be confirmed that the serum sample has been previously infected with a subtype of Influenza A virus.

Results & Discussion

The Hemagglutinin Inhibition (HI) Assay was only performed once using the panel of antisera by BEI resources, thus the assay's results are not statistically significant. Nevertheless, the results of the HI assay did show some further support for the presence of the pseudovirus. The hemagglutinin (HA) unit for H1 and H2 was calculated to be 100μL whereas the H3 HA unit was 50μL. After the 96-well plate sat for approximately 10 minutes, all wells for H1, and H2 showed varying strengths of hemagglutination inhibition (Figure 6). Although most of the H3 wells appeared to have hemagglutination inhibition, the first row did not and instead appeared to have hemagglutination. When there is hemagglutination instead of hemagglutination inhibition, it means that the antibody does not recognize the pseudovirus. This result is anticipated as the HA antiserum was diluted upwards, meaning that there is a weaker concentration of antiserum in these upper rows. Therefore,

the pseudovirus is able to infect the RBCs and retain suspension. This could also explain why there is not complete hemagglutination inhibition in the upper rows of the H1 and H2 samples. The controls also support the validity of this observation, as C1-C3 (HA sample with no antibody) have hemagglutination. A possible explanation as to why complete hemagglutination inhibition is not seen in H1 and H2 could be that there was a miscalculation of the HA units and so not enough pseudoviruses were added to the wells.

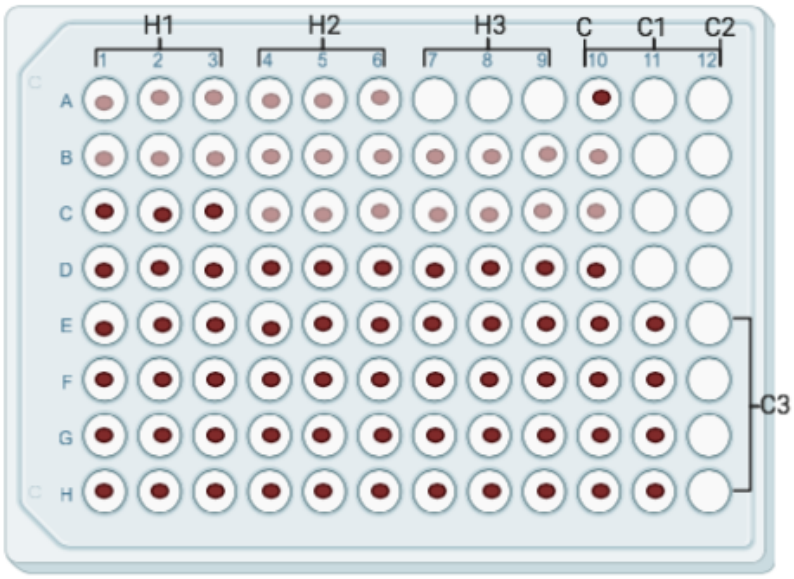

Figure 6. An attempt of Hemagglutination Inhibition (HI) Assay for Hemagglutinin 1 (H1), Hemagglutinin 2 (H2), and Hemagglutinin 3 (H3).

The diagram above of a 96-well plate is the first trial of an HI Assay for H1, H2, and H3. Each hemagglutinin was exposed to the antiserum: anti-H1, anti-H2, and anti-H3 (diluted upwards). There were four controls where C only contains PBS and RBCs, C1 is the H1, PBS, and RBCS, C2 is the H2, PBS, and RBCs, and C3 is the H3, PBS, and RBCs. The blank wells represent hemagglutination, the faded wells represent partial hemagglutination inhibition, and the red well represent complete hemagglutination inhibition.

With strong evidence of the presence of pseudoviruses from both the HA assay and the HI titer assay, our team would move onto ELISA method to measure the presence of a specific HA subtype. Depending on the results of the ELISA assay, we will be able to determine the presence of the anticipated HA protein on the pseudovirus. Then, we would move onto performing the HI titer assay again, but instead of using the provided antisera panel, we will use seal serum samples collected by the Runstadler laboratory at Cummings School of Veterinary Medicine at Tufts University. This will be able to test for the presence of antibodies with these pseudoviruses and permit the identification of a viral HA subtype.

Conclusion & Future Recommendations

Earlier, we hypothesized that by using this pseudo virus system, we will be able to identify the HA-subtype of a virus from serum samples of seals subjects and potentially derive which IAV strain it may be from. However, we only were able to gather enough evidence to suggest the presence of pseudo virus infection. If the methodology mentioned in 'Part 2' were followed, we could anticipate further findings to support this hypothesis. In order to determine any new emergence of avian influenza virus in the mammalian northeastern seal population, we recommend that future work be continued on this project and that the rest of the HA panel be complete. Avian influenza virus infections still remain a global epidemic, and if an antigenic drift can be found in the northeastern seal population, possible human infection could occur. With the pseudovirus system, it would not only ease the laboratory restrictions of testing animal serum samples, but also reduce the risk of cross-species infection.

Acknowledgements

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Supplementary Information:

Appendix A: Hemagglutinin (HA) Plasmid & Antisera Panel Information

The following reagents were obtained through BEI Resources, NIAID, NIH:
Plasmid Containing H1 Hemagglutinin (HA) Gene from Influenza A Virus, A/common teal/Netherlands/10/2000 (H1N1), NR-28993.
Plasmid Containing H2 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Netherlands/5/1999 (H2N9), NR-28994.

Plasmid Containing H3 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Sweden/50/2002 (H3N8), NR-28995.
Plasmid Containing H4 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Netherlands/1/1999 (H4N6), NR-28996.
Plasmid Containing H6 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Sweden/81/2002 (H6N1), NR-28998.
Plasmid Containing H7 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Netherlands/12/2000 (H7N3), NR-28999.
Plasmid Containing H8 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Sweden/24/2002 (H8N4), NR-29000.
Plasmid Containing H9 Hemagglutinin (HA) Gene from Influenza A Virus, A/Eurasian wigeon/Netherlands/4/2005 (H9N2), NR-29001.
Plasmid Containing H10 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Sweden/51/2002 (H10N2), NR-29002.
Plasmid Containing H11 Hemagglutinin (HA) Gene from Influenza A Virus, A/shoveler/Netherlands/18/1999 (H11N9), NR-29003.
Plasmid Containing H12 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Sweden/86/2003 (H12N5), NR-29004.
Plasmid Containing H13 Hemagglutinin (HA) Gene from Influenza A Virus, A/black headed gull/Sweden/1/1999 (H13N6), NR-29005.
Plasmid Containing H14 Hemagglutinin (HA) Gene from Influenza A Virus, A/mallard/Gurjev/263/1982 (H14N5), NR-29006.
Plasmid Containing H15 Hemagglutinin (HA) Gene from Influenza A Virus, A/shearwater/West Australia/2576/1979 (H15N9), NR-29007.
Plasmid Containing H16 Hemagglutinin (HA) Gene from Influenza A Virus, A/black headed gull/Sweden/5/1999 (H16N3), NR-29008.
Antisera Panel to Isolated Antigens of Influenza Virus, NR-10208.

Appendix B: HI Unit Calculations

H1 & H2 Hemagglutinins:
Titer: 1:16 = 1/16
1/16 * 4 = 4/16 = ¼ dilution contains 4 HA units
For 8 wells * 50µl/well = 400 µl total volume
400/4= 100µl virus

H3 Hemagglutinins:
Titer: 1:32 = 1/32
1/32 * 4 = 4/32 = ⅛ dilution contains 4 HA units
For 8 wells * 50µl/well = 400 µl total volume
400/8 = 50µl virus