An Exploration to Determine if Fab Molecules are Efficacious in Neutralizing Influenza H1 and H3 Subtypes

Nicholas Poulton

Briarcliff High School

Abstract

Causing upwards of 500,000 deaths worldwide each year, influenza infection is a major source of global morbidity and mortality that also poses an eminent threat of a pandemic. Thus, there is an urgent need for new antiviral therapies. Monoclonal antibodies (Mabs) present a promising new class of antiviral drugs due to their high specificity to influenza. Phage-display technology is a new method of developing Mabs that utilizes fragment antigen binding (Fab) molecules as an intermediate step in antibody development. This research sought to determine the neutralization activity of Fabs against influenza. Fabs of two published antibodies and several unpublished antibodies were produced in competent TGF- *E. coli*. Neutralization by both Fabs and Mabs was determined by examining protection of MDCK cells from several H1 and H3 viruses. Although the total number of antibodies tested is relatively small, the results suggest that some antibodies may work better in the Fab form and others may work better in the Mab form. Additional work needs to be done to fully determine the differences in neutralization between Fabs in Mabs. However, preliminary results indicate that certain Fabs may be more efficacious in neutralizing particular H1 and H3 viruses.

Introduction

Influenza is a major source of morbidity and mortality worldwide, causing between 200,000 and 500,000 deaths annually [1]. Along with the current damage that can be attributed to seasonal influenza infection, there is also an eminent threat of an influenza pandemic. It has been reported that there are already strains of avian influenza (H5N1) that are resistant to oseltamivir, a neuraminidase inhibitor that is the current standard in treating influenza infection [2]. As a result, there is a significant need for new immunization strategies as well as novel antiviral therapies [3-5]. Current research has been aimed at the development of monoclonal antibodies that neutralize influenza. A main target for these antibodies has been hemagglutinin (HA), the predominant protein protruding from the viral envelope [6,7]. Hemagglutinin is essential for influenza viruses to bind to the α 2,6 sialic acid receptor on human cells, allowing the virus to release its contents into the cell and, consequentially, replicate [8]. Thus, antibodies targeting Hemagglutinin may prevent viral entry into cells. There are several hemagglutinin subtypes, of which H1, H3, and H5 receive much of the research focus. H1 (swine) and H3 (seasonal) influenza subtypes are highly contagious and are important targets at present. H5 influenza subtypes, on the other hand, are highly pathogenic and present a pandemic threat [9,10]. Due to sequence variation between the different hemagglutinin subtypes, it is essential to find conserved regions within the HA molecule in order to prevent antigenic escape of the viruses from antibodies [11-13]. Because certain regions of the HA molecule such as the fusion peptide, the stem, and the head remain relatively constant within each HA subtype, antibodies targeting these regions are of high interest [14-16].

Monoclonal antibodies against influenza are typically developed through hybridoma, a process by which an isolated B cell, producing a desired antibody, is fused with a myeloma cell, allowing large amounts of a monoclonal antibody to be produced [17,18]. However, phage display technology is a new, alternative method of producing monoclonal antibodies that shows great promise. Phage display technology utilizes bacteriophages that project fragments of antibodies called Fab's from their viral surface. A Fab is a fragment of an antibody, containing the full-length light chain as well as the part of the heavy chain that is not in the Fc region. Each antibody contains two potential Fab molecules, one antigen binding site located on each. With phage-display technology, phages are panned against influenza proteins. Phages with influenza-specific Fabs are then selected and their genome is inserted into *E. coli* bacteria for expression of the Fab proteins. The *E. coli* bacteria typically do not secrete the Fab; therefore, it is necessary to lyse the bacteria to obtain the Fab. The ability of Fabs to bind to influenza can be determined from the bacterial lysate; however, the ability of Fabs to neutralize influenza cannot be determined from the bacterial lysate, due to interference from bacterial proteins. Therefore, it is necessary to determine the neutralizing capability of antibodies by purifying Fabs. Because the ultimate goal is to produce full monoclonal antibodies against influenza, purifying Fabs to test their neutralization capability is often overlooked. Alternatively, to assure accurate testing, full monoclonal antibodies are produced, in a long and difficult process using mammalian cells. Testing the neutralization capability of antibodies in the purified Fab form would save vast amounts of time and resources, and it would drastically increase the number of antibodies tested. This, in turn, would increase the likelihood of finding the most effective antibodies. Also, because the neutralization ability of Fabs against influenza has been largely undocumented, Fabs

may have the potential to be used as a new class of prophylactic and therapeutic drugs for treating influenza infection.

Study Aim: The main goal of this study was to assess how Fabs bind to and neutralize influenza in comparison to full antibodies. This was done in order to determine whether purified Fabs may be used as efficient and effective predictors of full monoclonal antibody efficacy. A secondary aim of this study was to determine if there is potential for therapeutic or prophylactic use of Fabs *in vivo* to treat influenza infection.

Materials and Methods

Preparation of Fab-encoding Constructs: Research regarding two influenza-neutralizing monoclonal antibodies has been published in multiple journals, describing their efficacy as both therapeutics and prophylactics. Published Antibody 1 (PA-1) is known to neutralize H1 influenza viruses and Published Antibody 2 (PA-2) is known to neutralize H3 viruses. The sequence for the Fabs of these antibodies is available to the public. For the purpose of this research, these Fabs were used as positive controls. The sequences that code for the various Fabs were obtained via reverse genetics and inserted into a His-tagged expression vector to aid in their purification. The expression of the sequence of interest with the tag was controlled via the *lac* operon. All Fabs described in this work were made with this vector.

The vector and insert were digested separately using the BlpI and XbaI restriction enzymes (New England BioLabs). Then, the vector and insert were resolved on an agarose gel and extracted

using a Qiagen Gel Extraction Kit. The expression vector was dephosphorylated using calf intestinal phosphatase (Roche) in order to prevent recircularization. After a second round of gel extraction, a ligation reaction with the relevant inserts was performed using T4 DNA ligase (Roche). Chemically competent DH5α *E. coli* cells (Invitrogen) were transformed and were plated onto LB plates containing CAM. Three mL starter cultures were produced in 2xYT with CAM and 1% glucose. From the starter cultures, large-scale cultures of the bacteria were produced, amplifying the Fab-coding constructs. The DNA constructs were then extracted and purified using a Qiagen Maxi-Prep and results were sent to GENEWIZ for sequence analysis. Results indicated that the Fab-coding DNAs were successfully inserted into the vector. Along with the Fabs of published antibodies, this study also included Fabs of unpublished Mabs (UFab-1 – UFab-8) produced via phage display. The constructs for these Fabs had previously been created in the His-tagged expression vector and were also transformed into competent DH5α *E. coil*. Bacteria containing each of the Fab constructs were cultured and the constructs were purified via Maxi-Prep. Using the purified Fab-encoding segment, TG1F$^-$ *E. coli* were then transformed via electroporation for optimal production of Fab.

Expression of Fab: After transforming TG1F$^-$ bacteria with the relevant Fab construct, 3 mL cultures containing 2xYT, CAM and 0.1% glucose were produced in order to express the Fabs. Once the cultures reached an optical density at 600nm (OD_{600}) of 0.6 they were induced overnight at 22°C with 4mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma). The next day, the bacteria were centrifuged (Avanti J26S XP, Beckman Coulter) at 3,900×g for 25 minutes. Bacterial pellets were resuspended into 25mL Lysis Buffer [1 mg/mL lysozyme, 0.2M sodium phosphate pH 7.4, 0.5M NaCl (all from Sigma), and protease inhibitor without EDTA (EMD Millipore)] releasing the Fab into the lysate.

ELISA: The binding of Fab was then tested for using an enzyme-linked immunosorbent assay (ELISA). Solid black 384-well ELISA plates (Nunc) were coated overnight at 4°C using 20μL of either purified influenza hemagglutinin protein (at 1μg/mL; Immune Technology Corp.) or sucrose purified influenza virus (used at 10μg/mL) provided by the lab at which this work was performed. The next day, plates were washed twice in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST). Twenty μL of the lysates were added to the wells to test their binding to both purified virus and purified HA after blocking plates for 1 hour with 5% nonfat dry milk in PBST (M-PBST). After 1 hour at ambient temperature, plates were washed 5 times in PBST. Bound Fab was then detected using 1/2000 dilution of goat Anti-Human IgG (Fab specific)-Alkaline Phosphatase (Sigma) in 0.5% milk in PBST. After 1 hour, plates were washed 5 times in 0.05% Tween 20 in Tris buffered saline (TBST), then once in TBS. 20 lambda of Atto-Phos substrate (Promega) was added and plates were read using an Infinite F200 Reader (TECAN) with PrimeScreen software.

Immunofluorescence Assay: Madin-Darby Canine Kidney (MDCK) cells (ATCC) were plated in 96-well flat-bottom plates (BD Falcon) at 2×10^4 cells/well and were incubated overnight in supplemented Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum. The following day the cells were infected with virus at 3×10^3 pfu (plaque forming units)/mL diluted in Opti-MEM for 1 hour at 37°C. The following stock viruses were used: A/Wisconsin/67/2005 (H3N2), A/Wyoming/03/2003 (H3N2), A/USSR/90/1977 (H1N1), A/Fort Monmouth/1/1947 (H1N1). After the incubation, the virus was decanted and cells were washed with PBS. Cells were then fixed with ice-cold methanol for 30min followed by several PBS washes. Plates were then incubated for 20 minutes on ice with blocking buffer [10% goat serum in 0.25% BSA, 0.1% Triton X-100 in PBS (SB+Tx)]. After blocking, the buffer was decanted

and bacterial lysates containing the Fabs were added neat at 50μL/well. Alternatively, some wells received 50μL of full Mab at 50μg/mL. After incubating on ice for 45 minutes, the plates were decanted and the wells were washed with SB+Tx. An Alexa Flour 488 goat anti-human IgG (H+L; Invitrogen) secondary antibody was diluted in SB+Tx at 1/500 and was incubated on ice protected from light for 20 minutes. Each well was then washed again with SB+Tx. Immediately following the washes, 50μL of a 5mg/mL DAPI stock was diluted 1/3000 in SB+Tx and added to each well. Plates were incubated for 15 minutes in dark conditions on ice. Plates were then washed with SB+Tx and finally with PBS. Images were then captured on an Eclipse T*i* with NIS-Elements software (Nikon).

Large-scale Production and Purification of Fab: Large-scale cultures (500mL 2xYT, CAM, 0.1% glucose) of Fabs were produced from 2mL starter cultures. Cultures were induced when an Optical Density $_{600\ nm}$ of 0.6 was reached. The cultures were grown overnight and were harvested the next day when an OD_{600} was approximately 6. Cultures were then centrifuged at 3,900×g for 30min and the supernatant was removed. The bacterial pellet was frozen on dry ice and then thawed. Lysis was then performed using Lysis Buffer (described above). Lysate was then centrifuged at 16,000×g (Optima XE, Beckman Coulter) for 30 minutes and supernatant was passed through 0.45 μm PES syringe filter (Millipore). 2mL of a 50% cobalt slurry was pre-equilibrated in buffer, then added to each supernatant and mixed at 4°C for 1 hour. Cobalt-bound Fab was then centrifuged at approximately 200×g for 5 minutes in a benchtop centrifuge (Eppendorf) at 4°C. The supernatant was removed and the cobalt-Fab complex was resuspended in 4mL cold PBS. The cobalt and Fab were then added to spin columns (Pierce) and mixed end-over-end before being eluted into 2mL elution buffer (0.3M imidazole, 0.15M NaCl in sodium phosphate pH 7.2). Fab eluates were then run on a 4-12% Bis-Tris NuPAGE gel (Invitrogen) in

1× MES and transferred to nitrocellulose (iBlot, Invitrogen) or coomassie stained (SimplyBlue™ SafeStain, Invitrogen) to assess the presence and purity of the Fab molecules.

Western Blot of Purified Fab: Fab samples on nitrocellulose were blocked for 1 hour in 5% M-TBST. Fabs were detected using an alkaline phosphatase-conjugated goat anti-human IgG secondary antibody (Sigma) at 1/2000 for 1hr in 5% M-PBST. Blots were washed several times in TBST, and once in TBS before development using 1 Step NBT/BCIP substrate (Thermo Scientific).

Coomassie Stain of Purified Fab: Fab samples on nitrocellulose gel were washed 3 times in distilled water to remove buffers and salts. The gel was then stained for 1 hour at room temperature in SimplyBlue™ SafeStain while gently shaking. The gel was then rinsed for three hours with distilled water before images were captured.

Microneutralization Assay: After determining that purified Fab was present, the neutralization efficacy of Fabs was then determined through a microneutralization assay. In a 96-well, V-bottom, polypropylene plate (Greiner Bio-One), Fabs or Mabs underwent two-fold serial dilutions in Opti-MEM resulting in concentrations ranging from 50 μg/mL to 0.78 μg/mL. Virus was then added at 3×10^3 pfu/mL in a volume equal to the antibody. The Fab/virus or Mab/virus mixtures were then incubated for 1 hour at 37°C to allow for neutralization. The mixture was then transferred onto wells of a 96-well plate containing confluent MDCK monolayers. Cells were incubated with antibody virus mixtures for 1 hour at 37°C before being rinsed with PBS. Cells were then incubated for 48 hours in Opti-MEM. After 24 or 48 hours, washes with PBS were performed to remove dead cells and debris from the wells. The remaining viable cells were

fixed to the plates using 3.6% formaldehyde (Sigma) before the plates were stained using Crystal Violet (HARLECO) to determine the neutralization activity.

Viruses Provided for These Studies: A number of influenza viruses were provided by the scientists at the institution where this work was performed. These were A/Hong Kong/1/1968 (2:6) (H3N2), A/Wisconsin/67/2005 (H3N2), A/Wyoming/03/2003 (H3N2), A/USSR/90/1977 (H1N1), A/Fort Monmouth/1/1947 (H1N1).

Results

Fabs were successfully produced and purified using a cobalt resin

Results indicate that the purification of each Fab using cobalt resin was successful. As shown the Fab molecules are present, but at a lower molecular weight (approximately 25 kD) than full Fabs (approximately 50 kD). Fabs produced via this method are held together by hydrophopic interactions instead of disulfide bonds. Therefore, it is typical for these Fabs to dissociate on western blot and coomassie stain. Coomassie stain, which shows the presence of all protein, indicates that the Fab eluates are very pure, with very little non-Fab protein. Purified Fab samples are present at about 50μg/mL.

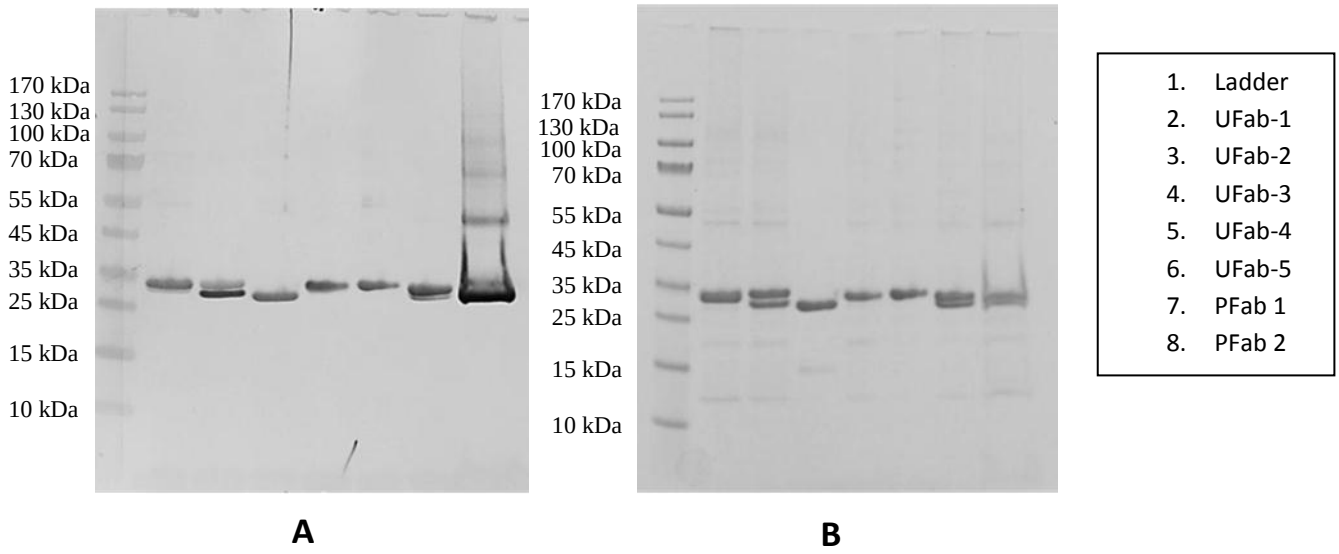
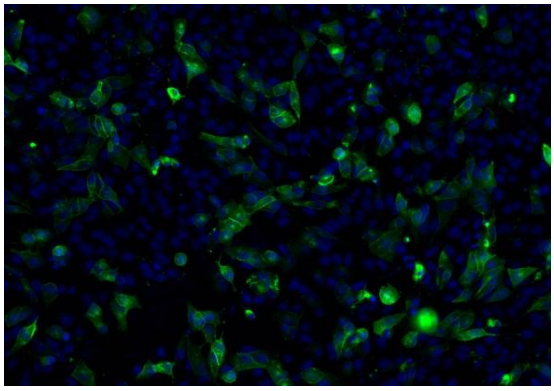


Fig. 1. Western blot (A) and Coomassie Stain (B) of Purified Fab eluates. Fabs purified with cobalt resin and buffered imidazole. Samples were run on 4-12% NuPAGE Bis-Tris gel with MES buffer. Western blot was probed with Anti-human IgG-Alk. Phos, 1/2000. Coomassie stain was developed using SimplyBlue™ SafeStain.

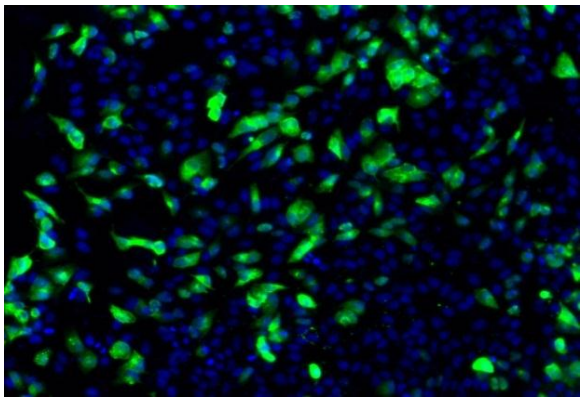
Fabs bind intended subtypes with high affinity

Published Antibody 2 has been well characterized to bind and neutralize H3 viruses. As shown, PA-2 Fab binds A/Wisconsin/67/05 (Figure 2A) and A/Wyoming/03/2003 (Figure 2C) very effectively in the Fab form. UMab-6 is an unpublished antibody also known to bind and neutralize H3 viruses. UFab-6 (Figure 2B and D) also binds A/Wisconsin/67/05 (Figure 2B) and A/Wyoming/03/2003 (Figure 2D) the same viruses with very high affinity in the Fab form.

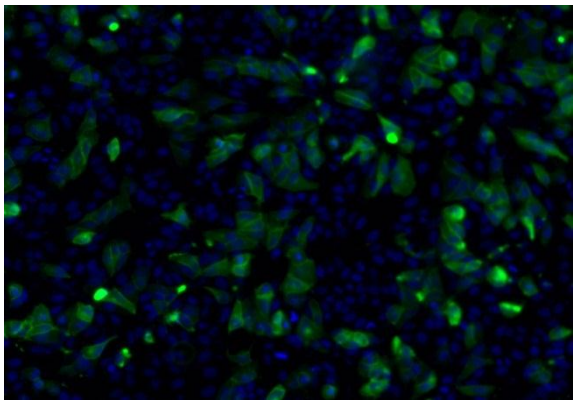
A.



B.



C.



D.

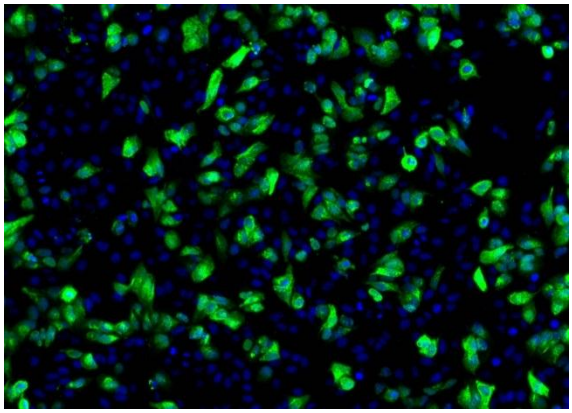


Fig. 2. Immunofluorescence staining of infected MDCK cells with Fabs. PA-2 Fab binding A/Wisconsin/67/05 (Image A) and A/Wyoming/03/2003 (Image C) and UFab-6 binding A/Wisconsin/67/05 (Image B) and A/Wyoming/03/2003 (Image D).

Fabs neutralize H1 and H3 viruses similar to Mabs

A 2-fold dose titration of relevant Fab or Mab from 50μg/mL was performed in the presence of A/Puerto Rico 8/1934 (H1) virus for 1 hour prior to addition of this mixture to an MDCK monolayer. After 1hr, cells were washed of free virus and allowed to incubate for 48 hr. Cells were then washed, fixed and stained with crystal violet. As shown in Figure 3, with the exception of a couple wells, PA-2 Mab fully neutralized A/Hong Kong/1/1968 (2:6) (H3) at

every concentration from 50 μg/mL to 0.78 μg/mL. PA-2 Fab, on the other hand, fully neutralized from 50 μg/mL to 0.78 μg/mL in every well. Both the Mab and the Fab appear to be highly efficacious due to their similarity with wells that did not receive virus (bottom row). UFab-2 and UFab-3 (Figure 4) are unpublished antibodies known to neutralize H3 viruses. Published antibody 3 (PA-3) is an antibody that has also been characterized as an H3 subtype neutralizer. When cells were challenged with A/Hong Kong/1/1968 (2:6) (H3) UFab-2 Fab appears to neutralize with full efficacy from 50 μg/mL to 0.78 μg/mL while UMab-2 weakly neutralized (Figure 5). UMab-1 was better at neutralization than UFab-1.mL. UFab-3 and PA-3 Mab, on the other hand, neutralize only at certain concentrations with no apparent dose-dependency. Unlike UFab-2, which neutralized fully at all concentrations, UMab-2 (figure 6) neutralized only at certain concentrations.

In Figure 6 cells were challenged with A/Fort Monmouth/1/1947 (H1N1) virus. UMab-2 is a known H3 neutralizer. However, western blot showed that UFab-2 Fab may cross react with H1 viruses (data not shown). Therefore, cross-neutralization was tested for. Due to time constraints, cells on this plate were incubated with Opti-MEM for 24 hours following infection, as opposed to 48 hours. It is more difficult to observe distinct differences between wells because of the shorter incubation period with the virus. However, it is evident that UFab-2 neutralizes this H1 virus effectively at several concentrations, although there is no apparent dose-dependency. Certain wells that received UFab-2 appeared to be similar to cells treated with no virus (bottom row). Cells in Figure 7 were challenged with A/Hong Kong/1/1968 (2:6) (H3). UFab-6 is appears to completely neutralize at all tested concentrations. UFab-7 also neutralizes, but only at certain concentrations and with no dose-dependency.

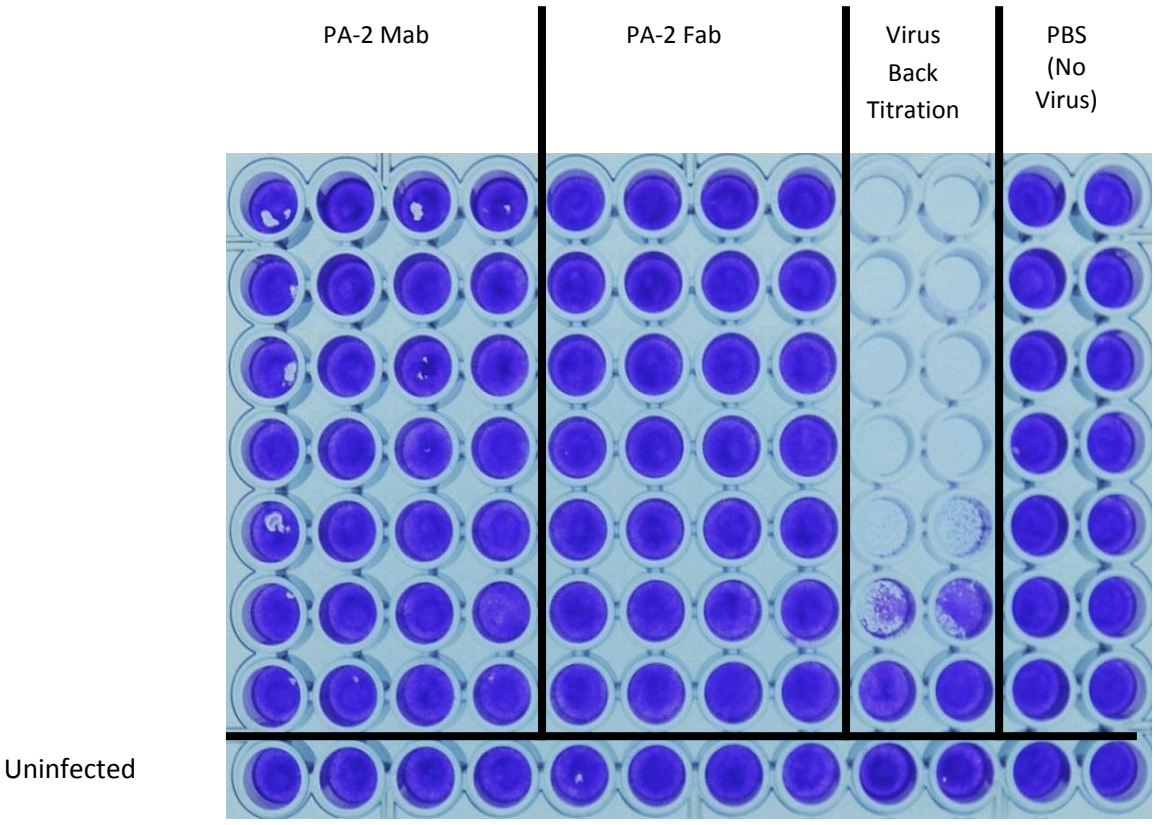


Fig. 3. H3 Virus Microneutralization Assay (1). A 2-fold dose titration of relevant Fab or Mab from 50μg/mL was performed in the presence of A/Hong Kong/1/1968 (2:6) virus for 1 hour prior to addition of this mixture to an MDCK monolayer. After 1hr, cells were washed of free virus and allowed to incubate for 48 hr. Cells were then washed, fixed and stained with crystal violet.

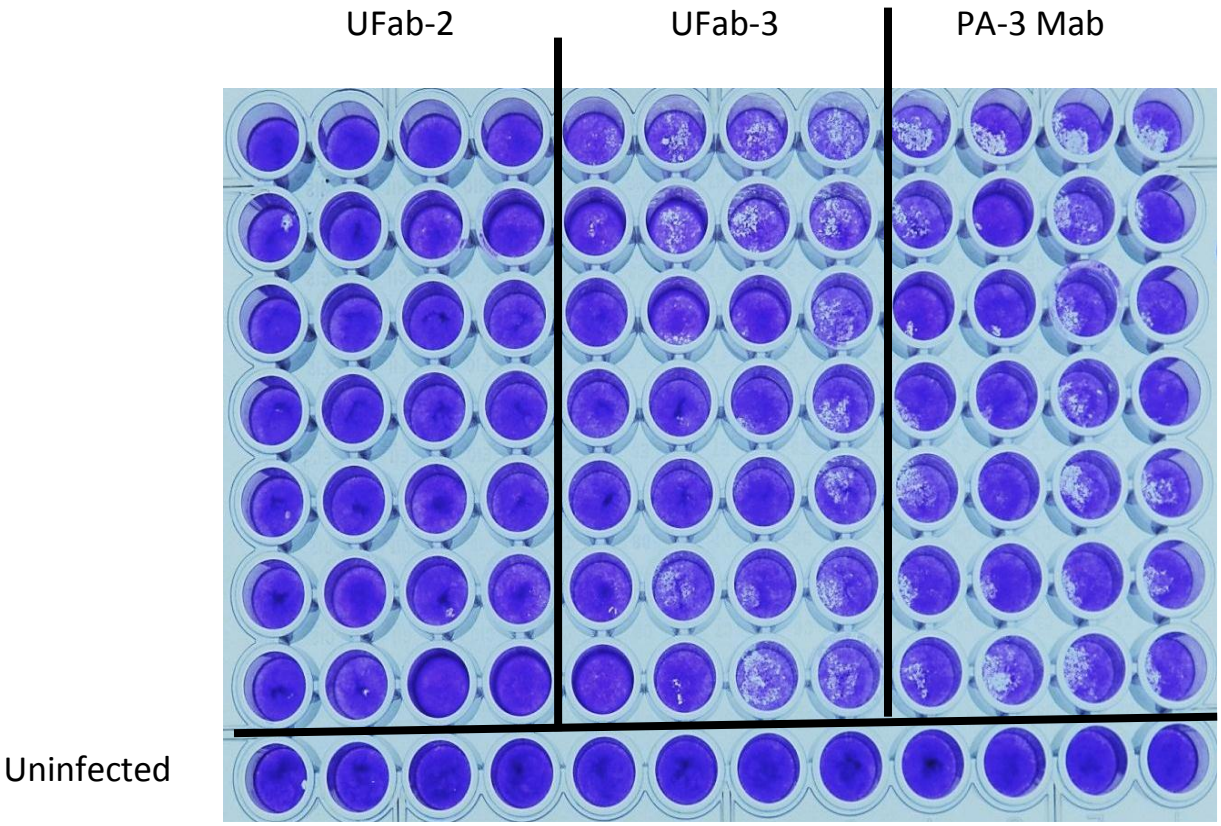


Fig. 4. H3 Virus Microneutralization Assay (2). A 2-fold dose titration of relevant Fab or Mab from 50μg/mL was performed in the presence of A/Hong Kong/1/1968 (2:6) (H3) virus for 1 hour prior to addition of this mixture to an MDCK monolayer. After 1hr, cells were washed of free virus and allowed to incubate for 48 hr. Cells were then washed, fixed and stained with crystal violet.

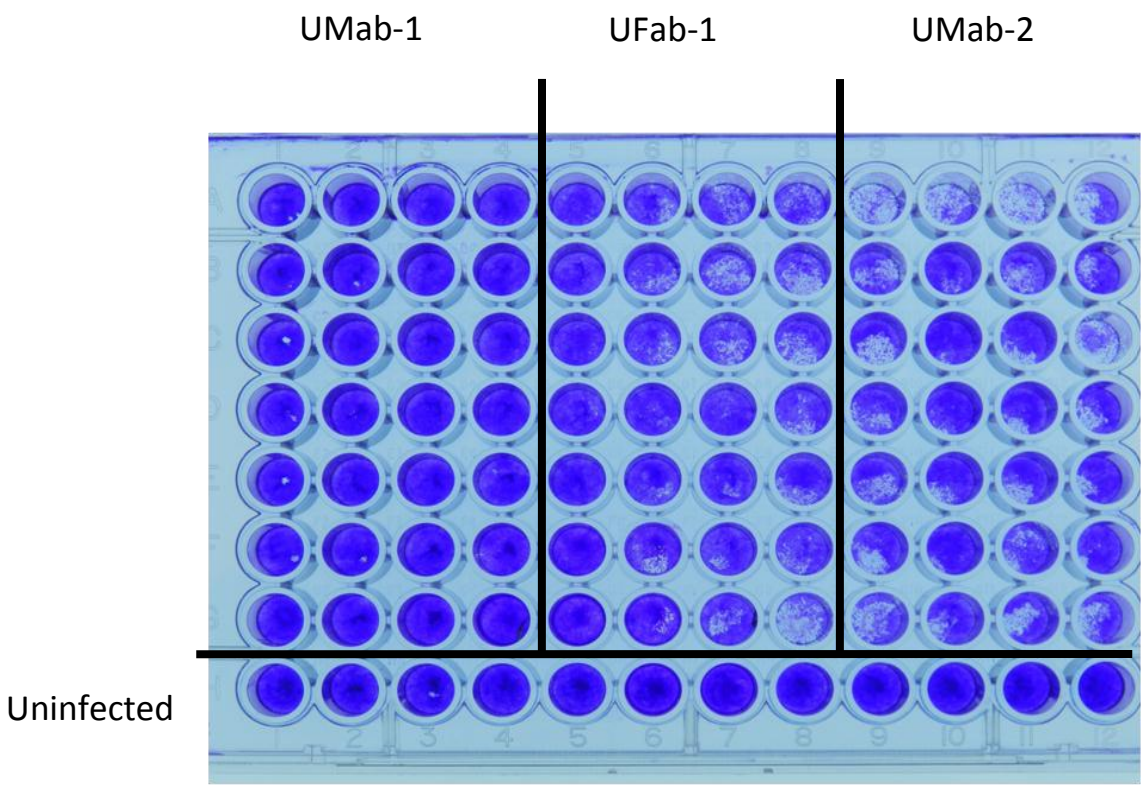


Fig. 5. H3 Virus Microneutralization Assay (3). A 2-fold dose titration of relevant Fab or Mab from 50μg/mL was performed in the presence of A/Hong Kong/1/1968 (2:6) (H3) virus for 1 hour prior to addition of this mixture to an MDCK monolayer. After 1hr, cells were washed of free virus and allowed to incubate for 48 hr. Cells were then washed, fixed and stained with crystal violet.

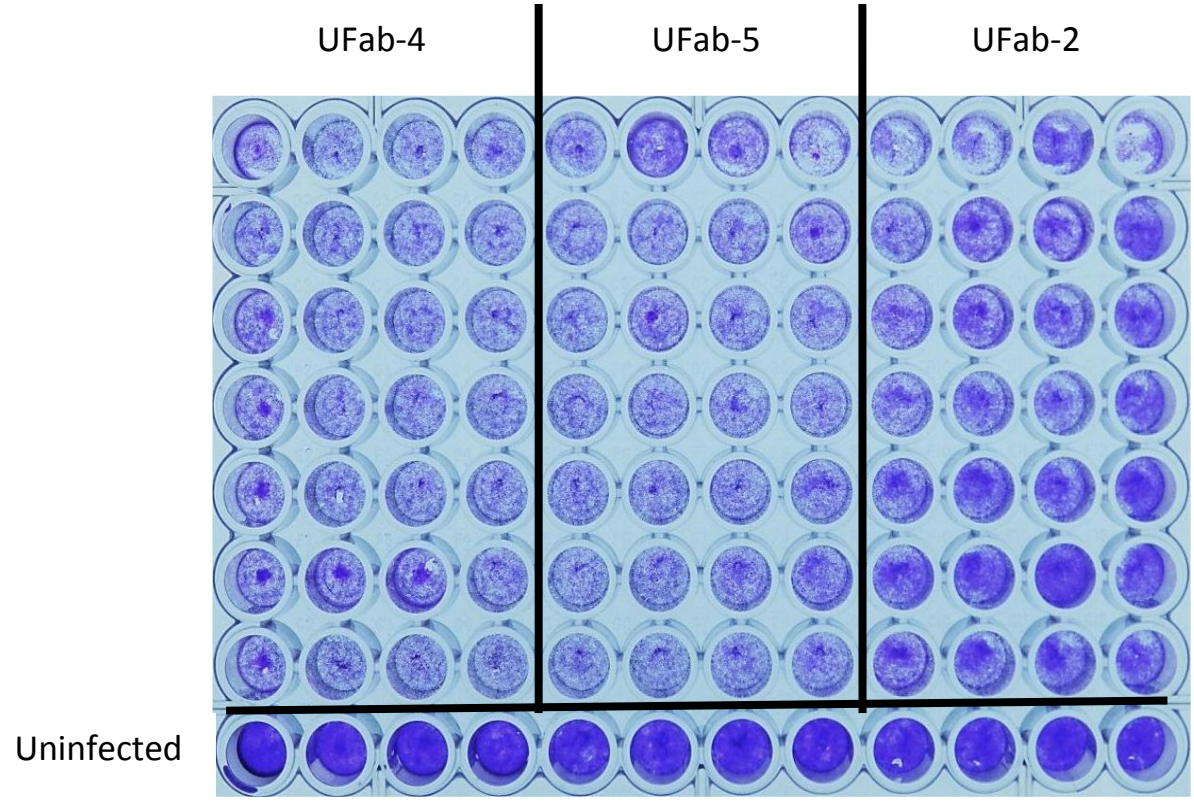


Fig. 6. H1 Virus Neutralization Assay (1). A 2-fold dose titration of relevant Fab or Mab from 50μg/mL was performed in the presence of A/Fort Monmouth/1/1947 (H1) virus for 1 hour prior to addition of this mixture to an MDCK monolayer. After 1hr, cells were washed of free virus and allowed to incubate for 24 hours, as opposed to 48 hours. Cells were then washed, fixed and stained with crystal violet.

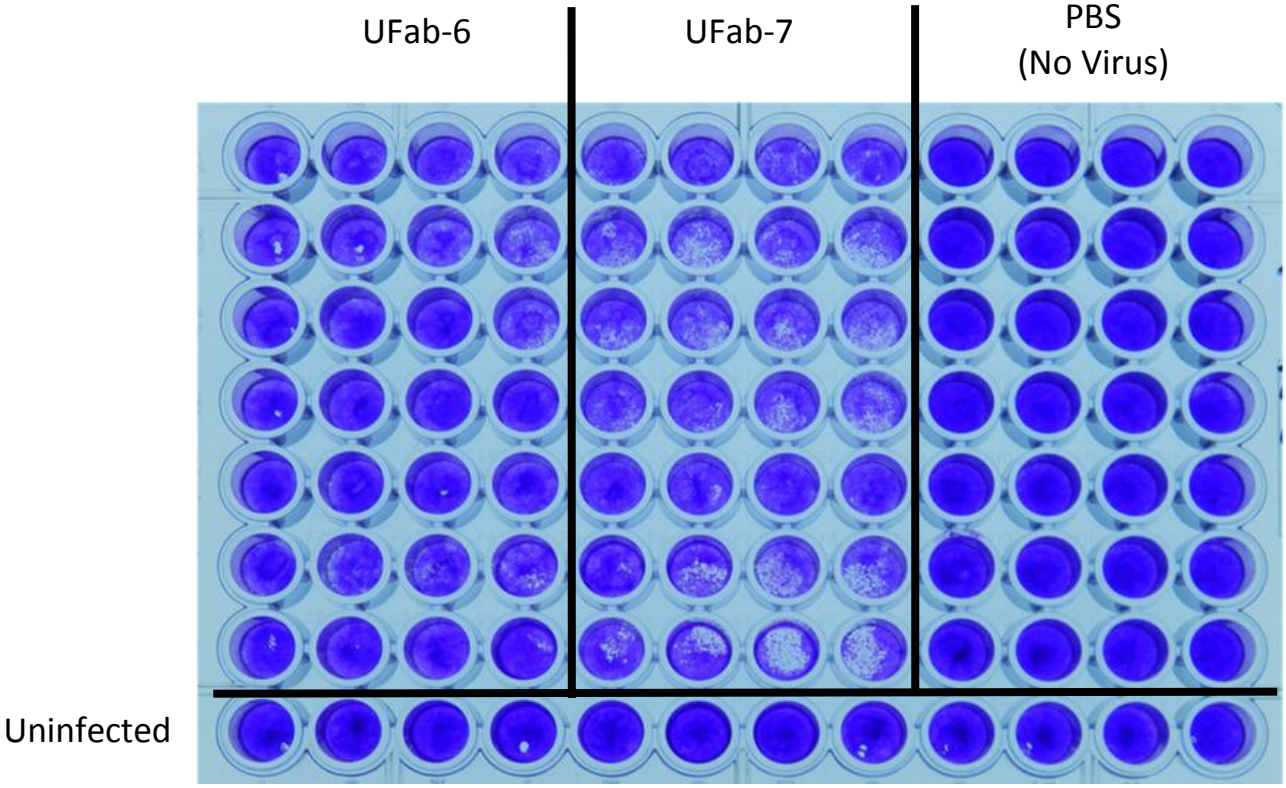


Fig. 7. H3 Virus Microneutralization Assay (4). Cells were challenged with A/Hong Kong/1/1968 (2:6) (H3) and were incubated with Opti-MEM for 48 hours following infection. Cells were stained with crystal violet, indicating viability.

Discussion

The results of this study show that certain Fab molecules are highly efficacious in neutralizing influenza viruses of the H1 and H3 subtypes. Initially, this study was mainly aimed at determining whether or not purified Fabs can be tested in neutralization assays to predict the efficacy of full monoclonal antibodies. From the results, it is evident that in some cases the Mabs and Fabs showed similar neutralization. Published Antibody 2 appears to neutralize A/Hong Kong/1/1968 (2:6) at all tested concentrations in both the Fab and Mab form. In instances like this, a Fab would appear to be an effective predictor of monoclonal antibody efficacy. However, in the case of Unpublished Antibody 2 (UMab-2), the opposite trend was observed. UFab-2 appeared to neutralize with increased potency at all tested concentrations, whereas UMab-2 weakly neutralized virus. In an instance like this, Fab neutralization activity is different from Mab neutralization activity, and the Fab would not be a good predictor. However, since it is possible that this difference could be explained by the differences in antigen binding sites per microgram of protein, additional investigration need to be performed.

This trend does help illuminate the answer to another aim of this study: determining whether Fabs show promise for *in vivo* use as either prophylactics or therapeutics to treat influenza infection. In the case of Unpublished Antibody 2, the results indicate that there could be a potential benefit for using Fabs *in vivo.* UMab-2 is an antibody that has been documented by this lab to be effective in targeting H3 viruses (data not shown). UFab-2 showed neutralization of H3 viruses at every concentration tested, whereas UMab-2 weakly neutralized at various concentrations. Not only did UFab-2 prove efficacious against H3 viruses, it also showed some neutralization activity against an H1 virus (Figure 6). UMab-2, on the other hand, was not observed to exhibit cross-reactivity toward H1 viruses (data not shown) . The cross-reactivity of UFab-2 presents the potential for one Fab to

target influenza of different subtypes, a very desirable attribute among antibodies. Along with UFab-2, UFab-6, documented by this lab to neutralize H3 viruses in the Mab form, neutralized H3 viruses at every concentration tested. This is another unpublished Fab that has potential for *in vivo* use.

Although these results indicate the potential for use of Fabs *in vivo*, there are several imperative steps before using the Fabs in a mouse model. Although this pilot study did document stronger neutralization by several Fabs, it is not yet conclusive enough to determine if this is unique to this set of antibodies or it is a characteristic of many antibodies. It is important to expand the sample size of Mabs and their respective Fabs in order to get a more comprehensive understanding of how neutralization differs between these two classes. Also, expanding the range of viruses against which the Mabs and Fabs are tested would help further dissect this trend. Other subtypes that pose a serious threat to public health include H2 and H5 [14]. Testing these additional subtypes as well as more H1 and H3 strains would indicate the true potential of Fabs to neutralize influenza viruses.

Another step in further understanding neutralization by Fabs is determining the kinetics of the neutralization. It may be that Mabs have an allosteric hindrance and Fabs can better reach the epitope for these antibodies. Characterizing such biochemical differences will also be helpful to fully understand the neutralization process. If further analysis indicates that Fabs are very efficacious in neutralizing influenza, the next step would be to test the most efficacious Fabs, such as PA-2 Fab, UFab-2, and UFab-6, *in vivo* in order to assess the true potential of Fabs as a new class of anti-influenza therapies. In the face of current and future influenza viruses, such novel therapies as this are critically important. This Fab-based approach could have the potential to meet this ever-growing challenge.

References

[1] Thompson WW, et al. (2004**) Influenza-associated hospitalizations in the United States**. *JAMA* 292:1333–1340.

[2] Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, et al. (2005) **Avian flu: Isolation of drug-resistant H5N1 virus**. **Nature** 437: 1108.

[3] O'Donnell CD, et al. (2012) **Antibody pressure by a human monoclonal antibody targeting the 2009 pandemic H1N1 virus hemagglutinin drives the emergence of a virus with increased virulence in mice**. mBio 3(3):e00120-12. doi:10.1128/mBio.00120-12.

[4] Crowe JE, Jr. (2012) **Influenza virus resistance to human neutralizing antibodies**. mBio 3(4):e00213-12. doi:10.1128/mBio.00213-12.

[5] Wei CJ, et al. (2010) **Induction of broadly neutralizing H1N1 influenza antibodies by vaccination.** *Science* (New York, N.Y.) 329.5995: 1060-4.

[6] Whittle JRR, et al. (2011) **Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin**. Proceedings of the National Academy of Sciences of the United States of America 108.34 (2011) : 14216-14221.

[7] Ekiert DC, Wilson IA. (2012) **Broadly neutralizing antibodies against influenza virus and prospects for universal therapies**. Current Opinion in Virology 2.2 (2012) : 134-141

[8] Samji T. (2009) **Influenza A: Understanding the Viral Life Cycle.** *Yale Journal of Biology and Medicine.* 82(4): 153–159.

[9] Kashyap AK, et al. (2008) **Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies**. *Proceedings of the National Academy of Sciences of the United States of America* 105.16: 5986-5991.

[10] Simmons CP, Bernasconi NL, Suguitan AL, Mills K, Ward JM, et al. (2007) **Prophylactic and therapeutic efficacy of human monoclonal antibodies against H5N1 influenza**. PLoS Med 4(5): e178. doi:10.1371/journal.pmed.0040178

[11] Burioni R, et al. (2010) **Monoclonal antibodies isolated from human B cells neutralize a broad range of H1 subtype influenza A viruses including swine-origin Influenza virus (S-OIV)**. *Virology* 399.1: 144-152.

[12] Ekiert DC, et al. (2009) **Antibody recognition of a highly conserved influenza virus epitope**. *Science* 324.5924: 246-251.

[13] Staneková Z, and Varečková E. (2010) **Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development**. *Virology Journal* 7.1: 351.

[14] Yoshida R, Igarashi M, Ozaki H, Kishida N, ToMabechi D, et al. (2009) **Cross-Protective Potential of a Novel Monoclonal Antibody Directed against Antigenic Site B of the Hemagglutinin of Influenza A Viruses**. PLoS Pathog 5(3): e1000350. doi:10.1371/journal.ppat.1000350

[15] Grandea AG, Olsen OA, et al. (2010) **Human antibodies reveal a protective epitope that is highly conserved among human and nonhuman influenza A viruses**. (2010) : n. pag. Print.

[16] Wang TT, Tan GS, Hai R, Pica N, Petersen E, et al. (2010) **Broadly Protective Monoclonal Antibodies against H3 Influenza Viruses following Sequential Immunization with Different Hemagglutinins**. PLoS Pathog 6(2): e1000796. doi:10.1371/journal.ppat.1000796

[17] Corti D, et al. (2011) **A Neutralizing Antibody Selected from Plasma Cells That Binds to Group 1 and Group 2 Influenza A Hemagglutinins**. n. pag. Print.

[18] Clementi N, De Marco D, Mancini N, Solforosi L, Moreno GJ, et al. (2011) **A Human Monoclonal Antibody with Neutralizing Activity against Highly Divergent Influenza Subtypes**. PLoS ONE 6(12): e28001. doi:10.1371/journal.pone.0028001

[19] Sui J, Hwang WC, Perez S, Wei G, et al. (2009) **Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses**. *Nature structural & molecular biology* 16.3: 265-73.

[20] The Centers for Disease Control. (2006**) Influenza : Propagation , Quantification**. 1-22. Print.

[21] Krammer F, Margine I, Tan GS, Pica N, Krause JC, et al. (2012) **A Carboxy-Terminal Trimerization Domain Stabilizes Conformational Epitopes on the Stalk Domain of Soluble Recombinant Hemagglutinin Substrates**. PLoS ONE 7(8): e43603. doi:10.1371/journal.pone.0043603

[22] Tan GS, et al. (2012) **A pan-h1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo**. *Journal of Virology* 86.11: 6179-88.

[23] Han T, and Marasco WA. (2011) **Structural basis of influenza virus neutralization**. *Annals Of The New York Academy Of Sciences* 1217: 178-190.