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Expression of HA1 fragment of H1N1 swine influenza viral protein, hemagglutinin, on the surface of yeast Saccharomyces cerevisiae using pYD5 shuttle vector

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ABSTRACT

Influenza viruses are enveloped viruses with segmented RNA genome surrounded by a helical symmetry shell. Due to genetic reassortment between avian, swine, and human influenza viruses, a global pandemic may emerge, calling for new methods of mass vaccine production. Yeast is an ideal organism to express viral antigens (e.g. hemagglutinin) because of its natural adjuvant activity, making the expressed proteins more immunogenic when administered to the human body [1,2]. In addition to the purpose of vaccination, yeast is economically convenient by expressing proteins in a fast, inexpensive manner [3,4]. In this study, the goal is to express the HA1 fragment of the hemagglutinin protein from swine influenza (H1N1) virus [A/Cali/4/09] on the surface of yeast stain *S. cerevisiae* using a reconstructed shuttle vector, pYD5. Development of recombinant yeast cells consisted of ligating genetic sequence of HA1 into pYD5, transforming into *E.coli* cells, and electroporating DNA plasmids into *EBY100 S. cerevisiae* cells. Primary antibody for the flu strain was used in conjunction with a fluorescing secondary antibody, allowing visual analysis under a microscope. In result, surface expression of HA1 fragment was demonstrated by immunofluorescence microscopy. This study represents the first steps in the generation of yeast-based vaccines for the protection of influenza viruses.

1. INTRODUCTION

In late March and early April of 2009, a swine-origin influenza virus emerged out of Mexico and within weeks, spread worldwide to 30 countries and in result, the World Health Organization (WHO) raised the level of influenza pandemic alert from phase 5 to phase 6 in June of 2009 [5,6]. The pandemic 2009 H1N1 virus is a swine-origin, influenza type A virus that encodes 11 proteins on eight segments of RNA [7]. The key protein that elicits prompt immune

response is hemagglutinin (HA) [8]. HA is responsible for binding to cellular receptors and fusion of the viral membranes, causing replication and transcription of viral RNAs and infecting the host [9]. More specifically, the HA protein binds to the host cell receptor, sialic acid [10]. Humans express sialyl-transferases in mucosal and respiratory tissues, resulting in N-glycans with α2,6 sialic acids. However, avian species contain N-glycans linked with α2,3 sialic acids [11]. The varying conformations restrict viruses to specific hosts, meaning that human influenza viruses do not replicate in birds, and vice versa. Swine, on the other hand, contain both α2,6 and α2,3 receptors in their tracheal epithelial cells, making them susceptible to human and avian influenza viruses [12-14]. Consequently, pigs can be seen as the "mixing" vessel for human, avian, and swine influenza strains, thus creating a triple reassortant virus [11,15]. The 2009 H1N1 swine influenza virus is an example of a triple reassortant virus, as in can be outlined in *Figure 1*.

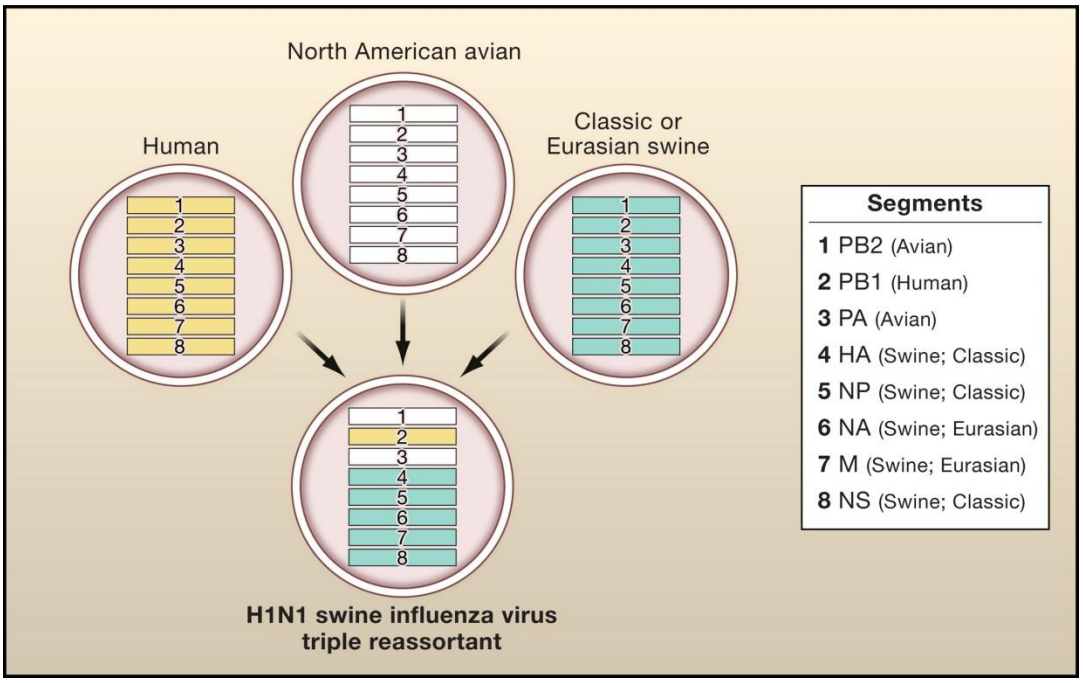

Figure 1. Lineage of the 2009 H1N1 Swine Influenza Virus. Influenza A viruses have RNA segments that encode 11 proteins including surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) and virulence factors NS1 (host interferon

antagonist) and PB1-F2 (proapoptotic factor). The 2009 H1N1 swine influenza virus contains proteins from swine, avian, and human influenza viruses, making it classified as a triple reassortant virus. Figure taken from Taia Wang [11].

The most severe influenza A pandemic occurred in 1918, causing 20-25 million deaths and affecting an estimated 50% of the world population [5,16,17]. Although also spread worldwide, the 2009 H1N1 was milder in nature than the 1918 pandemic, which caused similar mortality rates that of seasonal influenza viruses (affects 10% of the world population, causing 1 million deaths annually worldwide) [9,18]. Pandemics and/or outbreaks arise when novel HA proteins emerge to which humans have no immunity [19-22]. There are 15 different subtypes of HA that can be differentiated both antigenically and genetically [7]. Also, HA can undergo antigenic drift and antigenic shift. Antigenic drift is the gradual accumulation of point mutation in the protein and is responsible for yearly re-evaluation of the seasonal influenza vaccines [7,23]. Antigenic shift is a more complex form of genetic and antigenic change in which two or more viruses strains combine to form a new subtype. This may occur because of interspecies transmission or through genetic mutations between a current circulating virus and one or more new viruses [21,24,25]. Both methods of antigenic shift have been documented among influenza viruses [26-29]. With eight independents segments of RNA, there are 256 possible genotypes from two parental viruses, which make a strain with no human immunity a possible outcome. In case of a pandemic event, mass amounts of vaccines must be produced.

Currently, most influenza vaccines are produced in embryonated hens' eggs [30,31]. The viruses are allowed to naturally reassort in the embryonated eggs and reassortants with the desired characteristics (e.g. HA and neuraminidase gene) are selected [32]. This process is monitored by the WHO, which houses centers in Australia, Japan, the UK, and the USA. Investigators analyze the epidemiology of circulating influenza viruses that are isolated from humans and animals and make recommendations on which strains humans are likely to be

infected with [33,34]. The manufacturing processes of egg-based vaccines are time consuming and require millions of eggs, resulting in at least a six month production cycle [33]. It is estimated that in the event of a worldwide pandemic, current influenza vaccine strategies will only cover ~17% of the worldwide population in 1 year [35]. At this rate, it would take 5 years to cover 100% of the world population for *one* influenza strain. In addition, highly pathogenic avian influenza (H5N1) cannot be grown in embryonated hens' eggs [36,37] , prevailing new methods of mass production in case of a severe global pandemic [38].

A technology that has been used for production of human therapeutic proteins is yeast. Products such as Actrapid by NovoNordisk contain the therapeutic protein, insulin, utilizing *Saccharomyces cerevisiae* as an expression system [39,40] . Pharmaceutical companies like Genencor and Novoenzymes hoYeaRuse large-scale fermentation facilities for yeast propagation [39,41] . Also, the number of published studies using yeast as surface display systems for proteins has increased almost exponentially since the late 1990s [42]. Yeasts are commonly utilized because they meet safety issues (i.e. do not harbor pathogens, viral inclusions, or pyrogens [43]), have rapid growth rate in simple media, and are able to secrete and modify recombinant proteins [43-47]. Also known as baker's yeast, *S. cerevisiae* has been in use the longest, leading to a complete mapping of its genome in 1996 and the first eukaryote applied to heterologous gene expression [43,48,49]. *S. cerevisiae* will be used in this study as a host organism for surface display of the HA1 domain of the 2009 H1N1 HA protein, and more specifically the virus strain H1N1//Cali/4/09 (provided by St. Louis Children Hospital). The HA protein consists of HA1 and HA2 segments, but HA1 forms the large globular head of the protein that contains the receptor binding and antigenic sites, to which the immune system responds to [7,50-54].

For creation of recombinant yeast cells, "shuttle" vectors are generally used. Shuttle vectors are hybrids between yeast-derived and bacterial sequences [43,46]. The reason for this strategy is to maximize efficiency (e.g. DNA plasmids can be extracted from ampicillin resistant recombinant *E.coli* cells) [46,55-57]. *E.coli* has been used in previous studies to allow rapid propagation of recombinant cells and plasmids are then extracted from *E.coli* cells and electroporated into yeast cells [58-63]. An available yeast shuttle vector that is commercially available is vector pYD1, provided by Invitrogen, originally developed by Boder and Wittrup [64]. In the pYD1 vector, the protein of interested is flanked on the N-terminus by mating protein Aga2, which binds to surface membrane Aga1 protein by disulfide bonds. However, it was discovered by Dr. Z. Wang that the vector could be reconstructed to increase affinity to protein of interest by leaving the NH_2 terminus of the displayed protein of interest free, rather than protein of interest being sterically hindered [65]. The new yeast display vector is named pYD5. A visual image can be depicted in *Figure 2*.

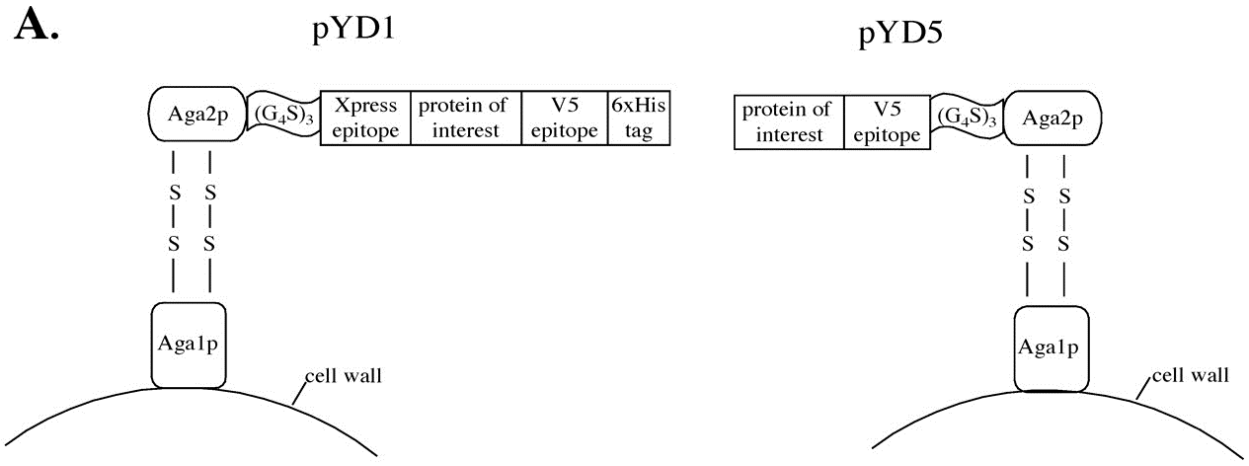

Figure 2. Schematic of the commercially available yeast display vector pYD1 (Invitrogen) in which the displayed protein is tethered at its N-terminus to Aga2p mating protein through the Xpress epitope and a $(G_4S)_3$ linker (left) and the rebuilt vector pYD5 in the reverse orientation, where the protein of interest's N-terminus is free (right). Picture taken from Z. Wang [65]**.**

This study describes the creation of an *EBY100 S. cerevisiae* strain expressing the HA1 domain of the hemagglutinin antigen of the H1N1 virus, A/Cali/4/09. The methods consist of (1) amplifying DNA of the HA1 domain gene sequence, (2) ligating plasmid DNA into expression shuttle vector, pYD5, (3) transforming of pYD5-HA1 into competent *E. coli* cells, and (4) electroporating plasmid DNA into *EBY100* cells. Because an important key factor for the production of heterologous proteins is the transcription efficiency of the genes, an inducible promoter, 2% galactose, will be used to maintain yeast cultures for optimal transcription maintenance [46,66].

2. MATERIALS AND METHODS

2.1 Plasmids, bacterial and yeast strains, antibodies

H1N1 HA plasmid fragment (pHW2000/H1N1/A/Cali/4/09) was obtained courtesy of St. Louis Children Hospital. Full sequence genome (available from NCBI Influenza Virus Resource database) of the HA protein can be found in *Appendix 1*. The HA1-domain is highlighted in yellow and is 981 base pairs long. *S. cerevisiae* strain cells, EBY100 was procured from

Invitrogen. pYD5 yeast display vector was obtained Dr. Z. Wang (NIH, Bethesda, MD). Competent 5-alpha *E.coli* cells were purchased from New England Biolabs. Monoclonal anti-influenza virus H1 hemagglutinin (HA) antibody was obtained from BEI Resources. Secondary Goat Anti-mouse IgG was purchased from Thermo Scientific.

2.2 PCR amplification of HA1 domain of H1N1 (pHW2000/H1N1/A/Cali/4/09) and gel electrophoresis analysis

With the help of Dr. Qing-long Liang, forward and reverse primers were designed. Forward primer, pYD5-H1N1-HA1-F read 5'-CTAGCTAGCGTTTTAGCAGCTGGTGACACATTATGTATAG-3' and reverse primer pYD5-H1N1-HA1-R read 5'CCGGAATTCTCTAGATTGAATAGAC-3'. The underlined portions are recognition sites for restriction endonucleases enzymes NheI and EcoRI, respectively. The kit used for PCR was Phusion High Fidelity PCR KIT (NEB, MA). According to the protocol, PCR optimization is enhanced when template DNA (plasmid pHW2000/H1N1/A/Cali/4/09) is at 10 ng/µL. The concentration of the plasmid was determined by a microplate spectrophotometer from BioTek and adjusted to 10 ng/µL. For 50 µL reactions: 10 µL of 5x Phusion HF buffer, 1 µL of 10 mM dNTPS, 2.5 µL of 10 µM forward and reverse primers, 1 µL of template DNA, 0.5 µL of Phusion DNA polymerase, and 32.5 µL of nuclease free water were added. Cycling conditions were as follows: 1 cycle of initial denaturation at 98 °C for 30 seconds, 35 cycles of 98 °C for 10 seconds, 45 °C for 30 seconds, and 72 °C for 1 minute, and 1 cycle of 72 °C for 10 minutes. The PCR product was then analyzed via gel electrophoresis (100 volts for 40 minutes) using 1x TAE buffer, 0.8% agarose, and ethidium bromide solution.

2.3 Extraction of DNA from gel and double enzyme digestion and ligation

The DNA fragment from the agarose gel was extracted using Qiagen Qiaquick Gel Extraction protocol. To digest the restriction enzymes and cut the double-stranded DNA to its proper sequence, restriction enzymes *EcoRI* and *NheI* were used. The protocol of double enzyme digestions consisted of: 2 µL of NEB Buffer 1 (10x), 0.5 µL of BSA (100x), 1 µL of NheI (10,000 U/mL), 0.5 µL of EcoRI (20,000 U/mL), and 16 µL of purified gel-extracted DNA products. The mixture was placed in a 37 °C water bath for 2 hours. The digested DNA product was then purified again using Qiagen Gel Extraction kit. The purified DNA product was then ligated into pYD5 yeast display vector in accordance to the protocol DNA ligation with T4 DNA Ligase (Fisher Scientific; USA).

2.4 Transformation of pYD5-HA1-H1N1into competent E. coli cells and PCR bacterial analysis

The ligation product was transformed to NEB 5-alpha competent *E.coli* cells using the High Efficiency Transformation protocol (NEB, USA). Recombinant *E. coli* cells were then spread onto LB plates (1.0% Tryptone, 0.5% Yeast Extract, 1% NaCl, and 100µg/mL of ampicillin). The plates were placed in a 37 °C incubator overnight. After overnight incubation, single colonies were isolated and mixed with 10 µL of nuclease-free water. 1 µL of the solution was then used as a template for PCR, while the remaining 9µL of positive clones solution tested by PCR were mixed with 5 ml of LB media (with ampicillin) and placed in a shaking (250 rpm) incubator at 37 °C overnight.

2.5 Extraction of plasmid from E.coli and double enzyme digestion and sequencing for confirmation of positive clones

To isolate the plasmid DNA from *E.coli*, the Pureyield Plasmid Miniprep System protocol was used (Promega; San Luis Obispo, CA). Product were then digested enzymatically again using EcoRI and NheI. Samples were sent to the department of Poultry Science for sequencing.

2.6 Electroporation of DNA plasmid into EBY100 yeast vector and induction

Plasmid DNA was electroporated into *S. cerevisiae* strain *EBY100* using the Mircopulser Electroporation Apparatus (Bio-Rad; Hercules, CA) following the "Electroporation of *S. cerevisiae"* protocol from the provided manual [67]. In the manual, it was suggested to 5-100 ng of plasmid DNA sample, and the selected amount was 100 ng. Electroporated product was then spread onto Minimal Dextrose (MD) plates that contained 0.01% leucine and incubated at 30 °C. This media allows for single yeast colonies to grow in 3 days (pYD1 Yeast Display Vector Kit; Invitrogen). After 3 day incubation period, two single yeast colonies (pYD5-HA1-1 and pYD-HA-2) were inoculated into 10 mL YNB-CAA growth medium containing 2% glucose as a carbon source (pYD1 Yeast Display Vector Kit; Invitrogen). Flasks were placed in a shaking incubator at 30 °C overnight. Plasmids were extracted from yeast cells and the extractant were used as templates for PCR to determine if the HA1 fragment was in the yeast clones. Protocol and kit used was from Zymoprep Yeast Plasmid Miniprepartion Kit I (Zymo Research; Irvine, CA). Cell cultures were then subjected to modification until correct OD_{600} readings resulted (following "Induction" protocol from pYD1 Yeast Display Vector; Invitrogen). Cell cultures were incubated at 20 °C with shaking. To determine the optimal induction time for maximum display, the cell cultures were assayed over a 68 hour time period and samples were stored at 4 °C for fluorescence immunostaining. Yeast cells were induced by 2% galactose (pYD1 Yeast Display Vector Kit; Invitrogen).

2.7 Test HA1expression using fluorescence immunostaining

After the 72 hour sample was collected, all time point samples were centrifuged at 3000-5000g for 10 minutes at 4° C. Cells were resuspended in 1x PBS buffer and centrifuged at same speed and time length After aspiration of PBS, the cell pellets were resuspended in 250 µL of 1X PBS, 1 mg/ml BSA, and 1 µg of monoclonal anti-HA (H1N1/Cali/4/09) antibody. Samples were placed on at 4 °C for one hour with occasional mixing. Samples were centrifuged again at same speed and time length. Cells were then washed with 1 mL of 1X PBS. After aspiration of PBS, cells were resuspended in 250 µL of 1X PBS, 1 mg/mL BSA, and 0.5 µL goat anti-mouse IgG conjugated with fluorescein isthiocyanate (FITC) secondary antibody. This secondary antibody will conjugate with the primary antibody and fluoresce under microscope, giving confirmation of expression of HA1 domain of HA protein. Samples were incubated at 4 °C for 30 minutes in the dark with occasional inverting of tubes. Samples were then washed two times with 1 mL of 1X PBS (no BSA) and were finally resuspended in 40µL of 1X PBS, where a 5 µL aliquot was placed on a slide and observed under a 400X fluorescing imaging microscope (Olympus; PA).

3. RESULTS

3.1 PCR results of HA1 domain of pHW2000/H1N1/A/Cali/4/09

The plasmid concentration of the HA fragment using the spectrophotometer was 167 ng/µL. A 2 µL aliquot was diluted with 33.4 µL of H_20 to result in an optimized concentration of 10 ng/µL. The HA1 domain of the strain is approximately 981 base pairs. The following figure (*Figure 3)* shows the gel electrophoresis product, with a specific band being right under the 1 kilo-base marker.

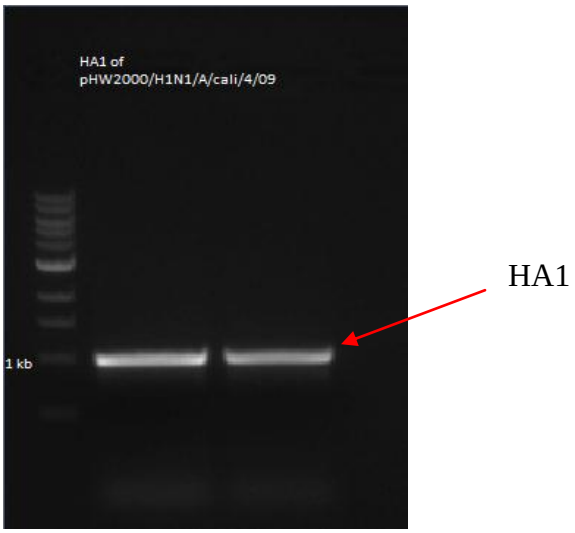

Figure 3. Gel Electrophoresis Analysis of HA1 domain of H1N1 strain pHW2000/H1N1/A/Cali/4/09
Agarose gel electrophoresis of PCR amplification of HA1 domain of H1N1 strain, pHW2000/H1N1/A/Cali/4/09. From the NCBI database, the HA1 domain 981 base pairs long, and the PCR product is right under the 1 kilo-base pair marker.

3.2 Double enzyme digestion of plasmid DNA extracted from E. coli

After the pYD5 vector containing HA1 had been transformed into *E.coli* and plasmid DNA had been recovered, double enzyme reaction was done to determine if a specific DNA band could be noticed after gel electrophoresis. *Figure 4.* shows the results of gel product. The specific band at right around 1 kb shows that HA1 fragment (981 bp) was able to be isolated from vector and *E.coli*. The genetic sequence (acquired from Poultry Science) is listed in *Appendix 2*. Alignment of obtained genetic sequence from Poultry Science and sequences from NCBI Influenza Virus Resource database is consistent and correct.

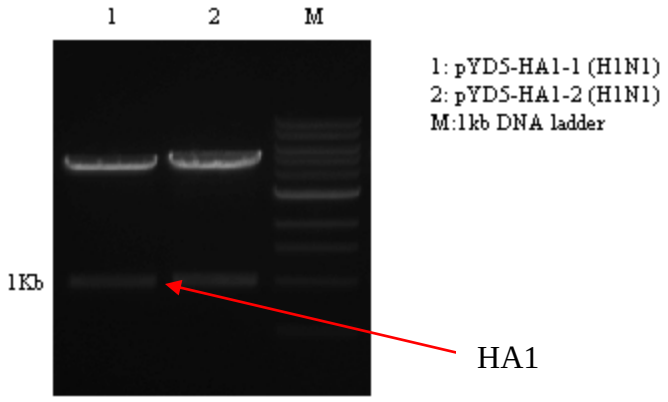

Figure 4. Gel electrophoresis after double enzyme digestion
Gel electrophoresis product after double enzyme digestion. The bottom specific band confirms that the HA1 fragment is detectable at around 981 bp.

3.3 Electroporation product and extracted plasmid from yeast

Data of the electroporated yeast cells and plasmid extraction from yeast were not obtained. However, PCR analysis of plasmid DNA extracted from the yeast cells did indeed have a specific band at 981 bp. *Image not shown*.

3.4 Immunofluorescence staining

Recombinant yeast cells expressing the HA1 fragment of HA protein are distinguished in fluorescence microscopy by emitting green color. Induction times were at 0 hr (control), 24 hr, 48 hr, and 68 hr. The images can be seen in *Figure 5*. The label FITC signifies only the cells that emitted protein expression. A FITC and BF image was also taken at time point to show the relationship between expressed cells and non-expressed cells. After induction with 2% galactose, it usually takes about 4 hours to detect expression, so the control sample at 0 hr does not show protein expression. The Yeast Display Vector Kit from Invitrogen had estimated that optical induction time would be between 24-48 hours. From the images, there is an increase in the number of expressed cells from 24 to 48 hours. However, from 48-68 hour, there was not an increase in expressed cells. To determine statistical analysis of expressed cells, flow cytometry

will need to be done, which is an experiment in the works and the results have not been documented.

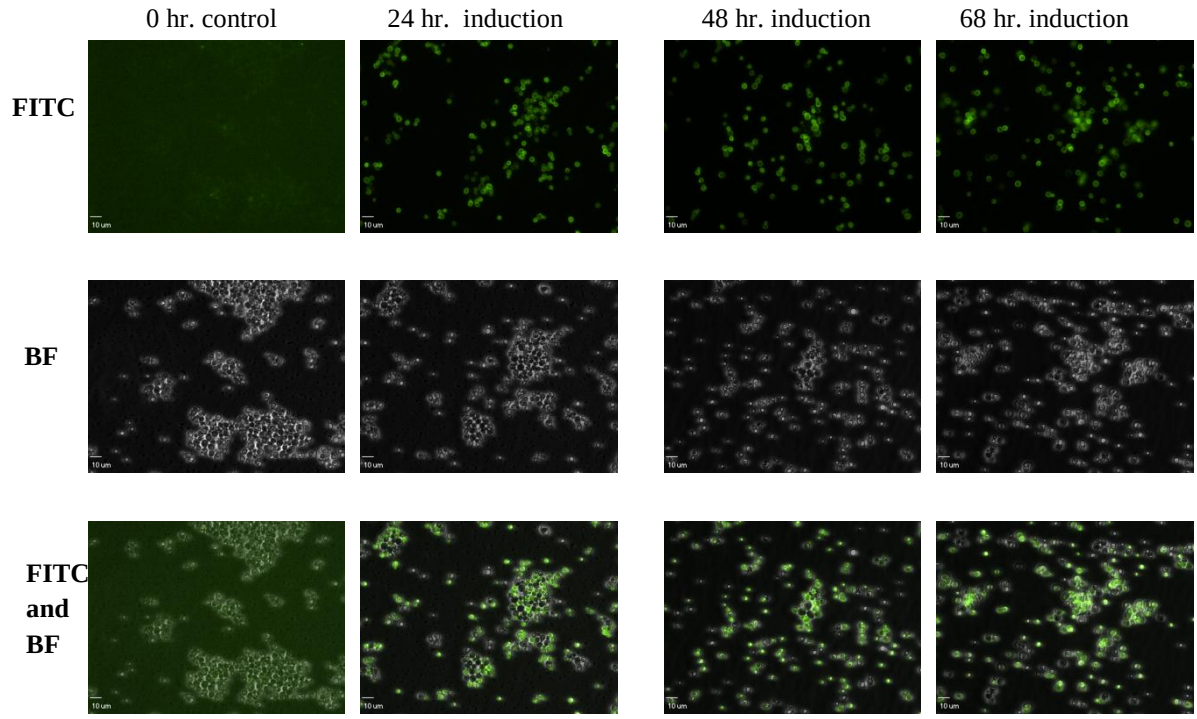

Figure 5. Immunofluorescence microscopy

Immunofluorescence using primary antibody that reacts with HA1 fragment of the HA protein of H1N1 virus and a secondary antibody that reacts with primary antibody. From the pYD1 Yeast Display Vector Kit from Invitrogen, it states that optimal detection should occur between 12 and 48 hours. The Aga2p fusion can be detected as early as 4 hours after the switch to galactose, so the 0 hour control confirms that there is no expression signal. The images suggest that cells at 48 hour induction time was slightly better than 24 hour induction time, but further analysis such as flow cytometry would need to be conducted. However, there is no statically significance between 48 hour and 68 hour induction time, suggesting that optimal expression is indeed between 12-48 hours. (BF: Brighfield)

4. DISCUSSION

Since the HA1 subunit of HA contains most of its antigenic sites, the work presented was to determine if the subunit could successfully be expressed in yeast for the purpose of developing vaccines. By determining the nucleotides and code length of the HA1 subunit, PCR analysis would confirm if HA1 could be detected after manipulations with *E.coli* and *S. cerevisiae.* DNA plasmid extractions from both *E.coli* and *S. cerevisiae* did contain the HA1 subunits in gel electrophoresis products, affirming that the strategy of using both hosts does increase efficiency.

The immunofluorescence staining results demonstrate that the primary antibody does attach to the HA1 fragment antigen. Moreover, it is likely that the express proteins in yeast cells do retain their proper confirmations, although further tests must be conducted for confirmation. Ideally, the goal is for all yeast cells to express the protein, which is not seen by immunofluorescence staining. To maximize cell expression, further research in modification of media should be conducted. Also, it was noted in the literature that optimal induction time was between 24 and 48 hr. To find the most optimal time, it would be a good idea to have several more time periods (e.g. 32 and 40 hr).

In summary, the hypothesis of yeast cells being able to express the HA1 fragment of HA was positive. Although further details, such as calculating the concentration of expressed protein and determining optimal concentration for immunization, this study represents a first step in the production of yeast-based vaccines for influenza viruses.

5. CONCLUSION

5.1 Current Status

In this study, HA1 subunit from a strain of swine influenza was expressed on the cell surface of *S. cerevisiae*. Immunofluorescence demonstrated the localization of the HA1 subunit on the surface of yeast. Although there are many more steps involved for the construction of a vaccine, the time-scale in creating recombinant yeast cells is significantly shorter than that of egg-based vaccines. Typically, *S. cerevisiae* fermentations take 5 days from start to finish: 2 days for inoculum development, 1 day for biomass accumulation on glucose, and ~2 days for galactose induction [66]. For egg-based vaccines, there is at least a 3-month lead time for the start of production [31].

5.2 Future Directions

To measure the ratio of expressed and non-expressed recombinant yeast cells, flow cytometry of the sample must be conducted. Also, to calculate the molecular weight of the HA1 fragment, a Western Blot assay must also be conducted. Then measures can be taken into calculating the concentration of the antigen secreted and construction of a proper dosage vaccine for mouse immunizations. The postdoctoral associates, Dr. Qing-Long Liang and Dr. Han Lei have constructed a similar expression system; however, they are using highly pathogenic avian influenza virus subtype (H5N1) In addition, they have constructed yeast cells that not only contain the antigenic HA protein, but also conjugated with CD154, a protein that is primary expressed on activated T cells (for increased immune response). They are currently undergoing mouse immunization.

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8. APPENDICES

7.1: Appendix 1: HA1 sequence of HA protein

A/Cali/4/09 (H1N1) – HA sequence from NCBI Influenza Virus Resource database

Highlighted portion is HA1 domain-981 bp

AGCAAAAGCAGGGGAAAATAAAAGCAACAAAA**ATG**AAGGCAATACTAGTAGTTCT
GCTATATACATTTGCAACCGCAAATGCAGACACATTATGTATAGGTTATCATGCGAA
CAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACACACT
CTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGGGGTA
GCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCCAGAG
TGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACACCTAGTTCA
GACAATGGAACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAGAGCA
ATTGAGCTCGGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTTCATG
GCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGAGCAA
AAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCCAAAG
CTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTATGGGGCATT
CACCATCCATCTACTAGTGCTGACCAACAAAGTCTCTATCARAATGCARATACATAT
GTTTTTGTGGGGTCATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCAATAAG
ACCCAAAGTGAGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGTAGAGC
CGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGATATGCA
TTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAGTCCAC
GATTGCAATACAACTTGTCAAACACCCAAGGGTGCTATAAACACCAGCCTCCCATTT
CAGAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTAAAAAGCACAAA
ATTGAGACTGGCCACAGGATTGAGGAATATCCCGTCTATTCAATCTAGAGGCCTATT
TGGGGCCATTGCCGGTTTCATTGAAGGGGGGTGGACAGGGATGGTAGATGGATGGT
ACGGTTATCACCATCAAAATGAGCAGGGGTCAGGATATGCAGCCGACCTGAARAGC
ACACAGAATGCCATTGACGAGATTACTAACAAAGTAAATTCTGTTATTGAAAAGAT
GAATACACAGTTCACAGCAGTAGGTAAAGAGTTCAACCACCTGGAAAAAAGAATAG
AGAATTTAAATAAAAAAGTTGATGATGGTTTCCTGGACATTTGGACTTACAATGCCG
AACTGTTGGTTCTATTGGAAAATGAAAGAACTTTGGACTACCACGATTCAAATGTGA
AGAACTTATATGAAAAGGTAAGAAGCCAGCTAAAAAACAATGCCAAGGAAATTGG
AAACGGCTGCTTTGAATTTTACCACAAATGCGATAACACGTGCATGGAAAGTGTCAA
AAATGGGACTTATGACTACCCAAAATACTCAGAGGAAGCAAAATTAAACAGAGAAG
AAATAGATGGGGTAAAGCTGGAATCAACAAGGATTTACCAGATTTTGGCGATCTATT
CAACTGTCGCCAGTTCATTGGTACTGGTAGTCTCCCTGGGGGCAATCAGCTTCTGGA
TGTGCTCTAATGGGTCTCTACAGTGTARAATATGTATT**TAA**CATTAGGATTTCARAA
GCATGAGAAAAACACCCTTGTTTCTACT

7.2 Appendix 2: Genetic sequence of DNA plasmid extracted from E.coli

Sample 1

NNNNNNNNNNNNTTNNTACNTTTTCATTAAGATGCAGTTACTTCGCTGTTTTTCAAT
ATTTTCTGTTATTGCTNNCGTTTTAGCAGCTGGTGACACATTATGTATAGGTTATCAT
GCGAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAAC
ACACTCTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAG
GGGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATC
CAGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACACCTA
GTTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGA
GAGCAATTGAGCTCGGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGT
TCATGGCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGA
GCAAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCC
AAAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTATGGG
GCATTCACCATCCATCTACTAGTGCTGACCAACAAAGTCTCTATCAGAATGCAGATA
CATATGTTTTTGTGGGGTCATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCA
ATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGT
AGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGAT
ATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAG
TCCACGATTGCAATACAACTTGTCAAACACCCNANGGGTGCTATAAACACCAGCCTC
CCATTTCANAATATACATCCGATCACAATTGGAAAATGTCCAAAATATGTAAAAAGC
ACAAANNGANACTGNNNNCNGNATTGANNNNNTCCCNNCTATTCNATCNANAANA
ANTNGGNANNNNTNCCNNNNCCNNNNCNNNGNNNNNNNATNNNNNNNNNNNNNN
NNNNNNNGNNNNNNNNGNNNNNNNNNNNNNNNNNNNNNNNNNNNGNNN

Sample 2

NNNNNNNNNNNTTNNNACNTTTTCATTAAGATGCAGTTACTTCGCTGTTTTTCNATA
TTTTCTGTTATTGCTAGCGTTTTAGCAGCTGGTGACACATTATGTATAGGTTATCATG
CGAACAATTCAACAGACACTGTAGACACAGTACTAGAAAAGAATGTAACAGTAACA
CACTCTGTTAACCTTCTAGAAGACAAGCATAACGGGAAACTATGCAAACTAAGAGG
GGTAGCCCCATTGCATTTGGGTAAATGTAACATTGCTGGCTGGATCCTGGGAAATCC
AGAGTGTGAATCACTCTCCACAGCAAGCTCATGGTCCTACATTGTGGAAACACCTAG
TTCAGACAATGGAACGTGTTACCCAGGAGATTTCATCGATTATGAGGAGCTAAGAG
AGCAATTGAGCTCGGTGTCATCATTTGAAAGGTTTGAGATATTCCCCAAGACAAGTT
CATGGCCCAATCATGACTCGAACAAAGGTGTAACGGCAGCATGTCCTCATGCTGGA
GCAAAAAGCTTCTACAAAAATTTAATATGGCTAGTTAAAAAAGGAAATTCATACCC
AAAGCTCAGCAAATCCTACATTAATGATAAAGGGAAAGAAGTCCTCGTGCTATGGG
GCATTCACCATCCATCTACTAGTGCTGACCAACAAAGTCTCTATCAGAATGCAGATA
CATATGTTTTTGTGGGGTCATCAAGATACAGCAAGAAGTTCAAGCCGGAAATAGCA
ATAAGACCCAAAGTGAGGGATCAAGAAGGGAGAATGAACTATTACTGGACACTAGT
AGAGCCGGGAGACAAAATAACATTCGAAGCAACTGGAAATCTAGTGGTACCGAGAT
ATGCATTCGCAATGGAAAGAAATGCTGGATCTGGTATTATCATTTCAGATACACCAG
TCCACGATTGCAATACAACTTGTCAAACACCCNANGGGTGCTATAAACACCAGCCTC

CCATTTCANAANATACATCCGATCACNATTNGAAAATGTCCAAAATATGTAAAAAG
CACAAAATNGANACTGNNNNCNGNATNGNNNNNTCCCGNCNATTCNNTCNNNAAN
AANNNNNNANNNNNTNNNNNNNNNNNNCNNNNNNNNNANNNNNNNNNNNNGGNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN