COLLEGE OF BASIC AND APPLIED SCIENCES

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CONSTRUCTION OF POTENT IMMUNOGENIC EPITOPES OF THE HAEMAGGLUTININS OF THE SEASONAL INFLUENZA A VIRUSES

BY

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DECLARATION

This work was done under the guidance of Professors Osbourne Quaye, William Kwabena Ampofo and Munir Iqbal. I hereby declare that the results in this submission are from my research work and illustrations from work done by others are duly acknowledged. I declare also that the content of my work has not been submitted to any other academic institution.

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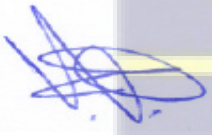
DATE: January 29, 2021 DATE: January 29, 2021



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ABSTRACT

Seasonal influenza viruses are renowned for recurring annual epidemics worldwide. The Influenza A subtypes H1 and H3 are the most dominant and prevalent in recent outbreaks in humans. As with other infectious diseases, vaccines are an important public health tool. However, influenza viruses continue to evolve evading pre-existing or transient vaccine-induced immunity in addition to antigenic pressures associated with antiviral drugs. For this reason, current seasonal influenza vaccines require annual review. Vaccine (immunogen) design, efficacy, and effectiveness presents a formidable seasonal influenza management issue.

Passive immunotherapy has been proposed to offer tremendous protection when appropriately used in the management of influenza, either as a substitute or to complement vaccines. A well-designed immunogen that elicits a strong antibody response towards the conserved domains of the surface Haemagglutinin (HA) protein would be critical to avert virus evolution.

A detailed analysis of the highly conserved regions spanning the fusion peptide, cleavage site, and the two heptad repeats for the HA gene in over 1000 and 21,000 H1 and H3 strains, respectively was therefore conducted. Chimeric haemagglutinins (cHAs) of these conserved regions were constructed by alignment with consensus sequences generated from exotic HAs (H5 and H9 for H1-based cHAs; H7 for H3-based cHA). These cHAs were successfully expressed in Drosophila S2 cell lines.

Mice were then immunized with these cHAs to determine protection against lethal doses in virus challenges against H1 and H3 seasonal viruses. Serum from seroconverted mice applied in challenge experiments indicated the presence of anti-HA specific antibodies with broadly cross-reactive potential against H5 and H7 viruses for H1 and H3-based cHAs, respectively.

This study offers an alternative approach whereby multi-subtype or pan-group immunogens could be utilized for the design and generation of cross-reactive antibodies of potential therapeutic value for influenza in humans.

DEDICATION

To all scientists contributing to the identification of novel therapeutics and universal influenza vaccine candidates.

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Finally, I thank my family for their great patience and love whilst I was at distance on countless occasions due to my academic endeavour.

LIST OF ABBREVIATIONS

AcNPV	*Autographa californica* nuclear polyhedrosis virus
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCL	Antibody-dependent cell lysis
ADCP	Antibody-dependent cell-mediated phagocytosis
APC	Antigen-presenting cell
AVMA	American Veterinary Medical Association
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCL 28	Chemokine (C-C motif) ligand 28
CDC	Centres for Disease Prevention and Control
CD	Cluster of differentiation
cHA	Chimeric haemagglutinin
COBRA	Computationally optimized broadly reactive antigen
CPMR	Centre for Plant Medicine Research, Mampong
cRNA	Complementary ribonucleic acid
CTL	Cytotoxic T lymphocyte
CVV	Candidate vaccine virus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay

FCS	Foetal calf serum
FDA	The Food and Drugs Authority
FRO	For research only
GFP	Green fluorescent protein
GISAID	Global initiative on sharing all influenza data
GISRS	Global Influenza Surveillance and Response Systems
GPI	Glycosyl phosphatidylinositol
H1/HA1	Haemagglutinin type 1
H2/HA2	Haemagglutinin type 2
H3/HA3	Haemagglutinin type 3
H5/HA5	Haemagglutinin type 5
H7/HA7	Haemagglutinin type 7
H9/HA9	Haemagglutinin type 9
HA	Haemagglutinin
HBV	Hepatitis B virus
hCRM1	Human chromosome maintenance 1 protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	Hepatitis E virus
HI	Haemagglutination inhibition
HIV	Human immunodeficiency virus

HPAI	Highly pathogenic avian influenza viruses
HPV	Human papillomavirus
HRP	Horseradish peroxidase
IAV	Influenza A viruses
IBV	Influenza B viruses
IEDb	Immune-epitope database
IFN ß	Interferon Beta
IFN γ	Interferon Gamma
IFV	Influenza viruses
IIV	Inactivated Influenza Vaccine
IRD	Influenza research database
IRR	Influenza/ International reagents and resources
I-TASSER	Iterative threading assembly refinement
LAIV	Live-attenuated Influenza Vaccine
LB	Lysogeny broth
M	Matrix protein
M1	Matrix protein 1
M2	Matrix protein 2
M2e	Matrix protein 2 ectodomain
MAFFT	Multiple alignment using fast Fourier transform

MDCK	Madin-Darby canine kidney cell line
MHC II	Major histocompatibility complex type 2
MHC	Major histocompatibility complex
mLD_{50}	Mouse lethal dose 50
mRNA	Messenger ribonucleic acid
MVA	Modified vaccinia virus Ankara
N1	Neuraminidase type 1
N2	Neuraminidase type 2
N10	Neuraminidase type 10
N11	Neuraminidase type 11
NA	Neuraminidase
NCBI	National Centre for Biotechnology Information
NEP	Nuclear export protein
NIC	National Influenza Centre
NKC	Natural killer cells
NMIMR	Noguchi Memorial Institute for Medical Research
NP	Nucleoprotein
NS	Non-structural protein
NS1	Non-structural protein 1
NS2	Non-structural protein 2

PA	Polymerase acidic
PAGE	Polyacrylamide gel electrophoresis
PB	Polymerase basic
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PBS	Phosphate-buffered saline
PBST	0.1% Tween-20 in Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein Database
PFU	Plaque-forming unit
PIV	Parainfluenza viruses
PLGA	Polylactic-co-glycolic acid
PMN	Polymorphonuclear cells
QIV	Quadrivalent Influenza Vaccine
RBS	Receptor binding site
RDE	Receptor destroying enzyme
RFLP	Restriction-fragment length polymorphism
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RSV	Respiratory syncytial virus

S2	Schneider's Drosophila Line 2 cells
Sf	*Spodoptera frugiperda*
SDS	Sodium dodecyl sulphate
SIA	Sialic acid
SOC	Super optimal broth with catabolite repression
TGM	Tris-glycine methanol
Th 1	T-helper 1 T-cells
Th 2	T-helper 2 T-cells
TIV	Trivalent Influenza Vaccine
TLR	Toll-like receptors
TM	Template modelling
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
UG-IACUC	University of Ghana - Institutional Animal Care and Use Committee
UK	The United Kingdom
US	The United States
UV	Ultraviolet
VLP	Virus-like particle
vRNP	Viral Ribonucleoprotein
WHO CC	World Health Organization Collaboration Centre
WHO	Word Health Organization

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CHAPTER 1: INTRODUCTION, AIM, OBJECTIVES, AND RESEARCH QUESTIONS

Introduction

Influenza viruses cause a global disease called influenza that is associated with cold-like symptoms. Unlike the common cold, influenza is associated with severer forms of coughs and fever as cardinal signs (Monto, Gravenstein, Elliott, Colopy, & Schweinle, 2000). Annually, the epidemics attributable to influenza is estimated to cause about half-a-million mortalities, plus several millions of morbidities (Iuliano et al., 2018). However, based on previous influenza pandemics, any future pandemics could cause over 200-300 % more mortalities or morbidities. This makes it a disease of major public health concern and one that requires potent means of intervention.

Vaccines and antivirals are available for the prevention or management of the disease; however, vaccine efficacies are suboptimal and there also exists an arms race that continuously renders the viruses resistant to a large number of the antivirals developed. A potent vaccine with long-term protection is all it takes to efficiently lead to the reduction in influenza epidemics and the prevention or delay of any unforeseen catastrophes. By this, a keen search for a more diverse better-reacting vaccine is fervently desired as a game-changer.

Aim:

The main aim of this work was to ascertain the conserved domains of the haemagglutinins (HAs) of the seasonal influenza A viruses and to exploit these domains in designing cross-reactive antibody-inducing immunogenic HA constructs.

Objective 1: Generation of cHAs with intact conserved domains

Active influenza vaccination regimens and platforms have driven -and are still driving- the constant evolution of influenza A viruses and the many other platforms mostly being developed are more likely to stagnate the problem.

Passive immunotherapy, using antibody administration, to control influenza infections, has been proposed to be relevant in influenza management, in the absence of the desired candidate vaccine (Kotey et al., 2019). Production of antibodies against conserved regions of the influenza A virus HA will be an effective tool that will serve both to inhibit viral replication and allow the immune system to develop an effective immunity to the presently infecting virus. More specifically, designing immunogens that could experimentally induce such diverse antibodies is necessary for this quest.

Therefore, this experiment was set up to employ *in silico* tools to construct seasonal HA molecules that are representative of seasonal H1 and H3 viruses.

Hypothesis: The HAs of seasonal influenza A viruses possess conserved structures that are potentially useful for the design of a recombinant immunogen.

Specific objectives:

1. To assess thousands of HAs of seasonal influenza A viruses and generate the consensus conserved sequences.
2. To employ the conserved sequences in the construction of cHAs for immunogenicity assessment.

Objective 2: Expression of potentially immunogenic cHAs

In recent times, a recombinant vaccine – the Flublok- exists which is expressed in the insect fall armyworm (*Spodoptera frugiperda, Sf*) cells (Cox, Patriarca, & Treanor, 2008). The specific cell type for this vaccine is referred to as ExpreSF+, derived from fall armyworm *Sf9* cells. Protein expression in this system has been greatly aided by the Baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV). However, it is thought that the *Sf9* cells are limited to the secretion of relatively smaller amounts of proteins that are expressed using the system. Also, the cabbage looper (*Trichoplusia ni*) cell derivative BTI-*TN*-5B1-4 cells are believed to express relatively higher amounts of proteins (Krammer et al., 2010; Palmberger et al., 2011). Though the recombinant influenza vaccines expressed in the ExpreSF+ require higher doses, about 3 times the dosage of the conventional egg-grown vaccines, their immunogenicities are known to be decent (Treanor et al., 2007).

The choice of vaccine expression system has been mostly driven by user-preference and to some extent, the robustness of the system: traditional diagnostic and screening methods requiring the expression of proteins, for instance, are probably, more interested in systems associated with both decent yields and physiologically native proteins. Similarly, immunogenicity and cross-reactivity studies may tend to focus more on the physiologically native version of an expressed protein; a good yield is often just an additional interest.

The *Drosophila* line 2 cells (S2) have also long been proposed as a versatile protein expression system, showing comparable protein amounts to that produced by both the *Trichoplusia ni* and *Sf9* systems under the AcNPV transfection (Lee, Chen, Hsu, & Juang, 2000). They have also expressed decent amounts of proteins when in ordinary plasmid-based transfections, without the intermediation of a baculovirus (Iwaki and Castellino, 2008). More recently, other schools of thought are that the mammalian cell systems are superior to insect-based systems, in terms

of similarities in protein modifications to the molecules on a mammalian-cell infecting virus, hence, being immunologically superior to the insect cell counterparts (de Vries et al., 2012; Ecker et al., 2020).

Unlike mammalian cells, like the HEK 293, that are associated with high-mannose modification of expressed proteins, the *Drosophila* S2 cells are known to modify proteins with paucimannose moieties and thereby depleting the requisite glycosylation. Furthermore, the depletion of glycans to mono-glycosylation of influenza consensus cHAs has been shown to increase the breadth of antibody-dependent responses, spanning cross-reactivity and infected cell lysis (because of enhanced CD 8+ T-cell responses) (Liao et al., 2020).

Hence, for this work, the *Drosophila* S2 cells were used for expression of the *in silico*-designed cHAs to enable exploration of their immunogenicity.

Hypothesis: cHAs expressed in S2 cells will allow for the exploration of their inherent immunogenic properties.

Specific objectives:

3. To express high yields of cHAs.
4. To assess the haemagglutinating capacity of the cHAs.

Objective 3: Assessment of cHAs to both induce broadly reactive anti-HA antibodies and confer protection to virus-challenged mice.

The head of the influenza A viruses' HA as described, presents variable host immune-focussed globular head regions that probably exert three main functions: facilitate virus-cell interaction that aids viral infection; or by adapting to a new host; or as a means of escaping or evading host immunity - the latter, being of critical interest in the development of several platforms that harness the immunodominance of the HA in the synthesis of influenza vaccines. However, the therapeutic use of discovered anti-HA antibodies recognizing relatively conserved domains of the HA molecule is of great interest (Kotey et al., 2019). Of this antibody collection, the anti-HA stalk antibodies have received great attention (Corti et al., 2010; Margine et al., 2013a; Nachbagauer et al., 2016; Sui et al., 2011; Wohlbold et al., 2015). Nevertheless, several other antibodies have been discovered recently that interact with conserved domains on the globular head of the HA (Benjamin et al., 2014; Boonsathorn et al., 2014; Krause et al., 2011; Yasuhara et al., 2018; Yu et al., 2017). The major challenge, however, has to do with designing immunogens that would achieve the induction of such protective antibodies. But noting the relevance of these anti-HA antibodies targeting the globular head conserved domains in inhibiting viral infection and transmission, therapeutic intervention or specific vaccine design is likely achievable. As such, emphasis needs to be placed on the development of influenza A virus-like HA antigens (or immunogens) that could have the potential of inducing a more promiscuous collection of antibodies in diverse non-human primates. Perhaps, in this collection, there will be polyclonal antibodies that could either inhibit conserved regions on the head of the HA or the stalk.

Hypothesis: cHAs expressed in a system with limited post-translational carbohydrate modification will induce antibodies directed to the exposed conserved domains

Specific objectives:

5. To assess seroconversion amongst mice immunized with the cHAs.
6. To assess the seroconverted serum to broadly-react with distant group-based HA-bearing viruses.
7. To assess the protection of mice immunized with the cHAs during a lethal virus challenge.

CHAPTER 2: LITERATURE REVIEW

Historical accounts on influenza as an ancient virus

History holds that influenza had long existed dating to as far back as before the 15th century. However, it was until the 15th century when an epidemic broke out in Italy that the disease was attributed to the "influence of stars" and thus named influenza. The Latin "influentia" meant "to flow into" and during those days, it was believed that stars emitted intangible fluids that could flow into and affect humans. By 1743, what the Italians called "influenza di catarro" meaning "outbreak of the catarrhal fever" had spread gradually to Europe and was soon referred to as Influenza in English. Since then, the name Influenza has often been abbreviated as flu, which is more common lately (Francis, 1960).

By the end of the first world war in 1918, another outbreak occurred, recording up to about 500 million morbidities and at least 20 million mortalities worldwide. The outbreak was termed the "Spanish flu" due to earlier thoughts that the virus had been spreading from Spain. It appears that the very first person who was diagnosed with the virus responsible for the outbreak was a military serviceman who was attended to in camp Funston located in Kansas. He was rushed to Europe by fellow American Servicemen; here, it was believed that the virus evolved to a highly virulent strain causing more havoc. This points to the notion that the outbreak had started in the US and not Spain or Europe as thought. This premise was further corroborated by testing of archived tissue samples in an American military hospital in South Carolina after almost 8 decades and realizing that this was then regarded as the swine flu (Kolata, 2001).

Amidst all the outbreaks that perhaps might have occurred centuries back, a lot of interest is shown in influenza viruses and outbreaks that occurred spanning the 18th century until date. The world war was almost subsiding when the unpropitious outbreak of influenza had occurred. During the period, history described a lot of troop camping and movements, denoting closeness

in space among military personnel and thus fostering the rapid expansion of the infection. As there were no vaccines or treatments, the outbreaks became a public health burden and numbers of cases multiplied day-by-day. Other key factors included troop celebration of the truce and because they were gradually disbanding, many more new contacts contracted the virus leading to the resurgence in the numbers of cases firstly in the US. The infection turned sporadic, involving the European continent (Erkoreka, 2010).

Discovery and isolation of influenza A virus

The infection was being referred to as Influenza or flu, but it was still not clear to microbiological scientists what kind of pathogen caused this pandemic. Therefore, around 1933, a team of researchers attempted to identify the cause of the flu. Foremost, Smith and colleagues 1933 played a crucial role in identifying that the cause of flu was a virus. They arrived at this premise after they had obtained throat washes from persons who were acutely infected with flu and subsequently filtered the washes with a collodion membrane with a pore size of about 0.6 microns. Filtrates when instilled into the nares of ferrets, yielded disease phenotypes indistinguishable from persons from which throat washes were obtained. As a control, they realized that when the nares of a new batch of ferrets were instilled with throat washes from healthy persons comprising 2 people who had recovered after a previous infection and 2 others that did not have the infection, the disease phenotype was not established. Also, filtered nasal wash from a patient suffering from a severe common cold could not cause disease in ferrets. At this stage, the scientists were convinced that the pathogen that caused the flu was filtrable providing a clue to suspect a virus. The study also confirmed the typical hallmark of the contagious virus pathogen, which is its ability to spread from an infected ferret to an uninfected when housed together within 24 hours or even when filtered materials from infected tissues had been used to instil the nares of otherwise uninfected ferrets. Besides the human strain of virus sourced from the throat washes, Smith and colleagues also realized that another

filtrable virus of the swine-origin yielded indistinguishable symptoms as that caused by the viruses of the human origin when they had infected ferrets. The swine virus was previously shown to cause a severer form of infection in swine in association with a haemophilic bacillus - one that was not realized in their experiments on the ferrets; however, one striking observation was the fact that ferrets that recovered from the swine virus challenge were protected from challenge with the human viruses, but not the vice-versa (Smith, Andrewes, & Laidlaw, 1933).

Previous experiments had involved lightly etherized ferrets due to the strong reflex of the animals to sneeze, compromising on the amount of infectious inoculum; Researcher Shope obtained 2 swine flu viruses and designed a setup that required a complete knock-off of ferrets using anaesthetics, to facilitate administration of a nearly appropriate dose of the inocula created with these viruses. He also confirmed the transmissibility of the virus from infected ferrets to uninfected ones, with retention of the classical symptoms. Several pathologies, including ungroomed fur, inappetence, thumping, etc., were thoroughly described. There was an earlier notion that the Haemophilus bacillus, *H. influenzae suis* exacerbated swine flu infections in swine, and this was observed even when serially passaged swine flu virus in ferrets, still showed the classical symptoms when pigs were infected. Also, amongst ferrets, bacteriological assessments of the respiratory tracts of sick swine flu inoculated ferrets confirmed the absence of bacteria and that, unlike the swine, ferrets did not require the presence of a bacteria to exacerbate flu infection. This was further proved when *H. influenzae suis* only or *H. influenzae suis* -spiked flu virus inoculum was used to challenge ferrets and observed that the bacillus alone could not establish an infection and was indifferent in the case of the two pathogens. Thus, in ferrets, the flu virus infection was independent of a bacillus as conversely established for swine. Shope's work debunked further the notion that the bacillus played a role in the infectivity and severity of the swine flu infection when he observed that a pig that was administered with both the bacillus and the virus at a certain passage developed only mild

symptoms. Shope demonstrated further that other than intranasal inoculation of ferrets with the swine viruses, ferrets could not show the disease phenotype of the flu owing to subcutaneous inoculation, whether an anaesthetic was used or not. This was used to clearly outline the route of infection of the virus. Further, It was necessary to completely prove that the virus that was used to induce disease in ferrets was also responsible for the disease in swine, using a cross-neutralization test; For this test, convalescent sera from ferrets were mixed (at different proportions) with different proportions of viruses from ferret and the mixture was used to intranasally inoculate pigs – the converse was also done, and it was observed that in both instances, certain ferrets and pigs that received a favourable amount of sera mixed with either the ferret/ swine-specific viruses were protected from infection compared with their controls (Shope, 1934).

Isolation of Influenza B virus

By 1933, pandemic influenza A virus had been isolated in ferrets; however, it became noticeable that not all the influenza disease had been caused by one virus. Thomas Francis performed immunological experiments that confirmed that more than one type of virus could cause influenza symptoms. During his investigations, he called this new virus the "Lee virus". He still could not clearly show that this new virus was different from the swine flu virus that caused the earlier pandemic, until in 1940, when he could use neutralization assays to show that a current virus causing an outbreak was similar to what he had detected before back in 1936 and that the virus that caused the 1918 pandemic and the current one was different. Thus, to avoid confusion, this "Lee virus" was termed influenza B as the classical one was termed the influenza A virus (Francis, 1940).

During the time when Thomas Francis had discovered the new virus (influenza B), Burnet who had had an enormous amount of experience in isolating certain viruses in eggs, adopted this

process to attempt to isolate the influenza B virus so named. In his experiment, stock viruses were prepared by inoculating ferrets and scraping off nares. Pathogenicity of these viruses was assessed by the inoculation of mice closely followed by the analysis of mice lungs for characteristic lesions. Subsequently, eggs were inoculated through several passages using viruses harvested from both ferrets and mice. The end of the egg adaptation process also indicated the characteristic lesions that led to the extent that the eggs were killed. For some of these viruses, it was realized that they could no longer induce symptomatic disease in ferrets; however, mice infections became milder, after inoculation with viruses severally passaged in eggs. In most of the cases, viral passages in ferrets warranted virus infectivity in mice, and this was retained even after egg passage, even though the egg-adapted viruses could only cause milder infections in mice but not ferrets. This phenomenon was one of the interesting findings that were observed during the passage in eggs. Noting that egg characteristics remained constant throughout the experiments, it most definitely was something about the viruses that caused them to gain high affinity and pathogenicity in eggs, after several egg passages. This phenomenon led Burnet to posit the likelihood of using egg-adapted viruses as the source of vaccine viruses for the vaccination of humans in the nearest future (Burnet, 1936).

Inception of influenza vaccines

Following Burnet's posits, it became very necessary to first understand the immunity against influenza viruses; But then, in several experiments, it was realized that working with one strain of the virus, a cognate serum would often neutralize the virus, whereas things got more complicated if several strains were employed. Insights were gained into this phenomenon when Andrewes (1939) reckoned that two influenza-like infections of a person in a short period may be underlined by different aetiological agents. Around this period, mind-boggling concepts encircled a lot of scientific issues involving the epidemiological profitability of vaccination as active immune responses were observed with matching strains but not with different strains in

a cross-neutralization test; 1. to what extent of protection can vaccines protect? 2. which is more appropriate – inactivated virus or live virus vaccines? 3. what should the composition of the vaccine be? (Andrewes, 1939; Smith & Andrewes, 1938).

Scientists feared that live viruses could revert to virulence, but attempts were still made using mice and ferrets to advance the development of influenza vaccines. Some of these studies principally identified subtle challenges stemming up from the right virus titres required for inoculation, the animal source from which the vaccine candidates are generated, the inoculation route, and even the state of the virus -whether living or dead. In one of these experiments, it became clear that homotypic vaccines – whether living or dead – induced some level of protection amongst mice or ferrets that were given the shots. However, vaccines from heterologous species were poor in immunizing performance as they contained a lot of interspecies material that interfered so much with immune responses against the minimal virus content (Andrewes & Smith, 1939).

Experiments gradually extrapolated to humans when a group of scientists tried the egg-adapted viruses as candidate vaccines to assess if that could induce some protective immunity amongst vaccinees. The viruses – moderately virulent in ferrets and mice – were intranasally administered to persons who had previously been immunized with a less virulent strain of the influenza virus and had low serum antibodies against the virus. Subsequently, nasal exudates collected from these persons were shown to exhibit viricidal activity against different strains of the influenza viruses. This would, later, be realized that even exudates from normal human nares exhibited a similar virucidal effect; hence, warranting further elucidating studies (Burnet, Lush, & Jackson, 1939).

During these times, other experiments either had been done or were being done to ascertain which form of vaccine was considerable. In most experiments, irradiated or heat-treated or

chemical-treated viruses had abolished viral infectivity and thus, no significant immunity could be observed. However, one leading experiment was the one by Kidd (1938), showing that the Shope papillomavirus could be inactivated by UV irradiation without affecting the antigenicity of the virus. This was observed when the UV-irradiated virus cultures were administered intraperitoneally. Most of the rabbits that received the shots were assessed both for the formation of papilloma and for the induction of neutralizing antibodies. Using complement fixation tests, neutralizing antibodies were detected even though no active viruses could be extracted from the papilloma on the immunized rabbits. Kidd (1938) noted also that inactivation of the papillomaviruses using strong acids and bases rendered the viruses both un-infectious and serologically unreactive.

Insights from Kidd's findings spurred on the prospects of redesigning the inactivated influenza vaccine employing UV irradiation as the tool for the inactivation. Therefore, Salk and colleagues attempted an experiment on the abilities of unirradiated active influenza virus and irradiated inactive virus to induce immunity via intraperitoneal inoculation of mice. They employed virulent mice-infecting influenza viruses cultured on minced chicken embryos in the presence of physiological salts. UV irradiations were conducted on viruses at different time intervals just to ascertain the minimum effective time and irradiated viruses were further used to immunize mice. Observations were that the optimal UV-irradiated inactive viruses had nearly equal immunizing capacity as their unirradiated active viruses. Further quantitation of the antigenicity of the irradiated viruses became necessary to authenticate the importance of UV- irradiated influenza viruses as potential vaccine candidates; so, Salk and colleagues looked at intraperitoneal immunization of mice with graded doses of the UV-irradiated doses of influenza virus. After several days, each of these mice received an intranasal viral challenge. Results were conclusive that optimal UV-irradiated influenza viruses maintained a decent level

of antigenicity, except that these viruses lose their immunizing capacity by about 100-fold (Salk, Lavin, & Francis, 1940).

Different aetiological agents underly influenza

All the vaccine preparations during earlier works up until 1940-1941 have been influenza A virus-based; however, these reports had given insights into the existence of yet another aetiology which is dissimilar to the influenza A virus but could produce a similar disease phenotype as does the influenza A viruses (Francis, 1940; Francis, 1940; Magill, 1940; Magill & Tyndall, 1941). Studies by Lennette and colleagues rather consolidated the presence of distinct viruses causing influenza in each of the outbreaks from which virus-infected samples were obtained. This experiment also showed to an appreciable degree, the presence of other uncharacterized viruses (Lennette, Rickard, Hirst, & Horsfall, 1941). Thus, by 1942, experiments were carried out to assess the importance of an influenza B vaccine.

Eaton and colleagues performed several mice inoculations and passages of an influenza B virus strain, and then the virus was subsequently inoculated on eggs, within either the embryo's amnion or the chorioallantoic fluids. Isolated viruses were then treated with formaldehyde, based on earlier experiments and the notion that the influenza A vaccine formulated inactive using formaldehyde reduced the incidence of the infection by about half (Brown, Eaton, Meiklejohn, Lagen, & Kerr, 1941; Horsfall, Lennette, Rickard, & Hirst, 1941; Martin & Eaton, 1941). Thus, in the experiments by Eaton & Martin (1942), the chorioallantoic fluids were examined for both bacterial and neurotropic virus contamination. One mouse, during the assessment of the presence of a neurotropic agent by intracerebral inoculation, died showing marked lesions in the brain. Wondering what may have caused this, it was not farfetched that the influenza B viruses, other than any neurotropic virus, may have been the cause of the brain destruction and the subsequent death. It, therefore, became indispensable to experiment on

active influenza B virus as a vaccine candidate, except to inactivate it before administration to animals or human subjects. Hence, inactivating all vaccine lots by treatment with formaldehyde. Tests for viral activity in mice were performed by either intranasal or intracerebral inoculation. This time, there were no records of lesions in the brains and subsequently, other mice receiving the shot intraperitoneally were protected against an intranasal virus challenge. This experiment paved the way to begin experimentation on human subjects combining both influenza A and B vaccine preparations. Both the new influenza B vaccine formula and the previous complex influenza A-distemper virus vaccine earlier reported by Horsfall and colleagues was administered subcutaneously on the left and right arms, respectively (Horsfall et al., 1941). Blood was collected from the participants before and 2 weeks post-inoculation for neutralization experiments. Sera from these blood specimens were heat-inactivated, serially diluted and mixed with active viruses, and intranasally inoculated into mice. Eaton & Martin (1942) observed that vaccination with inactivated influenza B vaccine yielded an appreciable amount of both neutralizing antibodies, as well as complement-fixing antibodies.

Several investigative experiments with human subjects begun to spring up. A typical one was by Bodily and Eaton, who studied the specificity of antibodies by either neutralization and agglutination experiments on sera (from acute or convalescent cases), in response to specific influenza virus -type A and B- strains due to either natural infection or vaccination (Bodily & Eaton, 1942).

An influenza epidemic broke out around 1947 post-World War II and then, the current vaccine formula that had been developed failed to immunize recipients due to some sort of antigenic changes on the epidemic-causing influenza virus. The vaccine failure was described as one of the principal causes of the spread amongst military personnel in the UK, US, France, etc.,

albeit, relatively less virulent to cause a pandemic (Chu, Andrewes, and Gledhill, 1950; Rasmussen, Stokes, & Smadel, 1948; Sartwell & Long, 1948).

Inception of coordination of global influenza

This current and other earlier influenza-related epidemic/ pandemic experiences drove the World Health Organization (WHO) to establish a World Influenza Centre, stationed in the laboratories of the National Institute for Medical Research in London. Subsequently, Regional and National level laboratories were established to collaborate with the World Influenza Centre. Other setups were established to enable the sharing of influenza-related information with both the WHO headquarters in Geneva and the Influenza information Centre in the US (Chu et al., 1950).

Around 1952, the Global Influenza Surveillance and Response System (GISRS) was set up by the WHO, to coordinate national laboratories' surveillance activities and monitor the gradual evolution of influenza viruses. Their network of surveillance and reporting laboratories has increased steadily from 26 to about 152 institutions in 113 countries at present (Zhang & Wood, 2018). Four years after the setup of the GISRS, the influenza studies facility of the CDC laboratories in the Atlanta, US, became designated as a WHO Collaborating Centre (CC) for Surveillance, Epidemiology, and Control of influenza. The WHO CC was tasked with influenza-related work such as the assessment of circulating viruses genetically, immunologically, and susceptibility to antivirals. Currently, there are a total of 7 WHO CCs: 2 in the US; 1 in Australia; 1 in the UK; 1 in China; 1 in Japan; and 1 in Russia. Each WHO CC has been involved in the global selection of candidate vaccines to produce the annual influenza vaccines. They also act to offer both technical assistance and diagnostic reagent support to all members of the GISRS networks. Lastly, they serve as a repository of influenza virus

specimens and isolates obtained due to global surveillance by the NICs (CDC, 2019; WHO, 2022).

Second pandemic during the 20th Century: The Asian flu

Just about a year after a WHO CC had been set up in Atlanta, another influenza pandemic had sprouted up. A collection of reports in the US indicated that there was an ongoing outbreak of influenza-like illness (Anderson, 1957; Jensen, 1957; Reyes, Serfling, & Tayback, 1957). The influenza virus causing the ongoing outbreak had been first identified as a type of influenza A virus in China during the earlier part of 1957. It appeared that the causative virus had spread progressively from China to Singapore and then to the US. Thus, all the occurrences in the cities of the US were confirmed to emanate from the Far East (Langmuir, 1961; Mc, 1958). Subsequently, virus isolation and classification attempts confirmed that the Far East infecting virus originated from avian as it had 3 proteins [including Haemagglutinin type 2 (H2) and Neuraminidase type 2 (N2)] that originated from avian, that had reassorted with other human-infecting influenza virus proteins (Meyer, Hilleman, Miesse, Crawford, & Bankhead, 1957). Before the year ended, the outbreak had affected almost all 6 continents of the globe (Mc, 1958). Recently modelled experiments indicated that even though the virus had spread rapidly, the mortality rate was moderate compared with the 1918 pandemic (Cécile Viboud et al., 2016).

Public acceptance of influenza vaccination in the US

Observations made from influenza-related outbreaks include higher morbidities and mortalities amongst persons with underlying chronic conditions, as is usually observed with the aged who are mostly associated with some of these diseases. Such immunocompromised persons are deemed worthy of enormous attention. In those days of experiencing the 1957 outbreak, in the US, the Surgeon General had advocated for active vaccination regimens for the public with an emphasis on the immunocompromised persons (persons with chronic diseases such as diabetes

mellitus, chronic bronchopulmonary diseases, cardiovascular diseases, renal diseases, Addison's disease), including pregnant women. Decisions put forward by the Advisory Committee for Immunization, culminated in the public acceptance of influenza vaccination as an adopted practice in the US. But the vaccination would not end up for this high-risk group of people only: subtle doses were also recommended for everybody else spanning children (3 months old into pre-school and 6 to 12 years old) to adults of ages 13 and beyond (ACIP, 1961; Rosenstock, 1961).

The possibility that influenza viruses might have originated from migratory birds.

In 1961, there were records of dying terns at the shores of South Africa, in 4 main coastal areas. Terns were known as migratory birds that occasionally frequent the shores of South Africa to breed. However, during the expectant breeding period, there was a massive decline in the number of birds that were present. Also observed was that over 1000 dead birds at the four main focal points at the shores of South Africa. This alarming decline in the numbers of the birds had warranted for the Veterinary services to engage in active research to identify the aetiology of the catastrophe. Utilizing electron microscopy, it appeared that the Tern virus was morphologically like an influenza A virus (Becker, 1963). Down the line, it became very necessary to isolate and characterize this tern virus. Becker collected some influenza A virus strains: one isolated from a Tern in South Africa; another isolated from a chicken in Scotland; a third one isolated from Cape Town; an Influenza B virus; an influenza C virus; and a Newcastle disease virus. The Scotland strain had previously been shown to be morphologically similar to the tern virus and so in the experiment, mice infection and cross-neutralization experiments with all the viruses used confirmed that only the two viruses might be variants of the same strain of the virus. The tern virus present in dead European terns were subsequently the cause of the influenza infection among the common terns, switching ideas to the perspective

that these migratory birds may be responsible for the inter-continental transmission of influenza viruses amongst local birds that they encounter (Becker, 1966).

Discovery of Amantadine: a potent inhibitor of cell entry by influenza A viruses

Whilst influenza research was becoming more prominent, concerns began to ensue about not just prevention using vaccines, but also the management of persons that get infected. Researchers initiated active searches for compounds of therapeutic value, one of which was called 1-Adamantanamine (Amantadine). Adamantane is the most stable member with the molecular formula, $C_{10}H_{16}$, and its name stems from the fact that it is made up of 3 cyclohexane rings that assume the shape -in terms of the spatial arrangement of the carbon atoms- of a diamond; hence, regarded as close to diamond in Greek as "adamantinos" (Senning, 2006). Adamantane was first discovered in petroleum in 1933, but synthesis of the compound was a big challenge even though the structure was proposed (Figure 1) (Landa & Macháček, 1933).

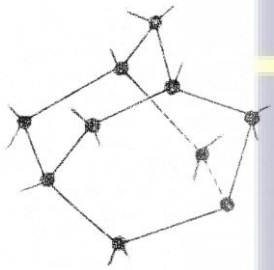

Image adopted from Landa & Macháček (1933).

Figure 1: Structure of the adamantane.

After several attempts had been made, Schleyer was successful at proposing a chemical equation (Figure 2) that would aid the easy synthesis of an appreciable amount of adamantane (Schleyer, 1957).

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A.

Image **A** adapted from Schleyer (1957)

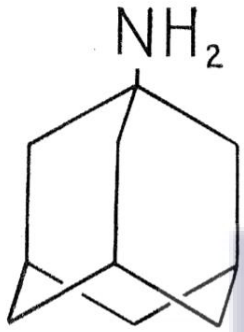


Image **B** adopted from Davies et al. (1964)

Image **C** adopted from Kirschbaum (1983)

Figure 2: Chemical synthesis of adamantane and structures of amantadine (1-adamantanamine)/ amantadine hydrochloride.

A. Adamantane was appreciably synthesized by the hydrogenation of the dicyclopentadiene in the presence of Platinum oxide (in ether) and further cyclised in the presence of aluminium chloride (a Lewis acid). B and C. The amantadine was notably administered as the amantadine hydrochloride.

Strides had been made in the synthesis of adamantanes and chemical synthetic methods have been applied to increase yields drastically, but how this became applicable as a drug for the treatment of influenza is still intriguing and unclear. Nevertheless, later, amantadine (1-adamantanamine) had been shown to exert therapeutic efficacy against influenza A viruses in a dose-dependent manner (Davies et al., 1964). Subsequent studies showed that treatment with Amantadine hydrochloride had caused retention of infectious influenza A viruses at the surface of an infected cell, indicating drug interference with the cell entry process by influenza A viruses (Hoffmann, Neumayer, Haff, & Goldsby, 1965).

Around 1966, the National Institutes of Allergy and Infectious Diseases (NIAID) recommended the use of Amantadine both to treat and prevent influenza A virus infections following approval (based on the susceptibility of the Asian flu viral strains to the drug) by the US Food and Drugs Authority (FDA) (Maugh, 1979). The drug had a minimal-to-no inhibitory effect on influenza B viruses and so was solely applied for the treatment of influenza A virus infections.

Eruption of the 1968 pandemic Influenza: The Hong Kong flu

Not too long after the introduction of the use of amantadine for the treatment and prevention of influenza A virus infection, another pandemic broke out: The Hong Kong flu pandemic. This time, it was a new virus subtype that had a new haemagglutinin (H3) and its neuraminidase gene was of the virus of the previous Asian flu pandemic (in 1957)- that is, the N2 (Ghendon, 1994). This influenza A (H3N2) virus was first noticeable in the Americas where it had been

reported to have caused over a hundred thousand deaths and then worldwide, over a million. History noted two typical waves: the first one which was quite benign -in mostly the Americas- and affected mostly aged people as was hypothesized that persons exposed to the Asian flu of 1957 might have had N2-specific antibodies that conferred some level of protection; and then a second wave that badly affected mostly Europe and Asia due to drifts on the N2 gene (C. Viboud, Grais, Lafont, Miller, & Simonsen, 2005).

Several reforms had been made following the successful containment of the A (H3N2) virus but as if that were not enough - by 1976, in the US, there were reports about a respiratory disease outbreak amongst military recruits living in Fort Dix, New Jersey. At first, it was thought to be an adenoviral infection -as confirmed by the Walter Reed Army Laboratory - during an earlier outbreak in the year in Fort Meade, Maryland. For a timely intervention, the causative agent needed to be identified. This warranted the collection and shipment of throat washes from infected recruits to a New Jersey Laboratory. In New Jersey, the specimens collected were shown to harbour influenza A viruses and this would be confirmed later (in the same year, though) in the CDC laboratories as influenza A viruses of the swine-origin (Neustadt, 1978).

Reformative approaches, such as vaccination, were necessary for consideration and so during this period, representative viruses were shared with Kilbourne's laboratory for virus growth and isolation attempts. In his laboratory, challenges were revolving around the growth of these viruses, leading to the resort of the tried and tested recombination approach. In this approach, Kilbourne's lab team mated the rapidly growing laboratory isolate PR8 (A/PR8/34) strain and one of the viruses from New Jersey (A/NJ/11/76), in the presence of antibodies that inhibited the HA and NA of the PR8 strain. Resultant recombinant (termed X-53) bore the internal genes of the PR8 strain and the surface proteins (HA and NA) of the New Jersey virus. Selected

recombinants were, therefore, employed in the development of vaccines against the swine influenza outbreak (Palese, Ritchey, Schulman, & Kilbourne, 1976).

The swine influenza outbreak had led to the full activation of reformative measures, chiefly, vaccination based on Kilbourne's X-53 virus. An approximated 25% of the US population (i.e., about 48 million people) were vaccinated by the 10th month upon the inception of the outbreak. However, a typical setback was the resurrection of a neurologic disease called the Guillain-Barre Syndrome, a condition that was often experienced with vaccination regimens, but this time, was more than what was expected. Seeing this disease creep into the homes of some vaccine recipients triggered a halt on the vaccination programme. But, overall, some appreciable dimension of vaccine-induced protection was observed (Neustadt, 1978).

Rimantadine – a more refined derivative of amantadine was generated.

One of the therapeutic milestones was the remodification of amantadine into an analogue drug called rimantadine. The remodification became necessary due to growing body of knowledge depicting that influenza A viruses had begun to develop some resistance to the amantadine. For instance, investigations by Hay and colleagues both buttressed other earlier posits that both the HA and Matrix protein 2 (M2) played a crucial role in the viruses that developed resistance to the amantadine. Findings from his team further pinpointed locations of mutations that led to resistance mainly on the hydrophobic amino acids that span a region of the M2 protein (i.e., amino acids between positions 25-43) and thereby positing the mechanism of action of the amantadine hydrochloride to be on the membrane-spanning domains. Roping of the HA into the mechanism of resistance was further explained to be due to the apparent functions of the M2 acting with other viral proteins during syntheses and assembly. Thus, its inhibition by the amantadine mostly led to the reduction in the numbers of the HA that were expressed by the virus and these HA proteins even suffered a consequence of an altered structure as this affected

reactivity with antibodies (Hay, Wolstenholme, Skehel, & Smith, 1985). Further studies by Hay and colleagues yielded informative results detailing mutations in the HA gene amongst resistance viruses as occurring less frequently and thus, specific mutations in the M2 protein may be responsible for the resistance to the amantadine (Hay, Zambon, Wolstenholme, Skehel, & Smith, 1986).

Rimantadine, another derivative of adamantanes was developed sometime to serve as a more refined pharmacokinetically favourable drug to either complement the use of amantadine or better still, as a substitute drug that would circumvent some adverse reactions amongst amantadine recipients. Just like amantadine, rimantadine (1-adamantane-methylamine hydrochloride) inhibits the *in vitro* infection of influenza A viruses. However, it was thought to be superior to amantadine (Koff & Knight, 1979): In cell culture, where studies by Wallbank (1969) showed that a relatively lesser amount of rimantadine (than amantadine) was required to inhibit 50% of Rous and Esh Sarcoma Viruses (Wallbank, 1969); and in animals, where the amount of rimantadine (similar to amantadine) lead to relatively lower titres of influenza A viruses in pulmonary lesions and lungs, in addition to relatively lower time-dependent HI titres, with the subsequent observation of a more reduced transmission of viruses from treated infected mice to non-treated sterile contact mice (Schulman, 1968). Conversely, in a clinical trial involving the management of a naturally occurring influenza infection during an outbreak in a US penitentiary, both amantadine and rimantadine showed comparable efficacies, even though in most instances, rimantadine (which was one-third dose more than amantadine) mainly performed relatively better: an indication that perhaps the apparent difference was due to the dissimilarity in drug concentration (Wingfield, Pollack, & Grunert, 1969).

In a study to investigate the prophylactic efficacy of rimantadine and amantadine, it was observed that an appreciable number of persons receiving amantadine had dropped out due to the development of side effects on their central nervous system; thus, selecting rimantadine as

the more preferred drug choice for the prophylactic or therapeutic management of influenza (Dolin et al., 1982).

Rimantadine (Figure 3) gradually gained some attention in the management of influenza: By 1986, it was used to treat against uncomplicated influenza A (H3N2) with the realization of a satisfactory efficacy, in terms of the reduction in rates of nasal secretions and the accelerated times for abatement of fever and symptoms (Hayden & Monto, 1986);

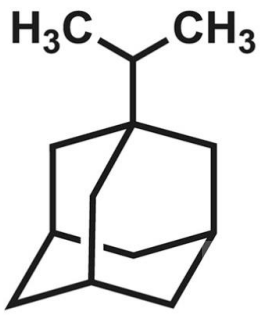


Image adopted from Suzuki et al. (2016).

Figure 3: Structure of rimantadine.

A more interesting finding in the then Union of Soviet Socialist Republics (USSR) buttressed the point that rimantadine is one of several influenza antivirals that had withstood the test of over 20 decades of use, and that in all these years, efficacy did not depreciate amidst the emergence of resistant strains (Kubar, Brjantseva, Nikitina, & Zlydnikov, 1989). Following several studies, rimantadine had received the US FDA approval for use as either prophylaxis or treatment for influenza A virus infections. However, due to adverse effects and the emergence of resistance strains (that may easily be transmissible), the use of both the amantadine and the rimantadine is, thus, subject to stringent guidelines, considering different age categories, persons with chronic illnesses, regulated dosages, and frequency of drug use (Arden, Cox, & Schonberger, 1994).

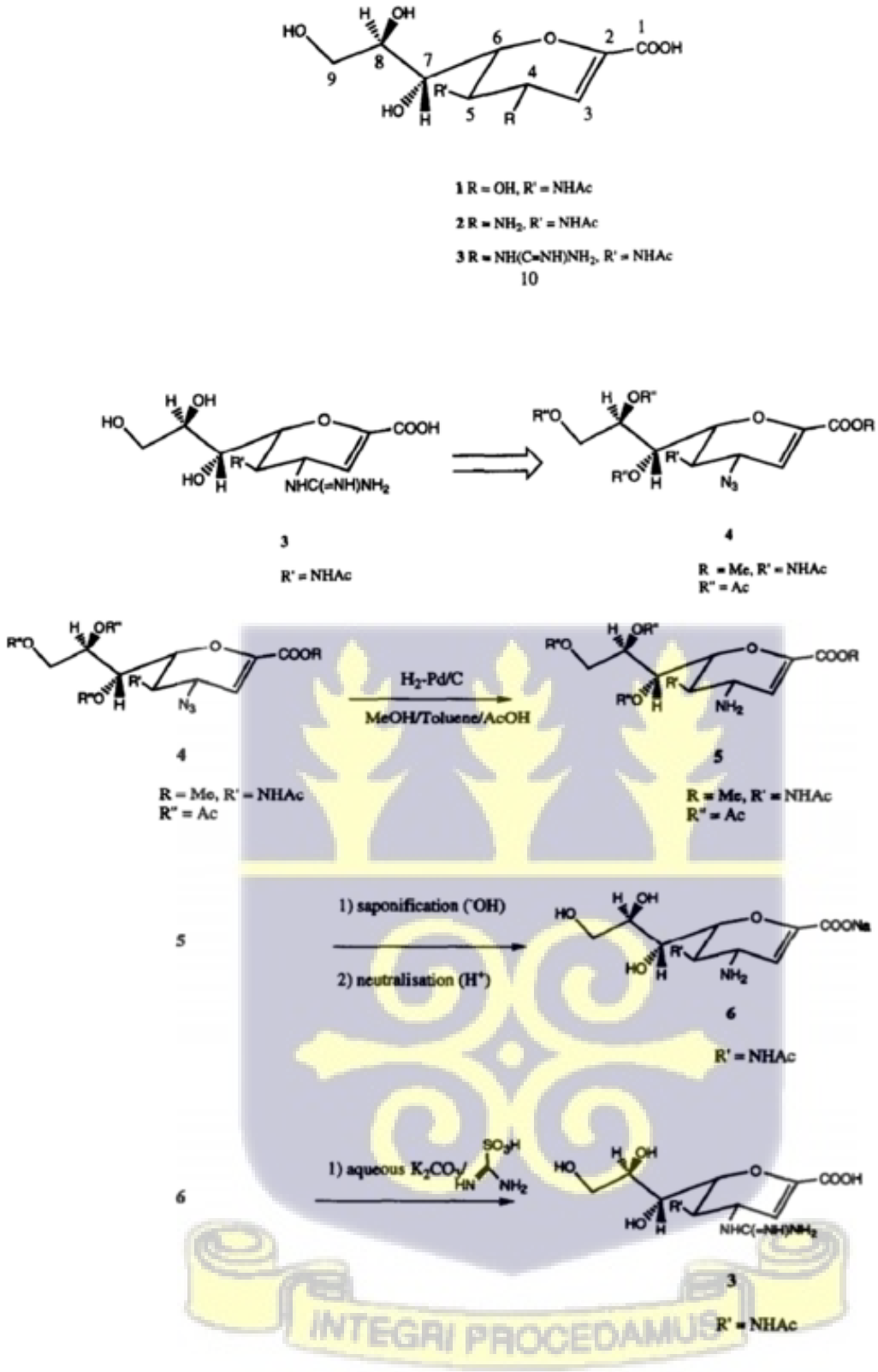
Identification and licensing of new influenza antivirals: Zanamivir and Oseltamivir

Influenza virus emergence and re-emergence had continued. All such continuing cases leading to the explorative discovery and licensing of new anti-influenza compounds: The Oseltamivir and Zanamivir. In addition to influenza virus emergence or re-emergence, influenza B viruses have not received much attention with the use of the M2 protein inhibitors (the amantadine and rimantadine). The identification of zanamivir and oseltamivir proved very useful, as this drug had a broad-inhibitory capacity against all influenza Viruses discovered as of then.

First, the rational design of zanamivir followed closely after the unravelling of the crystal structures of the haemagglutinin (HA) and neuraminidase/ sialidase (NA)- the two surfaces proteins of the influenza viruses (Colman, Varghese, & Laver, 1983; Varghese, Laver, & Colman, 1983; I. A. Wilson, Skehel, & Wiley, 1981). The identification of the sialidase (neuraminidase) notably generated a lot of attention as many of such sialidase-possessing microbes had been shown to exert some pathogenicity in man (Schauer, 1983, 1985). Several studies, following on, supported a previous posit that the influenza sialidase played a critical role in the disaggregation of new virions after successful cycles of replication in an infected cell (Palese, Tobita, Ueda, & Compans, 1974). Subsequently, it made sense to think of the influenza viruses as able to use the sialidase in the process of dissemination in the mucus lining respiratory tracts, which is known to be rich with lots of sialic acids (Colman & Ward, 1985; Klenk & Rott, 1988; Schulman & Palese, 1977). But more importantly, the known crystal structure of the influenza sialidase/ neuraminidase lead Itzstein and colleagues to apply the rational computer-aided drug design to design two analogues of sialic acids – the 4-Guanidino-2,4-Dideoxy-2,3-Dehydro-N-Acetylneuraminic and 4-amino-2,4-Dideoxy-2,3-Dehydro-N-Acetylneuraminic, with the former showing a relatively higher fold of inhibition (against both influenza A and B viruses in both *in vitro* and *in vivo*) than the amantadine (von Itzstein et al., 1993). The 4-Guanidino-2,4-Dideoxy-2,3-Dehydro-N-Acetylneuraminic (also known as, 4-

582 guanidino-Neu5Ac2en) would later be selected for being superior to the inhibition of a breadth
583 of influenza viruses (Woods et al., 1993).

584 By 1994, a workup for the synthesis of the 4-guanidino-Neu5Ac2en had been proposed (Figure
585 4) (von Itzstein, Wu, & Jin, 1994), and several other studies had verified the inhibitory capacity
586 of the drug in diverse experimental designs (Hayden, Rollins, & Madren, 1994; Ryan,
587 Ticehurst, Dempsey, & Penn, 1994; Thomas, Forsyth, Penn, & McCauley, 1994).



588

589 Image adopted from von Itzstein et al. (1994)

590 **Figure 4: Structure and synthesis of 4-guanidino-Neu5Ac2en.**

591

592 The drug had gained the name, Zanamivir by the time the first efficacy and safety studies were
593 being carried out, and here, Hayden and colleagues had seen great success in terms of the drug
594 intervention during the clinical trials (Hayden et al., 1997), receiving US FDA approval in 1999
595 for the treatment of influenza caused by the types A and B viruses (Oxford, 2000).

596 Oseltamivir, another sialidase/ neuraminidase-inhibitor was also being developed concurrently,
597 and this was because zanamivir had a very poor bioavailability via the oral route of
598 administration and was highly efficacious when administered via the intranasal inhalation
599 route. Oseltamivir (then called RO 64-0796 or GS4104) was designed based on the structure
600 of zanamivir and therefore exhibit great resemblance to each other (Figure 5), except that a
601 more stable carbocyclic template was used in place of the Neu5Ac2en dihydropyran ring such
602 that Oseltamivir would have better bioavailability and rapid excretion when orally
603 administered, in addition to desirable efficacy against influenza viruses (Kim et al., 1997).

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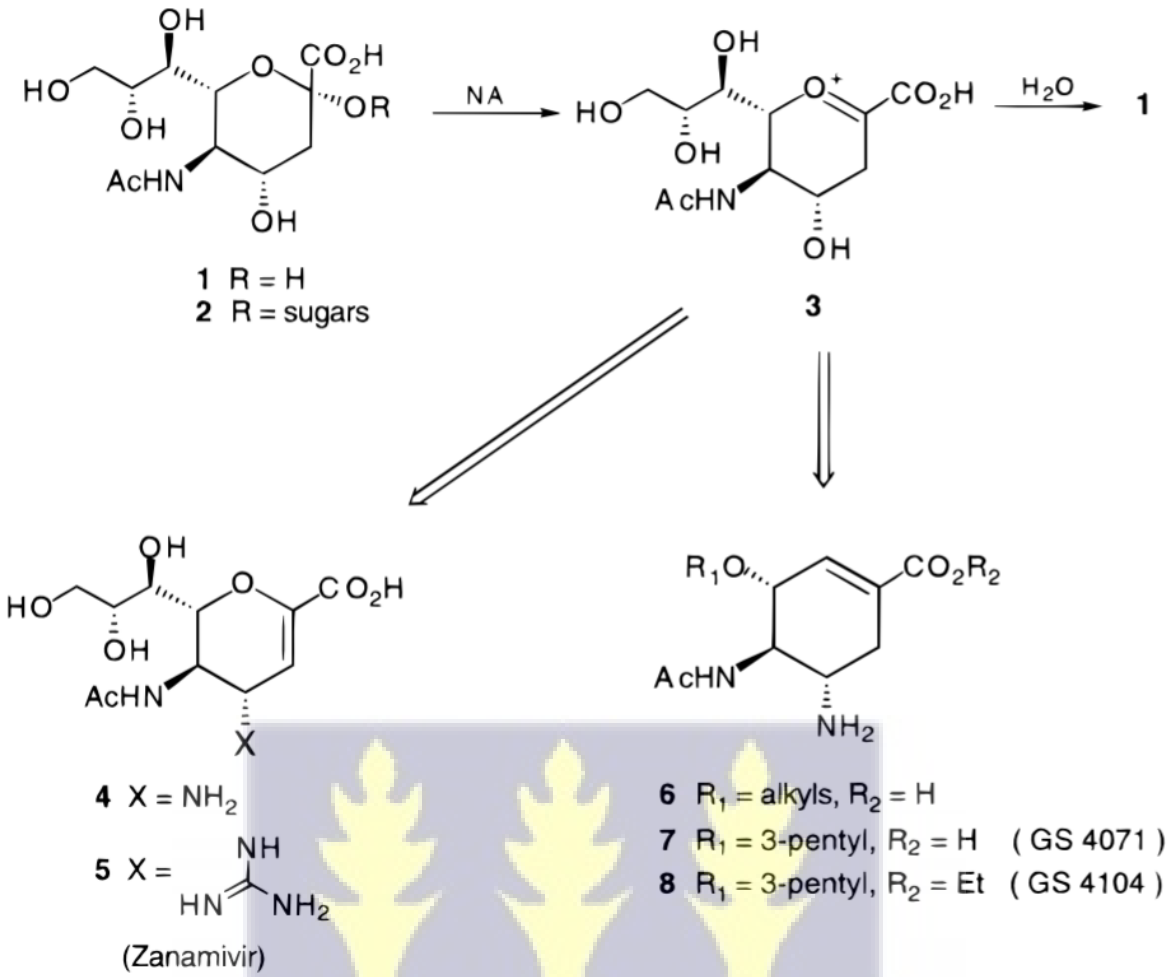


Image adopted from Kim et al. (1998)

Figure 5: Structural resemblance of Zanamivir to GS 4104 (Oseltamivir).

Oseltamivir (GS 4104) is the resultant metabolite of the GS 4071.

Oseltamivir was designed as a pro-drug that unleashes its active anti-influenza metabolite that subsequently exhibited acceptable pharmacokinetic values, besides tolerability and efficacy in adult subjects during clinical trials (Hayden et al., 1999; Massarella & Nieforth). With such appreciable efficacy against both influenza A and B viruses, the Oseltamivir was also approved for use by the US FDA in 1999, just a few months after the Zanamivir was approved (CDC, 1999).

It would not take too long for the use of the adamantanes to be discouraged for the treatment of influenza A virus infections. This was around 2006 when the US CDC gathered that there was an outrageous spike of adamantane-resistant influenza A viruses as captured by surveillance activities (Bright, Shay, Shu, Cox, & Klimov, 2006). The discovery of the anti-influenza properties of the neuraminidase inhibitors - zanamivir and oseltamivir- came in just handy to replace the adamantanes in the fight against the influenza viruses.

Systematic initiatives: the "one health" initiative, pandemic influenza operational plan, and full deployment of the PCR in the diagnosis of influenza

Soon, the fight against influenza viruses would turn robust, as all possible arms of health security reforms had been deployed:

The "one health/ one medicine initiative" in 2007 was of interest to the American Veterinary Medical Association (AVMA) with reasons being that there's a constant human-interface in all facets of human life and that these interactions could foster zoonotic disease "spill-overs" in humans. This initiative strategized to involve all arms of Veterinarians, Physicians, public health, food and agriculturists, and environmentalists, to draw attention to the public health importance of human-animal interactions which could be crucial in the eruption of either a zoonotic disease amongst humans or even, an anthroponotic disease amongst animals (Kahn, Kaplan, & Steele, 2007). This was going to benefit a lot in the global management of influenza due to the story about the origin of influenza viruses (i.e., from birds) and the fact that pigs acted as mixing vessels in the brewing of reassortants which could have a propensity to cause future pandemics.

The pandemic influenza operational plan, an initiative to give credence to the ability of newly isolated avian influenza virus amongst birds and humans to be the next likely cause of an

influenza virus-related pandemic, was birthed. The isolated virus was characterized to be highly pathogenic to birds and caused fatal infections in humans, thus, creating the scare that the next pandemic could be of avian influenza origin. The plan was, therefore, established both to evaluate national and global healthcare capacities in the detection and containment of any pandemic influenza if one should arise (Ortu, Mounier-Jack, & Coker, 2008).

At the time, one of the documented challenges was the heterogeneity in laboratory methods for influenza diagnoses – they were mostly less sensitive and undermined the burden of influenza. The pandemic influenza operational plan also advocated the adoption of the PCR system to enhance the sensitivity and specificity of detection so that such high-fidelity diagnosis would aid in the rapid containment of any influenza if detected at all (Simmerman & Uyeki, 2008). Not too long, the CDC would set up the Influenza Reagents and Resources (IRR) to support the national influenza centres in the PCR-diagnosis of influenza.

Eruption of another pandemic influenza virus: The H1N1pdm09

Interestingly, all these preparations in anticipation of a future pandemic were applied in 2009, when a triple reassortant (as illustrated in Figure 6) led to a new pandemic (Neumann, Noda, & Kawaoka, 2009). Before the end of 2009, a monovalent H1N1 vaccine was produced to combat the new influenza strain, 6 months post inception (CDC, 2009a, 2009b).

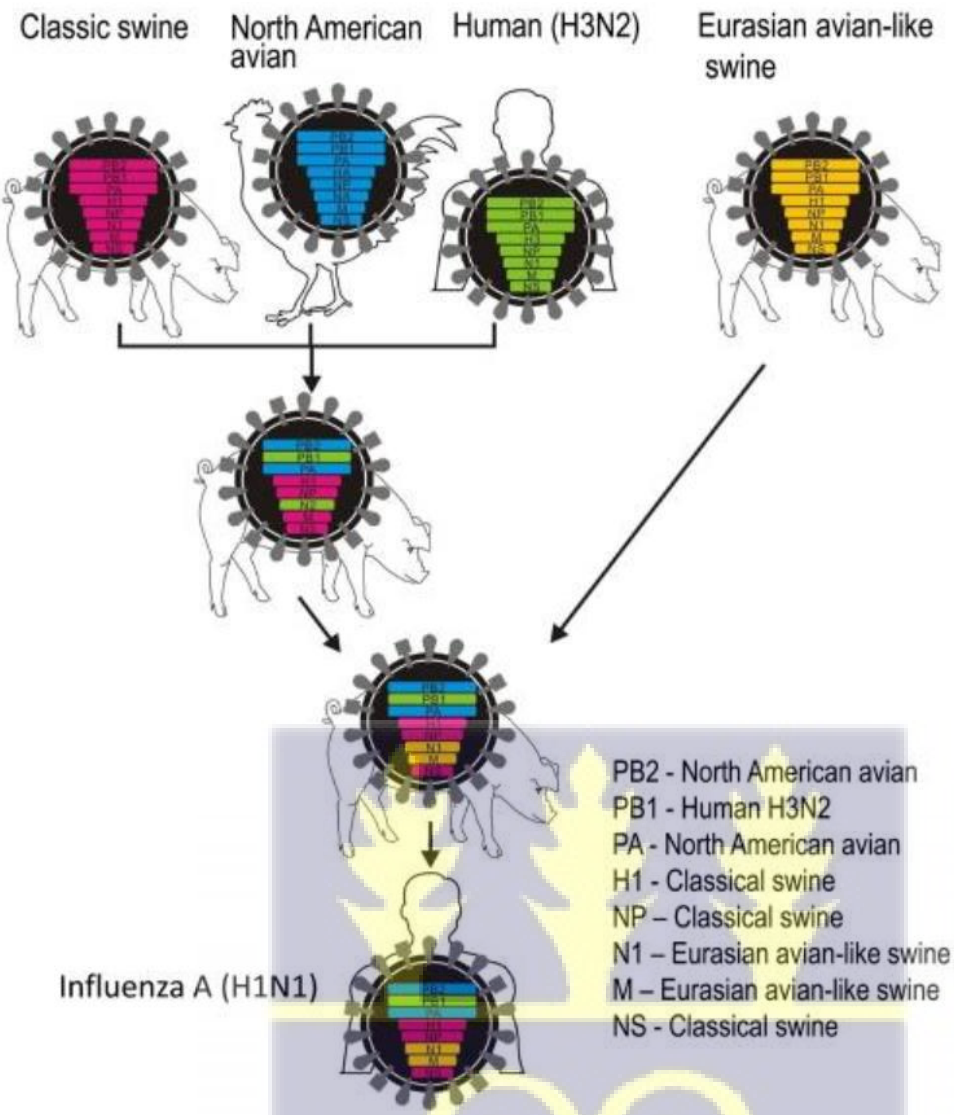
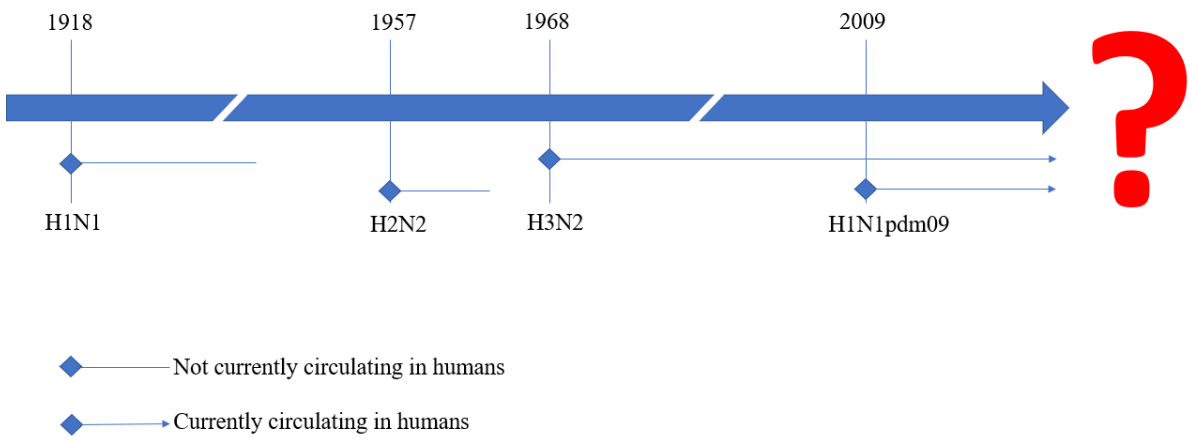


Image adopted from Neumann et al. (2009)

Figure 6: Systematic reassortment of the H1N1pdm09.

The report by Neumann and colleagues reveals the nature of the present virus as a reassortant of a Eurasian avian-like swine influenza virus and a previous triple reassortant virus (one comprising genetic segments of classic swine, North American avian, and human H3N2 influenza viruses).

The last influenza pandemic observed is the one caused by the H1N1pdm09 in 2009. Though, scientists fear occurrence of another influenza outbreak, it is still not quite certain whether there is going to be another pandemic anytime in the nearest future (Figure 7).



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669 **Figure 7: The 20th-21st Century influenza pandemic timelines and persisting seasonal**
670 **viruses.**

671 The chart here outlines the pandemics caused as a result of influenza viruses. There is still a lot of anxiety about
672 a possible forthcoming influenza-related pandemic and thus, warranting the continuous search for more potent
673 interventions spanning the discovery of universal influenza vaccine candidates and novel therapeutics.

674

675 ***A new neuraminidase inhibitor - Peramivir***

676 Both zanamivir and oseltamivir have been useful in the management of influenza viruses even
677 amidst the challenges with resistance. Peramivir (also known as, Rapivab) [Figure 8] is another
678 drug that was developed based on the rationally designed previous drugs. But this would
679 subsequently be administered intravenously to exert an almost similar or better efficacy against
680 influenza A and B viruses and that warranted the FDA approval in 2014 (Hata, Akashi-Ueda,
681 Takamatsu, & Matsumura, 2014).

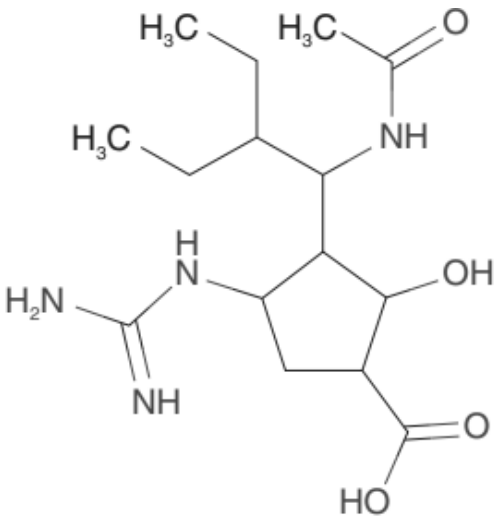


Image adopted from McLaughlin, Skoglund, & Ison (2015)

Figure 8: Structure of peramivir

Influenza viruses

Influenza is a contagious disease caused by orthomyxoviruses that comprise the genera influenza- A, B, C, and D -viruses. Influenza presents mostly as an acute upper respiratory tract infection that exerts significantly nearly a million morbidities, plus about half-a-million mortalities, all-year-round (WHO, 2014; Bouvier & Palese, 2008; Kimura et al., 1997; Su, Fu, Li, Kerlin, & Veit, 2017). Broadly, influenza viruses are known to infect both mammals (human, swine, canine, equine, etc.) and avian (such as ducks, chickens, etc.). Influenza A viruses (IAV) mainly exhibit a broad host range -infecting both mammals and avian; and each of Influenza B, C, and D viruses by far infect at least one mammal. As indicated, IAVs (modelled in Figure 9A) are of tremendous public health importance as they have been the main focal viruses responsible for all historical pandemics and many other epidemics recorded among both mammals and avian (Glezen & Couch, 1997; Stuart-Harris & Schild, 1976). Nevertheless, influenza caused by the influenza B viruses (IBV) also poses a significant burden

699 on public health systems (Paul Glezen, Schmier, Kuehn, Ryan, & Oxford, 2013). Hence, the
700 categorization of seasonal influenza viruses comprises both the IAVs and IBVs that mainly
701 infect humans. Two main influenza A virus subtypes that are currently co-circulating globally
702 are the A(H1N1) pdm09 and the A(H3N2), whereas two lineages of the IBVs (i.e., Yamagata
703 and Victoria) are equally co-circulating (Rota et al., 1990).

704 Cases of influenza establishment have been recorded in all ages of individuals (Dosseh, Ndiaye,
705 Spiegel, Sagna, & Mathiot, 2000), however, the high-risk population encompasses persons
706 with compromised immunity due to persisting chronic diseases, children less than 5 years,
707 pregnant women, caretakers of old people's homes, and the aged (Cheng, To, Tse, Hung, &
708 Yuen, 2012; Harper, Fukuda, Uyeki, Cox, & Bridges, 2005; Nair et al., 2011; W. W. Thompson
709 et al., 2009). Influenza has been managed mainly with antivirals and vaccines: common
710 antivirals being the neuraminidase inhibitors and the matrix 2 protein (ion channel) inhibitors
711 (Gubareva, Kaiser, & Hayden, 2000; McKimm-Breschkin, 2002; Van Voris, Betts, Hayden,
712 Christmas, & Douglas, 1981). The use of vaccines has also been relied on as both a preventive
713 and severity reduction measure. However, these antivirals exhibit either time-restricted
714 efficacy – must be administered within a very narrow period (about 48 hours) between onset
715 and diagnosis, or strain-type restricted efficacy - works against only molecularly susceptible
716 strains (Gubareva et al., 2000; McKimm-Breschkin, 2002). Matrix protein 2 inhibitor class of
717 compounds, also known as adamantanes has been eliminated due to both drug-associated side
718 effects and extensive resistance to seasonal IAVs (Deyde et al., 2007). Therefore, more-or-less
719 rapid emergency warrants for many other drug classes such as the influenza virus polymerase
720 inhibitors (i.e., Favipiravir, Baloxavir marboxil, and Pimodivir), the viral glycoprotein
721 haemagglutinin inhibitors (e.g., Arbidol and Nitazoxanide), the host-targeting sialidase (Das
722 181), and the inhibition of viral release due to drug interference with the influenza viral
723 nucleoprotein (e.g., Ingavirin) (Kotey et al., 2019). The use of conventional vaccines have also

had a major impact on the control of influenza (Pebody et al., 2018; Pepin et al., 2019; Sah, Medlock, Fitzpatrick, Singer, & Galvani, 2018; Shim et al., 2018); however, its suboptimal efficacy is a challenge that has been closely associated with "antigenic imprinting", waning of immunity, vaccine efficacy decline due to repeated vaccination and antigenic-drift or-shift driven mismatch (Belongia et al., 2015; Ferdinands et al., 2017; Gostic, Ambrose, Worobey, & Lloyd-Smith, 2016; Paules, Marston, Eisinger, Baltimore, & Fauci, 2017; Ramsay et al., 2019; Shim et al., 2018; Yewdell, Webster, & Gerhard, 1979). Given that influenza is a global burden and the active influenza vaccination platforms are currently limiting, it is important to explore unconventional vaccine development approaches.

Influenza contraction, viral transmission, and pathogenesis

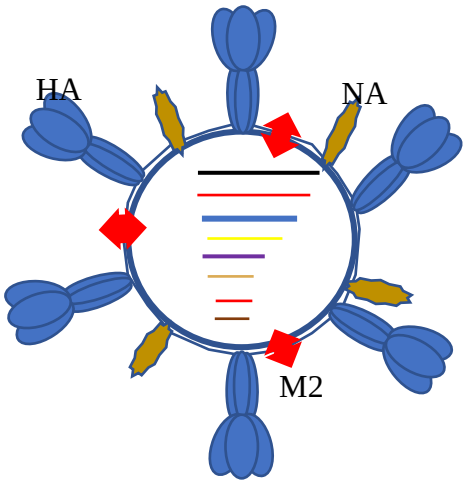
Influenza is established when any of the viruses infect mucosal surfaces on the respiratory airways, due to aerosol-transmission from an infected individual to a non-infected individual. Notably, wild birds are the reservoirs of the avian IAVs and are responsible for virus shedding throughout the globe, via both air- and waterways (Stallknecht & Brown, 2016). Seasonal influenza viruses, especially, the IAVs on the other hand, have been reported to persist due to both local and external seeding of epidemic strains (Nelson & Holmes, 2007; Nelson, Simonsen, Viboud, Miller, & Holmes, 2007; Nelson et al., 2006; Russell et al., 2008a, 2008b)

The viruses are characterized by glycoproteins -the Haemagglutinins - that both define their host specificity and partly play pathogenic roles: in that, a model influenza virus contacts a specific host by the interaction of its haemagglutinins (HA) with specific sialylated receptors on cells lining the airways (Klenk & Rott, 1988). Subsequently, aided by another surface glycoprotein - the Neuraminidase- virions replicate from the initial virus are dispersed to infect neighbouring cells (Liu, Eichelberger, Compans, & Air, 1995).

The influenza viruses are highly infectious and require a relatively small amount of inoculum to establish an infection in an exposed susceptible individual, with symptoms acutely manifesting within 3 days post-contraction. Affected individuals largely present with obstructed upper respiratory airways, coughs, high fever, fatigue, and myalgia amongst many other symptoms. Symptoms are estimated to prevail for up to about a week, but the situation may worsen in certain instances where affected persons fall under the high-risk category of persons (Cheng et al., 2012).

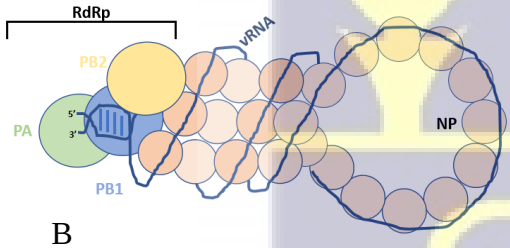
Influenza virus structure and genome organization

A model influenza A virus (Figure 1a) is either a spherical or filamentous particle with a size between 80 and 120 nm in diameter (Lamb & Parks, 2007; Noda et al., 2006). The structure takes shape due to reinforcement of the outer viral lipid envelope by the matrix protein 1 (M1). Embedded within the envelope is the HA, NA, and matrix protein 2 (M2) in declining order of abundance (Nayak, Balogun, Yamada, Zhou, & Barman, 2009). Within the core of the M1 are eight ribonucleoprotein complexes, each of which is made up of three polymerase subunits [i.e., Polymerase basic 1 and 2 (PB1 and PB2) and Polymerase acidic (PA)] and nucleoproteins (NP), all of which are closely associated with a negative-sense RNA segment (Area et al., 2004). An RNP particle assumes the structure of a returning twisted rod that has further been coiled to form a helical structure (Compans, Content, & Duesberg, 1972) (Figure 9B) and by function, is responsible for the transcription and overall replication of the influenza virus (Coloma et al., 2009). The size of the RNA segments is estimated to fall within 890 and 2,341 nucleotides in length (Lamb & Parks, 2007; Noda et al., 2006). More importantly, each component of the RNP is a prerequisite for a virus to thrive, in terms of infecting, replicating, and re-infecting neighbouring cells.



A

Image adapted and modified from Kotey et al. (2019)



B

Figure 9: Structural description of IAV.

A: Basic structure of IAV; B: Ribonucleoprotein complex

Life cycle of influenza A virus

Using the HA, the virus first makes contact and adsorb to a susceptible cell via interaction with sialylated receptors, which triggers the mechanism of endocytosis (Wenjie Zheng & Tao, 2013). Endosomal maturation by acidification is an immune process that aids the destruction of endocytosed pathogens; however, this is favourable for the influenza virus, in that the M2 -

an ion channel- conducts protons which leads to the activation of the fusion domain of the HA and subsequently facilitating fusion of the viral envelope with the endosomal membrane, thereby dissociating the vRNPs from the M1 (Das, Aramini, Ma, Krug, & Arnold, 2010). Viral RNPs (vRNPs) are then released and shuttled directly into the nucleus due to the recognition of nuclear localization signals (NLSs) on the nucleoproteins (Wu & Panté, 2009). Within the nucleus, two main viral replication pathways occur: in one pathway, the viral polymerase complex initiates the synthesis of a positive-sense strand complementary RNA (+cRNA) for each of the 8 negative-sense strand viral genomic segments, and these are required to further make more genomic segments (the viral RNP, vRNP) that get incorporated into the virions; the other pathway involves snatching the 5' cap of host pre-mRNA -aided by PB2 binding to the host 5' cap-(Ulmanen, Broni, & Krug, 1981) and PA using an endonuclease domain to cleave ~13 nucleotides away from the 5' cap (Plotch, Bouloy, Ulmanen, & Krug, 1981). The 5' cap with the 10-13 nucleotides act to prime the negative-sense stranded viral RNA for the viral polymerase to transcribe it to a positive-sense mRNA (+mRNA) -that doesn't require any 3' end processing, as in the case of the host mRNA- and gets shuttled into the cytoplasm for translation into viral proteins; the surface glycoproteins (i.e. HA, NA, and the M2) are processed in the endoplasmic reticulum, further glycosylated in the Golgi apparatus and transported to the surface of the host cell membrane (Hagen, Chung, Butcher, & Krystal, 1994; Plotch et al., 1981).

The non-structural protein 2 (NS2) contains a nuclear export signal motif and it is a truncation of the non-structural protein (NS) plays a crucial role by forming a complex with the M1: this complex directly associates with the vRNPs and are shuttled from the nucleus into the cytoplasm by the aid of the human chromosome maintenance 1 protein (hCRM1) interacting with the NS2 (also known as the Nuclear export protein, NEP) of the tripartite complex (Gabriele Neumann, Hughes, & Kawaoka, 2000).

Interestingly, the non-structural protein 1 (NS1) plays a critical role in the success of virus replication by inhibiting the process of 3' host pre-mRNA end processing and thereby suppressing or blocking host mRNA synthesis leading to a biased protein production from the viral +mRNA (Nemeroff, Barabino, Li, Keller, & Krug, 1998; Shimizu, Iguchi, Gomyou, & Ono, 1999). The blockage of host mRNA production inadvertently affects immune antiviral molecules such as the interferon ß (IFN ß) (Das et al., 2008).

vRNP complexes recruited by the M1-NS2 complex approaches the plasma membrane where the modified surface glycoproteins have been shuttled and subsequently through a complex assembly process as reviewed by Nayak, Hui, & Barman (2004), which culminates in bud formation and viral dissemination aided by the NA cleavage of virions from the host cell (Murphy & Webster, 1996; Wilson & Itzstein, 2003) [Figure 10].

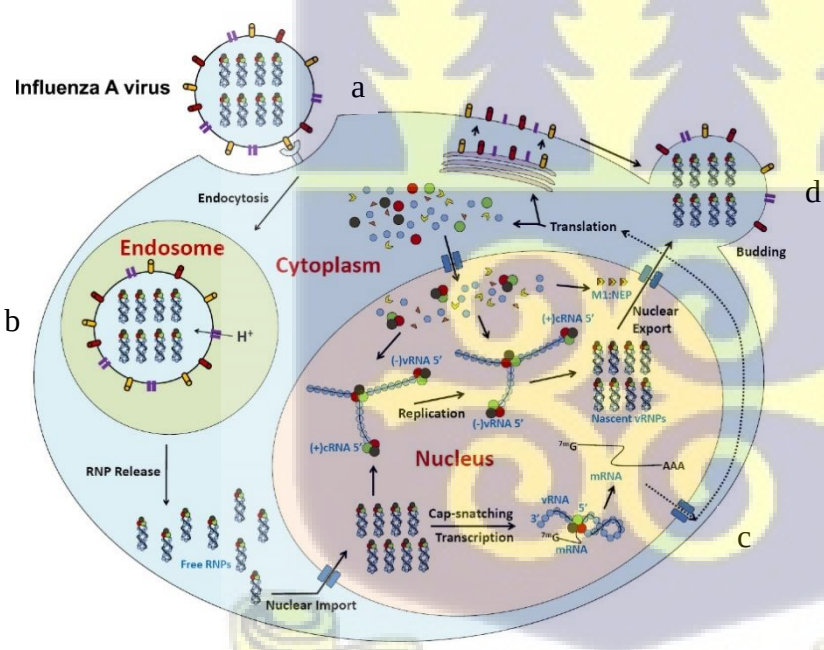


Image adopted from Zheng & Tao (2013)

Figure 10: IAV replication cycle.

An elaboration on the replication process of the IAV including virus attachment and endocytosis (a), acidification of virus-trapped endosome and vRNP release and shuttle into the host nucleus (b), production of viral mRNA -

for viral protein production in the cytoplasm- and production of -vRNA for packaging into viral genomic RNP (c). Assemblage and budding of virions (d).

Influenza A virus haemagglutinin, the focus of many vaccine platforms

Nature of the influenza HA

Influenza virus haemagglutinins are the dominant glycoproteins on the surface of the virus and play a crucial role in the determination of the success of establishing an infection in a host. Most importantly, an infectious virus with a full-length and functional haemagglutinin protein recognizes and "sticks" to a susceptible cell, such as cells lining the epithelium of the naso-/oro-pharynx or those of the lungs. As the haemagglutinin is itself a protein that interacts with specific sialic acids (which are carbohydrate moieties), they are biochemically termed as agglutinins. Also, experimentally, these agglutinins can agglutinate the haems of certain red cells bearing recognizable sialic acids; they were termed haemagglutinins (Gottschalk, 1957; Hirst, 1941).

HA Structure and sialic acid preference.

The functional haemagglutinin is a homotrimer (Figure 11) with each of the monomers assuming a shape consisting of a "globular head" and "stem regions" (Wilson et al., 1981). A monomeric HA is synthesized as a precursor molecule, HA_0, whose activation is by the action of host proteases. Activation is by cleavage of a serine-sensitive domain that leads to the release of HA_1 and HA_2, constituting the globular head region and the stem, respectively (Skehel & Wiley, 2000). This process of activation also paves the way for viral membrane fusion with the host endosomal membrane during an infection and the subsequent replication of the virus. The mature HA is also characterized as comprising multiple glycosylation sites that play major roles in the thrive of the viral (Skehel et al., 1984).

The establishment of infection by influenza viruses is dependent on two means in a sequel: the presentation of the receptor-binding site (RBS) on the haemagglutinin and contacting of a specific sialylated receptor on the respiratory airway (John J Skehel & Wiley, 2000). A virus-adsorbed cell then engulfs the virus (by endocytosis) and by a pH-dependent mechanism, the virus fuses its membrane with the endosomal membrane, and then subsequently results in the release of the vRNPs that gets shuttled into the host cell nucleus for the initiation of viral replication (Chu & Whittaker, 2004; Stegmann, Morselt, Scholma, & Wilschut, 1987; Yoshimura & Ohnishi, 1984).

Adapted from Graaf & Fouchier (2014)

Figure 11: Structure of a Vietnamese mutant influenza A H5 HA.

A typical structure of the influenza A virus HA trimer showing 3 important domains: the receptor-binding domain/ site (RBS), vestigial esterase subdomain, and membrane fusion.

Virus HA-dependent tropism

Based on haemagglutination experiments, it has been observed that human and avian respiratory or blood cells present different conformations of sialic acids, affecting the preferences or specificities of the influenza viruses (Rogers & Paulson, 1983). Human-infecting influenza A viruses, such as those of subtypes H1, H2, and H3 have been shown known to exhibit preferential binding for receptors possessing α2,6- sialylated receptors, whereas the avian viruses prefer α2,3- sialylated receptors (Gambaryan et al., 1997; Nobusawa et al., 1991; Rogers & Paulson, 1983). Pig-infecting influenza A viruses are known to possess a preference for both α2,3-/ α2,6-sialylated receptors, or mainly the α2,6- sialylated receptors. The viral receptor preferences are predominantly due to both the distribution and abundance of the variable sialic acids in the airways of the organisms they infect, and this is crucial for tropism and host-range and perhaps, reassortant generation (as in the case of pigs that can accommodate different viruses) (Costa et al., 2012; de Graaf & Fouchier, 2014; Nelli et al., 2010; Nicholls, Bourne, Chen, Guan, & Peiris, 2007; Q. Xu, Wang, Cheng, Zengel, & Jin, 2010).

Characterization of influenza A virus HA

Structural descriptions of influenza HA

On the virus, the HA molecules assume a homotrimeric structure, comprising three monomeric HAs (I. A. Wilson et al., 1981). Each monomeric HA comprises two main functionally active sections: HA1 and HA2, both linked up by two disulphide bridges (Boonstra et al., 2018; Di Lella, Herrmann, & Mair, 2016). The HA1 mostly composed of anti-parallel beta-sheets, whereas the HA2 comprises of 3 alpha-helices joined end-to-end by loops to form coiled coils (Boonstra et al., 2018); The HA1 region, making up the globular head, plays the host-cell

attachment role whereas the membrane fusion role is accorded to the HA2 (Di Lella et al., 2016).

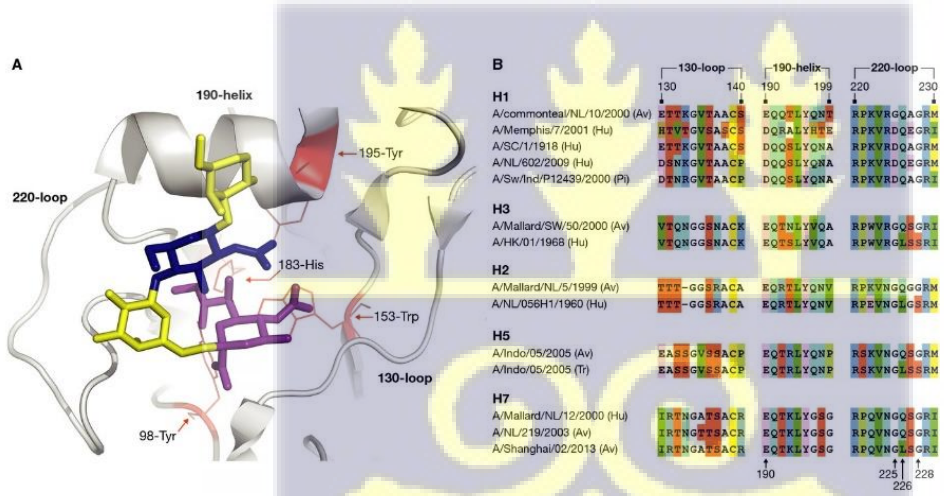
Subtypes of influenza A viruses

To date, 18 HA and 11 NA subtypes are known. Subtypes H1, H2, and H3 have been identified as infecting humans and other mammals, with the currently circulating seasonal human influenza viruses being the H1pdm09 and the H3. All except the bat influenza viruses' subtypes H17N10 and H18N11 are found in avian (Fouchier et al., 2005; Tong et al., 2012; Tong et al., 2013; Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). Interestingly, these bat viruses rather require the presence of MHC II as contact molecules before establishing infection (Karakus et al., 2019).

Typical sequence conservations in the HA of influenza A viruses

Even though influenza A viruses are rapidly evolving, there remain some key structural or genetic signatures that are relatively conserved (de Graaf & Fouchier, 2014). These conserved sites are critical, and they associate with the functional constellation of viral HA RBS (Figure 12), warranting exploitation in the development of appropriate interventions. If, for instance, conformational antibodies that potentially inhibit influenza A virus replication due to the recognition of these conserved loops or helical regions, then a revolutionary tool for the control of influenza would have been attained. However, antibodies found so far, do not interact with such loops or helical regions, and those that recognize any other conserved regions at all, result in poor antibody production, due to less frequent B-cell responses (Corti et al., 2010). For that matter, immunogens required to induce a broadly-neutralizing repertoire of specific antibody-producing B-cells against the relatively conserved regions of the HA are still being investigated (Berry, Penhale, & Sangster, 2014). As described, seasonal influenza vaccine components against the influenza A viruses, for instance, would typically induce antibodies targeting the

relatively variable globular head region of the haemagglutinin. This phenomenon is pivotal to vaccine efficacy decline against mismatching viruses (Berry et al., 2014). The anti-HA stalk antibodies are critically important, as the stalk domain remains relatively stable due to the crucial purposes of membrane fusion events that are paramount to the release of vRNPs into host cells (H. Zhang, Wang, Compans, & Wang, 2014). Inhibition of the stalk with anti-stalk antibodies has the advantage of inhibiting a broader scope of influenza A viruses, but the challenge being sub-immunodominance, and therefore requiring the development of appropriate adjuvants that could augment anti-HA stalk-specific B-cell induction (Berry et al., 2014).



Adopted from Graaf and Fouchier, 2014 (de Graaf and Fouchier, 2014)

Figure 12: Conserved sequence structure of selected influenza A viruses' RBS.

Here is a typical constellation (A) and conservation (B) of amino acids that form the loops/ helix of the influenza A viruses' RBS.

Onward fight against influenza

Several advancements are being put in place to better prevent or delay the occurrence of the next influenza pandemic. Novel and more potent antivirals are key to initiate a fight against any possible future catastrophes. Broadly acting influenza vaccines are more preferred to avert any forecasted catastrophes. Considering the kind of vaccine desired, other potentially encouraging platforms, such as the generation of broadly neutralizing antibodies, and their usage as a therapeutic intervention as well as exploring other unconventional vaccine development approaches may be useful for enhancing efficacy and reactivity of influenza vaccines.

CHAPTER 3: CURRENT AND NOVEL APPROACHES IN INFLUENZA MANAGEMENT

Chapter 3 was published as a review in the journal "Vaccines" in 2019. Kotey et al., 2019.

Abstract

Influenza is a disease that poses a significant health burden worldwide. Vaccination is the best way to prevent influenza virus infections. However, conventional vaccines are only effective for a short period due to the propensity of influenza viruses to undergo antigenic drift and antigenic shift. The efficacy of these vaccines is uncertain from year-to-year due to potential mismatch between the circulating viruses and vaccine strains, and mutations arising due to egg adaptation. Subsequently, the inability to store these vaccines long-term and vaccine shortages are challenges that need to be overcome. Conventional vaccines also have variable efficacies for certain populations, including the young, old, and immunocompromised. This warrants diverse efficacious vaccine developmental approaches, involving both active and passive immunization. As opposed to active immunization platforms (requiring the use of whole or portions of pathogens as vaccines), the rapidly developing passive immunization involves the administration of either pathogen-specific or broadly acting antibodies against a kind or class of pathogens as a treatment to corresponding acute infection. Several antibodies with broadly acting capacities have been discovered that may serve as means to suppress influenza viral infection and allow the process of natural immunity to engage opsonized pathogens whilst boosting the immune system by antibody-dependent mechanisms that bridge the innate and adaptive arms. By that, the passive immunotherapeutics approach assumes a robust tool that could aid control of influenza viruses. In this review, we comment on some improvements in influenza management and promising vaccine development platforms; with emphasis on the

protective capacity of passive immunotherapeutics especially when coupled with the use of antivirals in the management of influenza infection.

Introduction

Influenza viruses are highly contagious pathogens that are associated with a year-round global record reaching nearly a million morbidities and half-a-million mortalities. Four types of influenza viruses (i.e., A, B, C, and D) have been identified. Influenza viruses C (isolated in pigs and humans) and D (isolated from cattle) are less common: typically, influenza virus C is associated with less severe illness (Kimura et al., 1997; Su et al., 2017). On the other hand, influenza viruses A (infecting avian and mammals including human) and B (almost exclusively infecting humans and seals) account for the annual global burden of influenza (Hinshaw, Webster, Easterday, & Bean, 1981; Osterhaus, Rimmelzwaan, Martina, Bestebroer, & Fouchier, 2000). The persistence of influenza viruses A and B has been attributed to their ability to evolve rapidly. Antigenic variabilities are also common with influenza viruses A and B, and these are partly a result of a phenomenon called the antigenic drift, referring to amino acid changes that allow viral escape from neutralizing antibodies (Gerhard & Webster, 1978; Yewdell et al., 1979). Such immune-escape mutants often tend to have a higher host-cell avidity (compared to the wild-type virus) in exposed or vaccinated host and vice-versa, in naïve host (Hensley et al., 2009). Studies by Fergusson *et al* revealed that antigenic drifts in seasonal influenza viruses (H3, H1, and B) were estimated at fixation rates of 0.0037, 0.0018, and 0.0013 nucleotide substitutions per site per year (±0.001) respectively (Ferguson, Galvani, & Bush, 2003). This supports the idea that antigenic drifts occur more frequently in influenza A viruses than influenza B viruses. Also, high mutation rates cause a tremendous impact on the efficacy of the seasonal influenza vaccines which comprise forecasted strains (Carrat & Flahault, 2007). For instance, gain or loss of N-linked glycosylation sites in the haemagglutinin

can also participate in the antigenic drift: Skehel et al. (1984) showed that a single D63N substitution in HA1 created a novel N-glycosylation site that allowed an antigenic variant of an H3N2 to escape neutralization by a monoclonal antibody. In the same study, the authors further observed that the 1968 influenza epidemic strain (A/VIC/3/75) that had N63 (known glycosylation site), was also recognized (when un-glycosylated) by antibodies raised against viruses of two earlier epidemics. As illustrated, altering glycosylation patterns is one of the means used by viruses that result in the potential cause of vaccine failure.

The antigenic shift also allows influenza viruses to escape pre-existing immunity (Webster & Govorkova, 2014). This mechanism is reliant on the ability of the eight genomic fragments of influenza viruses to reassort with genomes of other influenza viral subtypes. It occurs when two or more of these distinct viruses infect a common host and generate novel viral subtypes or strains (Gething, Bye, Skehel, & Waterfield, 1980; Webster & Govorkova, 2014). Thus, antigenic shifts (principally underlying influenza A virus pandemics) and antigenic drifts (underlying vaccine mismatches against seasonal influenza A and B viruses) and a wide host-range (for influenza A viruses) all contribute to the recurring cases of influenza all year round (Donatelli, Castrucci, De Marco, Delogu, & Webster, 2016; Zambon, 1999). Furthermore, antigenic drifts and shifts are reasons why there is an immediate need for highly efficacious intervention. We review here vital influenza management strategies, novel vaccine, and antiviral development approach with deliberation on those with prospects.

Current influenza vaccines

Three types of vaccines against influenza are currently used worldwide including inactivated influenza vaccine (IIV), live–attenuated influenza vaccine (LAIV), and influenza virus subunit vaccine: each of which has its advantages and drawbacks. IIV is formulated with a replication-incompetent virus, due to whole pathogen inactivation usually achieved by formaldehyde

treatment or split virion vaccines generated by disruption of the viral membrane. Intramuscular administration of the IIV has been shown to induce both local and systemic immunity (Hoft et al., 2017). However, to maintain the antibody titers, booster vaccinations are required. Additional considerations on the vaccine efficacy were raised following metadata analysis suggesting only 40% of children being protected against influenza, with the percentages going a bit higher up to 65% for the adults (Osterholm, Kelley, Sommer, & Belongia, 2012; Shinjoh et al., 2018). LAIV comprise reassortant viruses generated from cold-adapted donor viruses (that contribute their internal genes) and identified virulent circulating strains of viruses [that contribute their HA and neuraminidase (NA)] as recommended by the WHO. Cold-adapted donor viruses are raised by several passages in embryonated chicken eggs with a gradual reduction in temperature during every round of passage. By this process, reassortants viruses that comprise the LAIV can grow at 32-33°C, the temperature range of cells lining the mucosal surfaces of the nasopharynx, when administered intranasally (Beyer, Palache, De Jong, & Osterhaus, 2002). Replication of LAIV viruses in the nasopharynx elicits an immune response that epitomizes a natural influenza infection. For this reason, LAIV has shown some superiority over the IIV in terms of the induction of mucosal immunity via secreted immunoglobulin A (Beyer et al., 2002). Use of the LAIV has proven to be safe in children (15 to 71 months) and immunocompromised persons (HIV-infected, chronic bronchitis, and cystic fibrosis) (Belshe et al., 1998; Keitel, Couch, Cate, & Maassab, 1994; King et al., 2000). The most spelt-out advantage is the "non-invasive" capacity of the attenuated viruses, and this had made it suitable to use for all categories of vaccinees, although LAIVs are not recommended for people with underlying chronic medical conditions (Galazka, Lauer, Henderson, & Keja, 1984). A typical setback to the use of the LAIV is the possibility of the attenuated virus undergoing some genetic modifications and consequently reverting to virulence, a case which has not been reported for the LAIV (Hensley et al., 2009). Furthermore, since vaccine viruses are grown in eggs, there

have been several concerns about allergic reactions among certain vaccinees: whereas the LAIV ovalbumin contents (responsible for the vaccine allergies) are variable, other studies have shown that the IIV contains a tolerable ovalbumin content of about 0.7 µg/ mL (Kelso et al., 2012; Li, Rank, Squillace, & Kita, 2010; Owens & MacGinnitie, 2011). The development of the subunit influenza vaccines, which often comprise influenza virus HA that has been purified following protein expression in cells, could be a means to avoid adverse reactions in people with egg allergies (Grohskopf et al., 2014). Besides, the subunit vaccine also offers desirable protection against seasonal influenza viruses; but its downside being a higher dosage requirement at multiple times for full potentiation of immune protection comparable to that elicited by whole-virus vaccines (Stephenson et al., 2003).

It is worth noting that both the LAIV and IIV are cocktails of circulating seasonal influenza viruses. Mainly three viruses i.e. A (H1N1) pdm09, A (H3N2), and the pre-determined dominant influenza B lineage (whether Yamagata or Victoria) are the constituents of the seasonal trivalent influenza vaccines (TIV). Subunit vaccines are also formulated as TIV, containing the HA of all representative vaccine strains (Giezeman, Nauta, De Bruijn, & Palache, 2009). However, it became necessary to feature both lineages of influenza B viruses based on the current global epidemiology of influenza as recommended by Ambrose and Levin in 2012. This resulted in the advancement of quadrivalent influenza vaccines (QIV) containing the pre-determined representatives of both Yamagata and Victoria influenza B virus lineages, in addition to the two pre-determined circulating seasonal influenza A subtypes (Ambrose & Levin, 2012). This approach of vaccine preparation thus requires a constant reformulation to maintain desirable efficacy limits during an influenza season (Ambrose & Levin, 2012; Kumar, Meldgaard, & Bertholet, 2018).

Use of the seasonal influenza vaccines.

Due to the weight of the burden of influenza, the US CDC advocates the use of seasonal influenza in all persons > 6 months before the winter (Fiore et al., 2010). On the other hand, the WHO extends recommendations for the use of influenza vaccination in persons categorized as high-risk, which comprises children > 6 months, persons with chronic diseases, pregnant women, health care, and nursing workers (Organization, 2011). However, in some parts of the world, mostly Africa and Asia, there is either limited or no established influenza vaccination policies. Thus, the restricted availability of influenza vaccines makes vaccinations quite uncommon to these populations. Perhaps, such vaccination policies might not have been considered due to the cost of acquiring vaccines annually or still, the reduced efficacy of the influenza vaccines, as have been critically assessed by Xu et al. (2017) where recommendations have been made for twice-dose vaccination due to frequencies of seasonal influenza occurrences, all year round. Although poor vaccine coverage in African countries was previously reported by Duque *et al* upon investigation on the availability of seasonal influenza vaccines, there are still no clearly underpinned core reasons (Duque, McMorrow, & Cohen, 2014). Therefore, the improved efficacy of influenza vaccines would also contribute to enhanced vaccine coverage in Africa and Asia.

Novel influenza vaccine platforms

Rapid influenza virus evolution and yearly vaccine reformulations make the stockpiling of vaccines for the future use a complicated issue. This subsequently delays preparation against any unforeseen epidemics. Therefore, lots of research now focuses on the development of novel broadly protective vaccine platforms, with hopes of enhancing both immunogen delivery and consequent immune response to select antigens. Some of these platforms include virus-like particle vaccines (VLP), synthetic virus vaccines, epitope vaccines, antigen-presenting cell

inducible vaccines, COBRA vaccines, nanoparticle-based vaccines, and viral-vectored vaccines (Table1).

1093 **Table 1: Summary of novel influenza virus vaccine platforms.**

Vaccines	Design	References
VLPs	Self-assembling viral matrices that express a single or multivalent viral surface protein	(Gao et al., 2013; Grgacic & Anderson, 2006; Kapczynski et al., 2016; Mohan et al., 2017; Roldão et al., 2010; Wang et al., 2008; Q. Zhao et al., 2013)
COBRA	VLPs bearing computationally optimized viral surface proteins	(Carter et al., 2016; Crevar et al., 2015; Giles et al., 2012; Giles & Ross, 2011; Wong et al., 2017)
Synthetic virus	Generation of replication-incompetent viruses bearing genetically attenuated genomic sequences	(Baz et al., 2015; Fan et al., 2015; Holzer et al., 2018; Mössler et al., 2013; Nachbagauer, Liu, et al., 2017b; Osterholm et al., 2012; Pica et al., 2012)
Epitope	Epitope-rich proteins of viruses, designed to induce protective epitope targeted antibodies	(Atsmon et al., 2012; Correia et al., 2014; Impagliazzo et al., 2015; McLellan et al., 2013; Thompson et al., 2018)
APC inducible	APC-targeted delivery of immunogenic viral proteins to induce quicker and T cell responses	(Abdel-Motal et al., 2007; Fonteneau et al., 2003; Grødeland et al., 2013)
Nanoparticle-based	Self-assembling nano-molecules that carry a single or	(Chahal et al., 2016; Deng et al., 2015; Deng et al., 2018; Harding & Heaton, 2018; Hiremath et

	multivalent viral surface protein	al., 2016; Kanekiyo et al., 2013; Tao & Gill, 2015)
Viral-vectored	Mainly involves the use of dissimilar viral matrices as carriers of specific viral protein	(Antrobus et al., 2014; Berthoud et al., 2011; Draper et al., 2008; Kim et al., 2017; Lingel et al., 2017; Tripp & Tompkins, 2014)

Virus-like particle (VLP) vaccines

Virus-like particles are non-infectious multimers of viral surface glycoproteins that have the propensity to self-assemble (Grgacic & Anderson, 2006). VLPs are designed to maintain their native viral structure but without their complete set of genetic materials. A typical influenza VLP has the HA, NA, and matrix protein 1 (M1). Typically, plasmid constructs of the HA, NA, and M1 are used to transfect cells: resulting in the formation of the capsid displaying surface proteins HA and NA (Roldão, Mellado, Castilho, Carrondo, & Alves, 2010). VLPs have been proposed to be efficient vaccines against a range of viruses including human papillomavirus (HPV) and hepatitis B virus (HBV) or hepatitis E virus (HEV) as discussed by Zhao et al. (2013). Although these examples have completely different "biologies" when compared with the influenza virus, rapid advances and the need for a new vaccine platform are generating some promising data for influenza VLPs. For instance, to overcome challenges raised by rapid influenza evolution Gao et al. (2013) attempted to generate VLPs with HBV backbone containing matrix protein 2 ectodomain (M2e) together with the epitope of highly conserved nucleoprotein (NP). Mice immunization with chimeric VLPs induced humoral as well as cell-mediated immunity and resulted in cross-protection against several strains of the virus (Kapczynski et al., 2016). Another approach for the generation of VLPs is via a combination of distinct HAs. Such technique has been described by Kapczynski et al. (2016) who upon co-

expression of three different clade H5 HAs, a single NA protein and retroviral gag protein managed to generate triple-clade VLPs that were shown to protect chickens against lethal challenge. For heightened immunity (involving both innate and adaptive), VLPs may be adjuvanted with various Toll-like receptor (TLR) ligands, as demonstrated with the modified Salmonella flagellin acting as a TLR5 ligand described by Wang et al. (2008) which resulted in highly specific immunoglobulin response. Also, a GPI-anchored CCL-28 that was incorporated into the VLPs boosted IgA secreting cell migration, which increased murine mucosal immunity to both drifted and homologous influenza A (H3N2) viruses, as well as the longevity of protection (Mohan et al., 2017). VLPs thus provide a platform for improved formulation of multivalent (containing heterologous epitopes) influenza vaccine.

COBRA vaccines

Computationally optimized broadly reactive antigen vaccines (COBRA) comprise VLPs that carry a computationally designed HA. Ted Ross' group first generated consensus amino acid HA sequences of clade 2 highly pathogenic A (H5) involved in human infections and formulated VLPs to express this HA (Giles & Ross, 2011). H5 COBRA VLPs potently induced HA-neutralizing antibodies, which provided efficient protection of both immunized mice and ferrets in a pathogenic H5N1 challenge experiment (Giles & Ross, 2011). A similar approach also demonstrated the protection of cynomolgus macaques (Giles, Bissel, DeAlmeida, Wiley, & Ross, 2012). The ability of the COBRA VLPs has since been demonstrated as a powerful system that induces a strong broadly neutralizing antibody response against multiple clades of H5N1 of viruses and multiple isolates of H1N1 viruses (Carter et al., 2016; Crevar, Carter, Lee, & Ross, 2015). Similarly developed H1N1 and H3N2 COBRA vaccines have also been shown to induce broadly neutralizing antibodies in either mice or ferrets, against a broad spectrum of H1N1 and H3N2 viruses, respectively (Carter et al., 2016; Wong et al., 2017).

Synthetic influenza virus vaccines

Several approaches have been tried to generate attenuated viruses using reverse genetics technology. A suggested technique to downregulate viral protein synthesis is via biased virus codon sequences. Average codon frequency alteration can result in attenuation of the virus in mice models, as shown by Fan et al. (2015) suggestive that the avian codon–biased vaccine candidate that was fitter in eggs, is good news for the generation of influenza vaccines in eggs. Alternatively, replication-incompetent viruses can be generated upon truncation or knockdown of non-structural viral protein 1 (NS1) - a notable inhibitor of the host-protective interferon-induced immunity (Osterholm et al., 2012). This has led to a phase I/ II clinical trial of a trivalent vaccine which revealed the protection of vaccinees against the seasonal influenza viruses (Mössler et al., 2013; Pica, Langlois, Krammer, Margine, & Palese, 2012). This platform has also paved the way for the generation of single cycle replicating influenza viruses as vaccine candidates and has shown similar or higher protection than the conventional LAIV (Baz et al., 2015; Holzer et al., 2018; Nachbagauer, Liu, et al., 2017b). The main reason synthetic influenza vaccines remain promising is due to their ability to alter viral immunomodulatory traits and their amenability to the rapid production of vaccines.

Epitope vaccines

Epitope-based vaccines can serve both the immune refocusing role as well as targeting integral virus-specific epitopes. Remarkable work inspired by the stabilization of respiratory syncytial virus fusion protein (F) has shown that the relatively conserved HA stem could induce broadly neutralizing antibodies that are protective in mice and non-human primates against several virus subtypes harbouring group I HAs (H1, H2, H5, and H9) and group 2 HAs (H3 and H7) (Correia et al., 2014; Impagliazzo et al., 2015; McLellan et al., 2013). To characterize an epitope of limited variability that is located on the HA head, Thompson et al. (2018) observed

that sera collected from young children during a pandemic revealed a cross-reactivity pattern to historical influenza A (H1N1) isolates. A conserved epitope situated on the HA head was confirmed further, demonstrating the protective capacity of this epitope in a murine challenge against diverse strains of the influenza A (H1N1). Another universal vaccine candidate currently undergoing phase III clinical trials are based on *Escherichia coli*-expressed artificial recombinant protein consisting of the concatenation of nine linear epitopes (five of which are specific to HA; three, to NP and one, to M1) of several influenza virus strains, and this vaccine has been shown to induce both cellular and humoral immunity in mice and it is envisaged as able to overcome high virus mutation rates (Atsmon et al., 2012). The epitope-based vaccination, therefore, affords the direct involvement of B and T lymphocytes that are both required for effectual control of viruses during an infection.

Antigen-presenting cell (APC) inducible vaccines

Recently, more focus is drawn to increasing the abilities of antigen-presenting cells (APCs) to efficiently involve the T-cell arm of immunity to influenza virus clearance. One of the examples is the work of Fonteneau and colleagues who showed that after exposure to influenza virus, dendritic cells (DCs) (both $CD11c^+$ DCs and plasmacytoid DCs) induced an expansion of anti-influenza virus cytotoxic T lymphocytes (CTLs) and T helper 1 (TH1) $CD4^+$ T cells (Fonteneau et al., 2003). Inspired by the previous findings, Abdel-Motal et al. (2007) also demonstrated that grafting the alpha-Gal epitope onto HA promoted its opsonization thereby enhancing the uptake of the vaccine virus by APCs. Importantly, work by Grødelang et al. (2013) showed that it is possible to target the HA to different surface molecules on antigen-presenting cells and thereby orient the immune response towards either an antibody/Th2 response or a $CD8^+$/Th1 T cell response. There is a variety of approaches to target APCs

including antibody, nanoparticle, or ligand-mediated methods that emphasize APC inducible vaccine universality.

Nanoparticle-based influenza vaccines

Continued efforts to develop a universal influenza vaccine have driven the use of self-assembling monomeric ion-carrier molecules, called Ferritin for the administration of multivalent vaccine constructs. *In vivo* assessment of nanoparticle-based vaccines displaying multivalent HA from 8 diverse strains of H1N1 influenza A viruses, were shown to induce broadly protective antibodies in mice, whose protection spanned strains from 1918 through 2009. The breadth of protection by the nanoparticle-induced antibodies was also shown to be more profound in comparison to the individual components of the conventional multivalent vaccine (Kanekiyo et al., 2013). Tao and Gill (2015) also immobilized the matrix protein 2 extracellular domain (M2e) that resulted in increased induction of M2e-specific antibodies affording protection of mice challenged with a virulent strain of an influenza virus (Deng, Cho, Fiers, & Saelens, 2015; Tao & Gill, 2015). Intranasal administration of polylactic-co-glycolic acid (PLGA) nanoparticle conjugated to influenza A (H1N1) conserved peptides as a vaccine were also shown to induce protection in the lungs of pigs, via the induction of antigen-specific $CD4^+$ and $CD8^+$ T cells (Hiremath et al., 2016). A similar approach by Chahal et al. (2016) also demonstrated the induction of both $CD8^+$ and antibody responses in mice; this was separately challenged with either virus (i.e., H1N1 and Ebola) or a parasite (*Toxoplasma gondii*) after immunization with nanoparticle formulation that involved a single or combination of gene-specific RNAs encapsulated in a dendrimer. Recently, a double-layered protein nanoparticle developed using tandem expressed M2e (comprising human, avian, swine, and domestic fowl), with or without recombinant HA stalk proteins from H1 and H3, showed homosubtypic and heterosubtypic protection in mice that were immunized before challenging

with specific influenza A viruses (Deng et al., 2018). Though a promising influenza vaccine platform, high-throughput nanoparticle-based vaccines approaches that will facilitate the replacement of the seasonal influenza vaccines are still to be developed (Harding & Heaton, 2018).

Viral - vectored vaccines

Viral-vectored vaccines platforms are designed to mimic natural infections, in that viral molecules are displayed on either similar or dissimilar virus. This approach has been shown to involve both humoral and cellular immunity (Draper et al., 2008). Use of the modified vaccinia virus Ankara (MVA) as a vector, incorporating influenza NP and M1 proteins, has shown potent induction of both influenza virus-specific humoral and cell-mediated responses leading to the phase II clinical trial (Antrobus et al., 2014; Berthoud et al., 2011). An Adenovirus vectored influenza vaccine has equally shown a strong induction of influenza virus HA-stalk cross-reactive antibodies in mice (Kim, Han, & Nguyen, 2017). Recently, Lingel et al. (2017) expressed multivalent adenovirus vectored influenza virus vaccine (that comprise the consensus sequences of divergent H1, H2, H3, and H5 HAs) and demonstrated that low dose administration in mice conferred protection of mice in a lethal influenza virus challenge experiment. This provides an avenue for scaling up appropriate doses for later use in seasonal vaccine development efforts. The alphavirus, baculovirus, Newcastle disease virus, poxvirus, parainfluenza virus, and vesicular stomatitis virus, are other vectors that have also been proposed to be applicable in vectored vaccines (Tripp & Tompkins, 2014).

Current influenza managing antivirals

Management of ongoing influenza infection currently requires the use of antiviral drugs. Two drug classes approved for the control of influenza infections include the adamantanes (Amantadines and Rimantadines) and NA inhibitors (Oseltamivir, Zanamivir, Laninamivir, and Peramivir). Whereas Adamantanes target the ion channel M2, which is involved in the release of viral ribonucleoprotein complexes in the host cell, NA inhibitors act by competitively engaging viral NA protein that is otherwise responsible for newly generated virion dissemination (Gubareva et al., 2000; Ma et al., 2009; Pinto et al., 1997; Pinto, Holsinger, & Lamb, 1992). However, influenza viruses resistant to both the adamantanes and neuraminidase inhibitors have emerged rapidly (Aoki & Boivin, 2009; Bean, Threlkeld, & Webster, 1989; Deyde et al., 2007; Hussain, Galvin, Haw, Nutsford, & Husain, 2017; McKimm-Breschkin, 2002; Rameix-Welti, Enouf, Cuvelier, Jeannin, & van der Werf, 2008; Samson, Pizzorno, Abed, & Boivin, 2013; Van Voris et al., 1981). This shows a need for search of either novel antivirals or other viral or host targets, that can be used for the development of next-generation drugs (Noah & Noah, 2013). Additionally, lack of data suggesting antiviral efficacy against highly pathogenic avian influenza viruses (HPAI) i.e., H5N1 remains an important issue in areas with possible spillover events from avian into humans (Nguyen et al., 2013). It is imperative to note that there (Malakhov et al., 2006) is still NA inhibitor-sensitive influenza A viruses in circulation, and this drug class can be extremely helpful in the management of an outbreak or even, a pandemic in the absence of highly efficacious vaccines.

Novel influenza management therapies

Next-generation antivirals against influenza

The burden of influenza requires the identification of novel compounds that have the potential to alleviate the symptoms and reduce viral shedding. New research focuses are developing not only virus-targeting antivirals but also the ones that target the host organism (Table 2).

1262 **Table 2: Influenza antiviral drugs approved or in clinical trials.**

Antiviral	Mechanism of action	Clinical phase	Country of development/ trial
Das181 (Fludase)	Sialic acid removal in the respiratory airways	II (IFV), III (PIV) not yet recruiting	USA
Nitazoxanide	HA maturation inhibition	III completed	USA
JNJ-63623872 (Pimodivir)	Small molecule inhibitor of influenza A virus PB2	III recruiting	Belgium
T705 (Favipiravir)	RNA-dependent RNA polymerase inhibitor	IV	Japan
Baloxavir marboxil	Small molecule inhibitor of cap-dependent endonuclease (PA)	III recruiting children <1 year. *Approved for the treatment of acute uncomplicated influenza among ≥ 12 years	Japan
Arbidol (Umifenovir)	HA resistance to conformational changes triggered by pH	III recruiting in China / IV unknown status in Russia	China; Russia
Ingavirin	Interaction with NP and inhibition of viral genome release	IV completed	Russia

1263 Note: Drugs and their clinical statuses were adapted from the clinicaltrials.gov.

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For instance, DAS-181-F03/F04 is a recombinant host-sialic acid-targeting molecule acting as a sialidase at the surface of the host's susceptible cells such as the epithelial cells of the airways (Malakhov et al., 2006). This inhibits the initial attachment of the influenza virus HA that recognizes Neu5Ac in the α(2, 3) - and α(2, 6) - linked configurations of sialic acids (Baum and Paulson, 1990). The universality of DAS-181-F03/F04 is due to its ability to act on both types of sialic acids and, therefore, it can be used in avian - carrying α(2, 3), and mammalian - containing α(2, 6), hosts and was shown to inhibit H1N1pdm09, H3N2 and H5N1 viruses (Belser et al., 2007; Chan et al., 2009; Triana-Baltzer et al., 2010). Besides influenza viruses, other sialic acid-dependent viruses such as human metapneumovirus and parainfluenza III virus were also shown to be inhibited by DAS-181-F03/F04 (Thammawat, Sadlon, Adamson, & Gordon, 2015). Another host targeting antiviral is the Nitazoxanide which falls under a category of thiazolides that are known to produce active metabolites following deacetylation. These metabolites have been shown to inhibit the maturation of influenza HA by blocking the trafficking and insertion of HA onto the host cell surface (Rossignol, 2014; Rossignol, La Frazia, Chiappa, Ciucci, & Santoro, 2009). Nitazoxanide is a licensed anthelminthic drug that has been repurposed to ameliorate influenza due to its broad range of protection efficiency against influenza viruses in phase II b/III clinical trial (Rossignol, 2014). The antiviral, JNJ-63623872 (Pimodivir) is a non-nucleoside influenza virus PB2 inhibitor, which binds a conserved domain on the polymerase subunit, PB2 of influenza A viruses and thereby inhibiting host cap-snatching (a prerequisite for the initiation of viral replication). Pimodivir is efficacious in nanomolar concentration during both prophylaxis and treatment or in co-administration with the neuraminidase inhibitor (oseltamivir) in mice models (Byrn et al., 2015; Smee, Barnard, & Jones, 2016). Phase II clinical trial has also confirmed the efficacy of Pimodivir when used as a single drug or when co-administered with Oseltamivir. In addition to this, the drug effect was not associated with any detectable interference of any cellular

processes (Finberg et al., 2019; Fu et al., 2016). There is currently ongoing recruitment for phase III interventional trials of Pimodivir among adolescents, adults, and the aged with non-hospitalized participants with chances of developing complications. Meanwhile, a pre-approval trial (of Pimodivir) for the treatment of patients with influenza virus A (H7N9) infection, has been allowed.

In 2002, Furuta et al. (2009) discovered the anti-influenza virus drug T-705, during the screening of anti-influenza compounds by plaque reduction assay. T-705 was shown to have a selective index of over 2,000 for influenza viruses, with no detectable cytotoxicity *in vitro*. Trials of T-705 in mice confirmed selectivity to influenza viruses and protection as an anti-influenza virus therapeutic agent. Along the same lines, Furuta and colleagues further observed some inhibitory action of the drug to some other RNA viruses, but not in DNA viruses. The mechanism of action of the drug has been attributed to the inhibition of viral RNA-dependent RNA polymerase by the active phosphoribosylated T-705, which acts as a nucleotide analogue and, hence terminating viral replication (Baranovich et al., 2013; Furuta et al., 2005; Jin, Smith, Rajwanshi, Kim, & Deval, 2013; Naesens et al., 2013). These have warranted further experiments on many other RNA viruses possessing either negative-strand segmented RNA genomes such as arena-and bunya-viruses or positive-strand RNA such as noroviruses and flaviviruses (Gowen et al., 2007; Morrey et al., 2008; Rocha-Pereira et al., 2012; Safronetz et al., 2013). In summary, T-705 is effective against influenza viruses in group 1 such as H1N1pdm09, H5N1 and group 2 such as H7N9 and drug-resistant strains of these viruses and has been exploited for the treatment against other viruses e.g., Ebola virus (Bai et al., 2016), and till date, no known resistance has been reported, except for a purposeful mutation that conferred resistance to a laboratory H1N1pdm09 virus strain (Goldhill et al., 2018). The efficacy of T-705 for the treatment of influenza has thus warranted its advancement through phase III and II trials in Japan and the US respectively (Furuta et al., 2013).

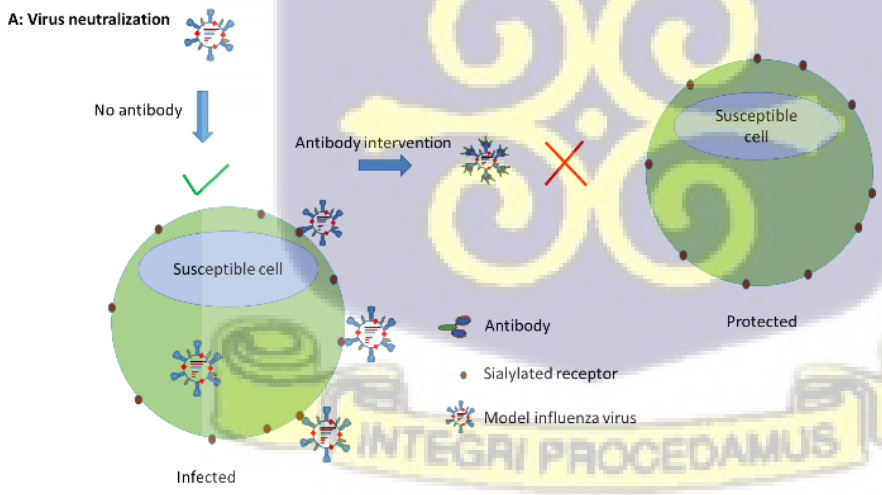
Baloxavir marboxil is another antiviral that was first developed in Japan and was shown to act as a selective cap-dependent endonuclease inhibitor of influenza viruses' (both A and B) polymerase subunit PA. The drug had exhibited preferable safety, tolerability, and pharmacokinetic properties in a phase I trial (Koshimichi et al., 2018). The overall optimal performance against uncomplicated influenza among adults and adolescents was shown during phase II and III trials that involved Japanese and Americans respectively (Hayden et al., 2018). The drug has currently been approved and marketed in the US as Xofluxa, for the treatment of acute uncomplicated influenza among ≥ 12 years (O'Hanlon & Shaw, 2019).

Arbidol (Umifenovir) is another influenza-limiting drug that had previously been licensed for use in both China and Russia, for almost several decades (Boriskin, Leneva, Pecheur, & Polyak, 2008; Gagarinova et al., 1993). It was originally developed in Russia and was found to potently inhibit influenza virus fusion with susceptible cell membranes, followed by interferon induction (Brooks et al., 2012). Like other broad-spectrum antivirals discussed earlier, besides influenza viruses, Arbidol has been shown to efficiently suppress other viral infections caused by paramyxoviruses and picornaviruses, bunyaviruses, rhabdoviruses, reoviruses, togaviruses, hepaciviruses, Ebola virus, arenaviruses, herpesviruses and the flaviviruses (Zika virus, West Nile virus and Tick-borne encephalitis virus) (Blaising, Polyak, & Pécheur, 2014; Haviernik et al., 2018; Pécheur et al., 2016). Currently, phase III trial of the Arbidol in ongoing in China and the drug is also due phase IV trial (with unknown status) in Russia.

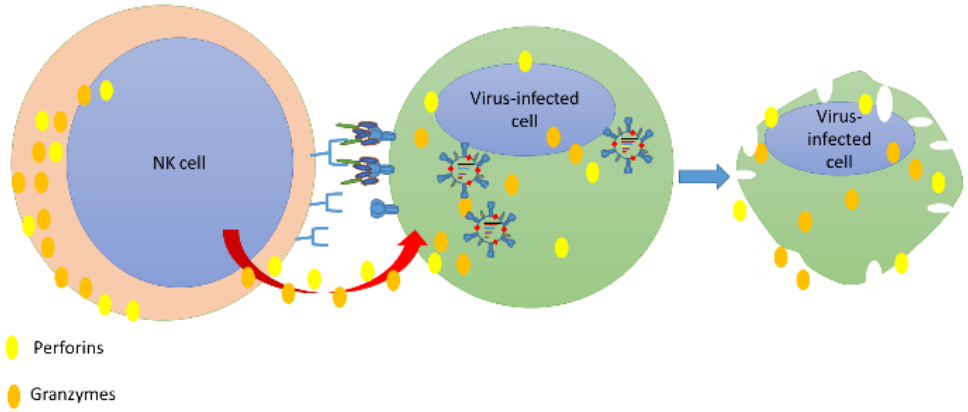
Ingavirin, a drug developed in Russia, adds to the current list of influenza-limiting antivirals due to its direct interference with the transportation of newly synthesized viral NP (Galegov, Andronova, & Nebol'sin, 2009; Loginova, Borisevich, Maksimov, Bondarev, & Nebol'sin, 2008; Zarubaev et al., 2011). As of 2017, Ingavirin has been approved for the treatment of influenza and other viral causes of acute respiratory illness, in Russia, following the completion of phase IV trial.

Passive Immunotherapeutics for management of influenza

The need for new strategies to control influenza infections has led to the investigations of antibody therapy potential. Such an approach is based on neutralizing monoclonal antibody (mAB) expression and delivery into the host pre- or post-exposure to the pathogen (Figure 13). Several clinical trials are testing mAB efficacy against infectious pathogens, including the TNX-355 (Ibalizumab) which has been successfully approved for the use in HIV-infected patients, and Palivizumab for the treatment of respiratory syncytial virus infections (Jacobson et al., 2009; Simoes et al., 2007). The feasibility of immunotherapy for rapidly evolving influenza was attained upon the discovery of broadly neutralizing antibody C179 isolated from a mouse immunized with H2N2 antigen (Okuno, Isegawa, Sasao, & Ueda, 1993). Further characterization showed the binding of C179 to the stem of HA, thus providing a structural basis to its ability to inhibit fusion (Dreyfus, Ekiert, & Wilson, 2013; Okuno et al., 1993).



B: ADCC



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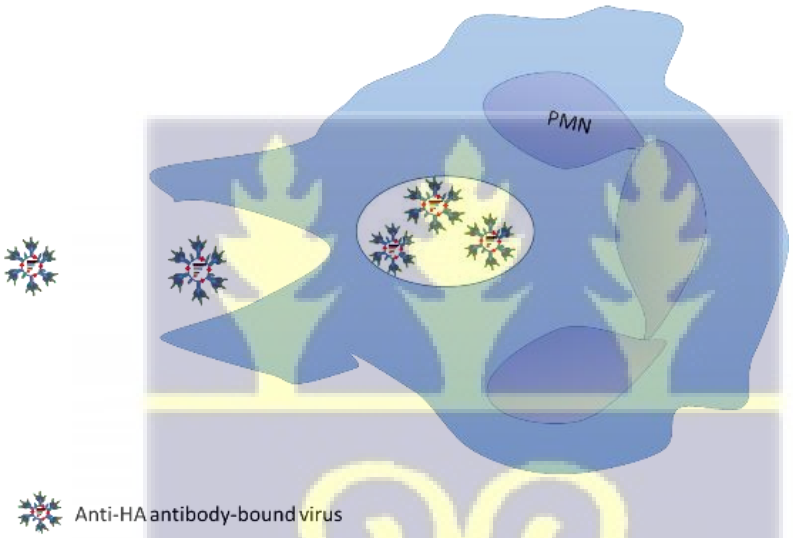
C: ADCP



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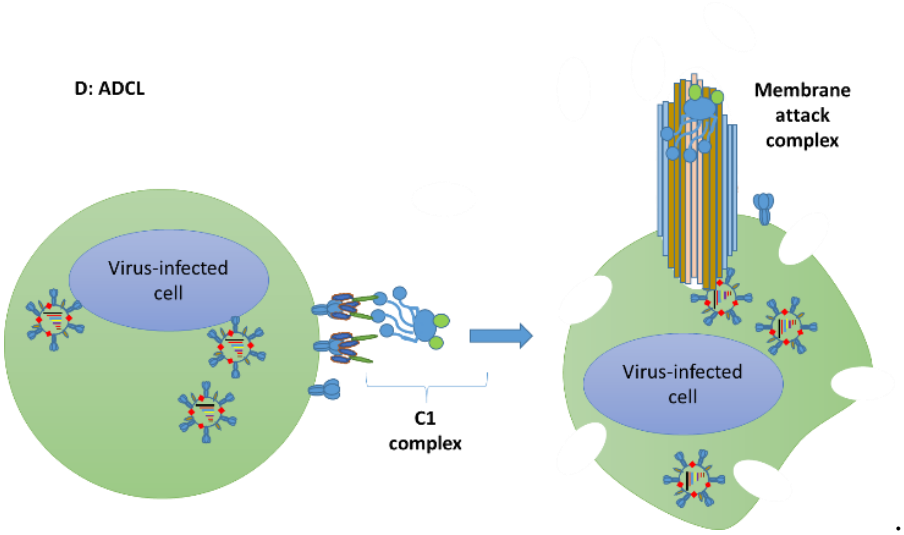

Figure 13: Mechanisms of antibody protection via passive immunization.

This figure outlines the possible mechanisms by which antibodies could mediate instant protection when administered either as prophylaxis or treatment. (A) Broadly neutralizing antibodies interact with HA interfering with the virus attachment to the host cell. (B) Opsonized infected host cells attract natural killer (NK) cell destruction via the process of antibody-dependent cellular cytotoxicity (ADCC). (C) Opsonized virus particles activate their phagocytosis by polymorphonuclear cells (PMN) via the process of antibody-mediated cell phagocytosis. (D) Virus-infected cells displaying the surface proteins of replicating viruses attract the assembly of the classical complement proteins forming a membrane attack complex that destroys the cell by osmosis in a process called antibody-dependent cell lysis (ADCL).

Techniques to fully recover human antibodies were further improved by Throsby et al. (2008) who used human memory B^+ cells and phage panning to recover thirteen mABs one of which CR6261 entered clinical trials. CR6261, as well as C179, was later found to neutralize the only influenza A viruses with group 1 HA. Similarly, another potential mAB MEDI8852 inhibiting cleavage of HA0 was also isolated from human memory B^+ cells and has completed phase IIa clinical trials (Ali et al., 2018; Kallewaard et al., 2016). This antibody can not only neutralize viruses of different phylogenetic groups but can also overcome amino acid changes due to its binding flexibility as shown by X-ray structures. Following on, the repertoire of broadly

neutralizing antibodies was characterized, using plasma cells (Pappas et al., 2014). Discovery of MAbs that can cross-neutralize multiple viral subtypes could lead to novel passive immunotherapy treatments for human infections and could also have the potential to abrogate spillover infection events from zoonotic species. Several example antibodies with the highest potential for use in the management of influenza, are listed in Table 3.

1385 **Table 3: Antibodies undergoing clinical trials.**

Antibody	Target/ mechanism of action	Clinical phase status	Country
MEDI8852	HA stem	IIa completed	USA
MHAA4549A	HA stem	II completed	USA
VIS410	HA stem	II completed	USA
Intravenous hyper-immune immunoglobulin (IVIG)	Antigen-specific antibody pool with neutralizing potential	III recruiting	USA
Ergoferon	Suppression of non-specific immune activation (by any virus)	IV completed	Russia
CR6261	"Highly conserved membrane-proximal stem of H1 and H5 viruses' HA1 and HA2"	II completed	USA

1386 Note: Information was retrieved from clinicaltrials.gov.

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Experiments by Lu and colleagues demonstrated that the Fragment antigen-binding, F(ab') 2 regions of an equine anti-H5N1 antibody could protect mice against a lethal challenge of influenza H5N1 (Lu et al., 2006). Such an example shows that the use of passive immunotherapy during influenza outbreaks could complement the use of available antivirals that would increase the survival of both humans and animals.

Most antibodies tend to be elicited against the more antigenically variable head domain of the major surface glycoprotein HA. On the other hand, the less variable stalk domain is characterized as sub-immunodominance and is associated with minimal immune response (Corti et al., 2010; Sui et al., 2011). Experiments by Margine and colleagues employing the variable heads but constant H3 stalk domain as a vaccine in mice triggered broadly cross-reacting stalk-based antibodies (Margine et al., 2013b). Wohlbold et al. (2015) and Nachbagauer et al. (2016) also realized the importance of the influenza HA stalk as a good vaccine target when they ascertained the transmission-blocking capacity of stalk-directed vaccines in ferrets. Also, studies by El Bakkouri et al. (2011) used an immune serum of mice (previously immunized with three tandem copies of M2e, fused with the – Hepatitis B virus (HBV) core fusion protein) to show Fc-dependent immunity against the M2e. Intranasal administration of monoclonal anti – NA antibody resulted in total protection (in mice) with significantly lower virus titers and no viral escapes as determined by deep sequencing of viral genomes (Wilson et al., 2016).

Passive immunotherapy has demonstrated rapid relief and life-saving capabilities in the treatment of viral infections like measles, rabies, HBV, and others (Audet et al., 2014; Qiu et al., 2014; J. Zhao et al., 2015). As established for RSV with the commercial immunotherapy palivizumab, antibody-based therapies with influenza antibodies could aid most susceptible populations including infants that often suffer from influenza-related complications. Considering this, prophylactic use of antibodies should be considered as a part of immediate

management and care procedures. One of the approaches considered for antibody delivery is via recombinant virus vectors. Balazs et al. (2013) developed an adenoviral-vectored human broadly neutralizing antibody (F10 or CR6261) which conferred protection in mice (who received the construct intramuscularly) during a pathogenic influenza virus (H1, H2, and H5) challenge. Their experiments also showed equally protective amounts of the intramuscularly expressed F10 antibodies in the sera of both young and old mice and non-obese diabetic/ severe combined immunodeficiency/ interleukin receptor subunit gamma null mice, suggesting how efficacious passive immunotherapy could be for both the aged and immunodeficient (Balazs et al., 2013). More so, antibody-based immuno-prophylaxis and -therapeutics have more prospects in effective intermediation of influenza outbreaks and production of specific influenza vaccines, noting that the broadly reacting antibodies have the capacity to both inhibit virus replication and shedding (Berry et al., 2014). More consolidating evidence of passive immunotherapeutic management of a case infected with A(H5N1) which was described to have encountered rapid relief in less than two days after convalescent plasma administration, and interestingly, resulted in no detectable viruses by real-time polymerase chain reaction (Zhou, Zhong, & Guan, 2007). Furthermore, it is worth noting the diverse protective mechanisms by which antibodies can exert their functions directly on pathogens or on pathogen-infected cells: virus neutralization, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell phagocytosis (ADCP), and antibody-dependent cell lysis (ADCL) are well-studied in the context of influenza viruses (Figure 13). ADCC, which mainly involves the destruction of infected cells chiefly by natural killer cell (NKC) activity (via perforin and granzyme B secretion into infected cells leading to cell lysis with the destruction of an intracellular pathogen), via recognition of antibody Fc that cross-links NKC Fc receptors (FCRs), has been observed for murine antibodies weakly interacting with cognate influenza M2e (Jegerlehner, Schmitz, Storni, & Bachmann, 2004). Similarly, virus replication in mice

was shown to be suppressed due to neutrophil-antibody-based enhanced phagocytosis (ADCP) in pulmonary infected mice that either received anti-influenza serum before or after influenza infection, when neutrophils were retained and not inhibited by antibodies (Fujisawa, 2008). In direct virus neutralization, antibodies that bind the receptor-binding site (or nearby site) of haemagglutinin prevent the viral attachment to the host cell: these antibodies, that are mainly targeted against the globular head of HA, are elicited by either vaccination or natural infection (Berry et al., 2014). ADCL is another mechanism that could augment the killing of influenza viruses as observed by Terajima et al. (2015) realizing that mostly neutralizing human monoclonal antibodies (either recognizing the globular head or stalk of HA) exerted this kind of effect, contrary to previously associated stalk-specific antibodies only.

Above all, we emphasize the therapeutic capacity of novel monoclonal antibodies in combating influenza infection and speculate that, in addition to the use of effective antivirals, passive immunotherapy against both influenza A and B viruses might be the way-forward for influenza virus management amongst all class (be it high- or low-risk) of patients. We envisage that the involvement of passive immunization will culminate in an accelerated relief through any of the mechanisms previously described, and this provides allowable time for the full activation of the adaptive immune system via the conventional antigen-presenting mechanisms. Additionally, infected persons who were passively immunized could develop a natural immunity to the specific viruses and this immunity could be long-lasting giving protection to other antigenically matched strains (Yu et al., 2008). Also, the association of passive immunization with rapid relief increases the chances of abating the evolution of escape mutants suggested to arise due to vaccination and its consequent herd immunity (Krammer, García-Sastre, & Palese, 2017).

Conclusions

Improving vaccine delivery and efficaciousness are paramount to combat the continuous circulation of epidemic influenza; however, in addition to the ongoing deployment of novel anti-viral strategies which include the highly promising immune therapies for treatment and prophylaxis are vital to limit and protect against the on-going threat of pandemic influenza.

CHAPTER 4: THE HAEMAGGLUTININS OF SEASONAL INFLUENZA A VIRUSES (H1 AND H3) POSSESS TARGETABLE CONSERVED DOMAINS

Abstract

Influenza causes a lot of epidemics that often lead to loss of productive hours worldwide. This is since conventional influenza vaccines do not offer long-term protection and requires constant updates. Current investments are into the search of potent vaccine candidates that can offer both long-term protection and transcend other novel strains.

The haemagglutinin, which is the dominant surface protein is one of the approaches for the design of a universal influenza vaccine. This is because it contains both immunodominant and sub-immunodominant epitopes that are targetable. The main challenge stems from the immunodominant epitopes that are under constant pressure and so keep evolving due to subtle mutations and thus rendering the seasonal vaccines useless for the next season after current use.

Maintaining the conserved domains and designing chimeric or mosaic haemagglutinins have revealed a new dimension of enhancing antibody induction to the conserved sub-immunodominant regions or increased breadth of antibody responses, respectively.

In this study, the conserved domains that mainly categorize under the sub-immunodominant regions (in the seasonal H1 and H3 HAs) were ascertained by means of consensus sequence building. Subsequent attempts were made to insert foreign epitopes or sequences from consensus sequences of foreign haemagglutinins into polymorphic regions of the consensuses of the seasonal HAs, to generate 3 chimeric haemagglutinins that were relatively loaded with

more epitopes and had relatively higher antigenicity, by in-silico prediction, when compared to control haemagglutinins of the 2018/ 2019 candidate vaccine viruses.

Introduction

Seasonal influenza viruses are highly infectious and have been estimated to cause several thousand to millions of morbidities and mortalities yearly throughout the globe (Iuliano et al., 2018). The seasonal influenza viruses comprise the two main lineages of influenza B viruses - the Yamagata and Victoria lineages- and two main subtypes of the influenza A viruses -the A (H1N1) pdm09 and A(H3N2). Of the influenzas, those associated with the influenza A viruses are a major cause of epidemics – including subtypes spelt to cause most infections in avian (De Luna and Hartshorn, 2017; Mostafa & Pleschka, 2018). Of great interest is the control of epidemics directly associated with humans. Hence, the availability of several antivirals that are currently approved for the treatment (Grohskopf & Munoz, 2018). However, the continuous evolution of these viruses both to adapt to new hosts and/ or resist the drug pressure, because of continuous use or misuse of the approved antivirals has become an issue also of immense concern (Dancer, 2004; Handel, Longini Jr, & Antia, 2009; Levy & Marshall, 2004; Livermore, 2005). Vaccines are generally the best means of controlling infectious diseases; however, current seasonal influenza vaccines require continual update due to the previously described mechanisms of shift and drift -associated with host adaptation or vaccine/ drug-induced pressure (Krammer & Palese, 2015; Paules et al., 2017; Paules, Sullivan, Subbarao, & Fauci, 2018). Thus, it is of immediate global interest to explore novel means of vaccine designs, and this extrapolates to the search for highly immunogenic constructs that would effectively induce immunity against both currently circulating seasonal influenza viruses and divergent viruses (Berry et al., 2014).

The desire for a universal influenza vaccine is not just limited to an ephemeral efficacy against seasonal or drifted viruses, but also geared towards long-term protection of vaccinees over several seasons with little or no update of the vaccine; otherwise, only booster shots may be required intermittently (Krammer, García-Sastre, & Palese, 2018). An intense area of immense research is the development of a universal influenza virus vaccine that will provide sustained and effective protection against all influenza viruses (Nachbagauer, et al., 2020).

The treatment of infections with antibodies or convalescent sera (Passive immunotherapeutics) has recently been proposed to be an effective tool in the control of influenza viruses (Berry et al., 2014; Kotey et al., 2019; Zhou et al., 2007). Several antibodies have been promising for the treatment of influenza in both animal models and humans (Fujisawa, 2008; J. R. Wilson et al., 2017; Zhou et al., 2007). More desired antibodies are those that could exert both homotypic and heterotypic protection against the seasonal influenza A viruses. Such antibodies offer to use as a therapeutic tool in the immediate control of influenza. However, only a few of such antibodies are being advanced into clinical trials. This offers an avenue to explore into means of generating highly effectual antibodies: especially, those that interact with influenza A viruses on highly conserved viral regions. Already known is the immunodominance of the influenza A virus HA globular head, and most antibodies raised against it are known to primarily affect the five main antigenic sites (Ca1, Ca2, Cb, Sa, and Sb) (Caton, Brownlee, Yewdell, & Gerhard, 1982; Walter Gerhard, Yewdell, Frankel, & Webster, 1981). But it is obvious that these constellations are continuously evolving to evade immunity (Retamal, Abed, Rhéaume, Baz, and Boivin, 2017), making it expedient to explore into diverse HA conserved sites that have the potential to elicit 'promiscuous' broadly cross-reacting antibodies which would circumvent vaccine-induced viral evolution.

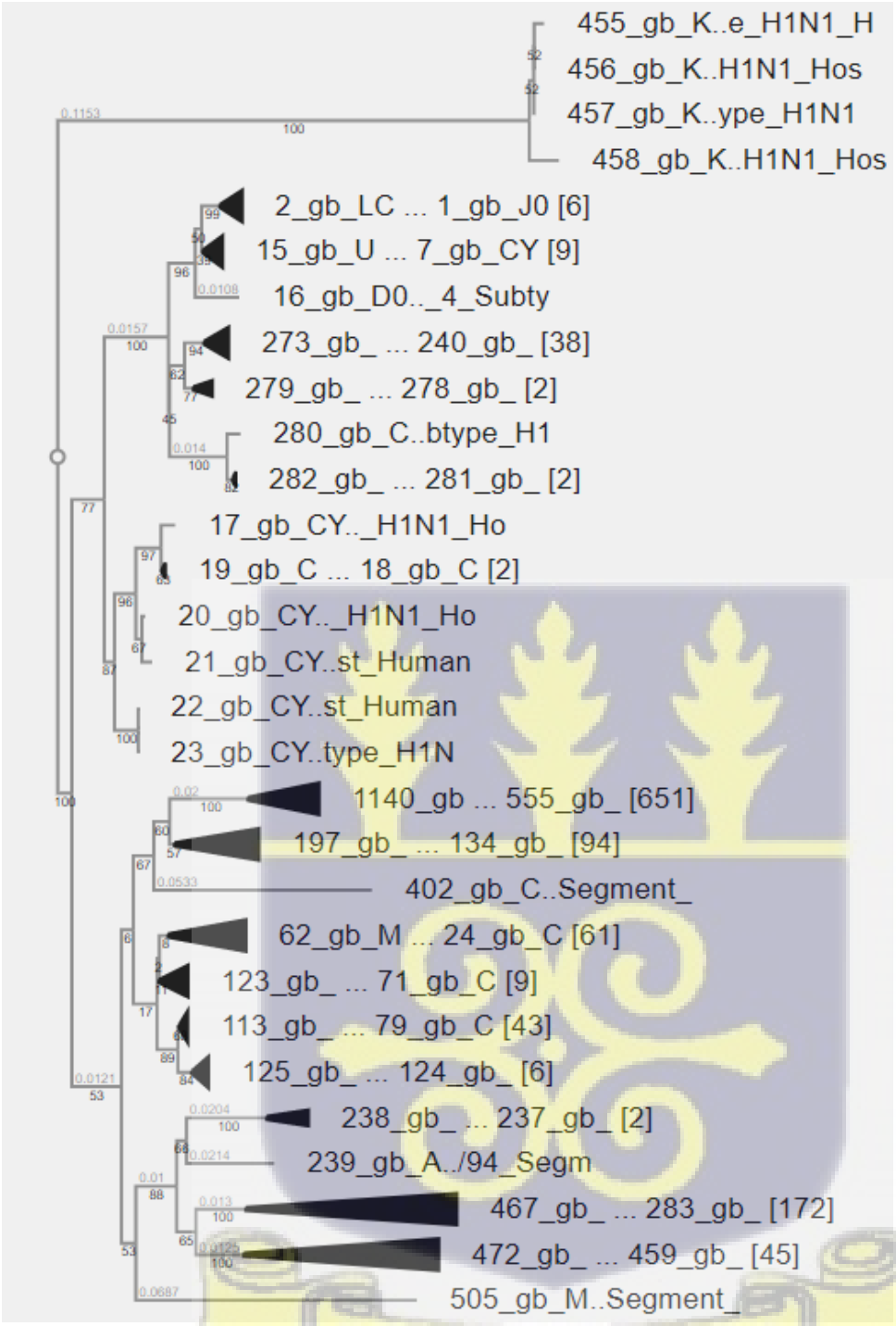
We, therefore, set out to identify commonly conserved regions on seasonal influenza A viruses' haemagglutinins -both H1 and H3 viruses.

Results

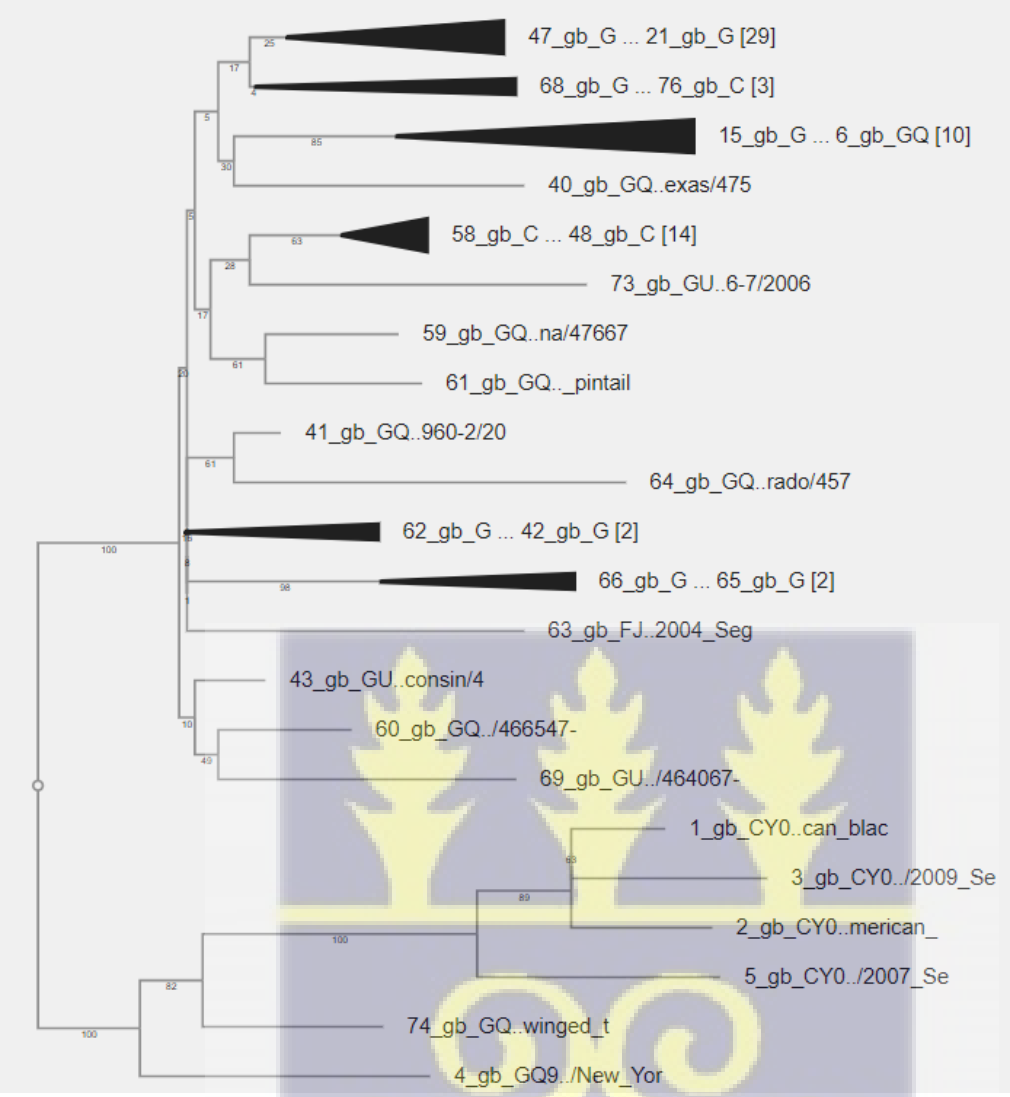
H1 and H3 cHAs constructed.

Diverse sequences -1,156; 21,749; 76; 463 and 3,771 sequences – available on the Global initiative on sharing all influenza data (GISAID) platform were selected for the generation of singular consensuses of H1, H3, H5, H7, and H9 HAs, respectively. Phylograms (Figure 14) confirms the diversity of sequences selected following multiple sequence alignment using the online MAFFT programme. Consensus for each of the HA sequences were subsequently generated by BioEdit (version 7.2.5) and finally aligned in MEGA (version 7.0.21), where cHAs were then constructed by blending two sequences (consisting of a seasonal HA consensus and an avian HA consensus from the same HA group). Thus, the 3 cHAs were designed to predominantly represent seasonal HA sequences and are named H1/H9 (cHA-E), H1/H5 (cHA-C), and H3/H7 (cHA-A) [Figure 15].

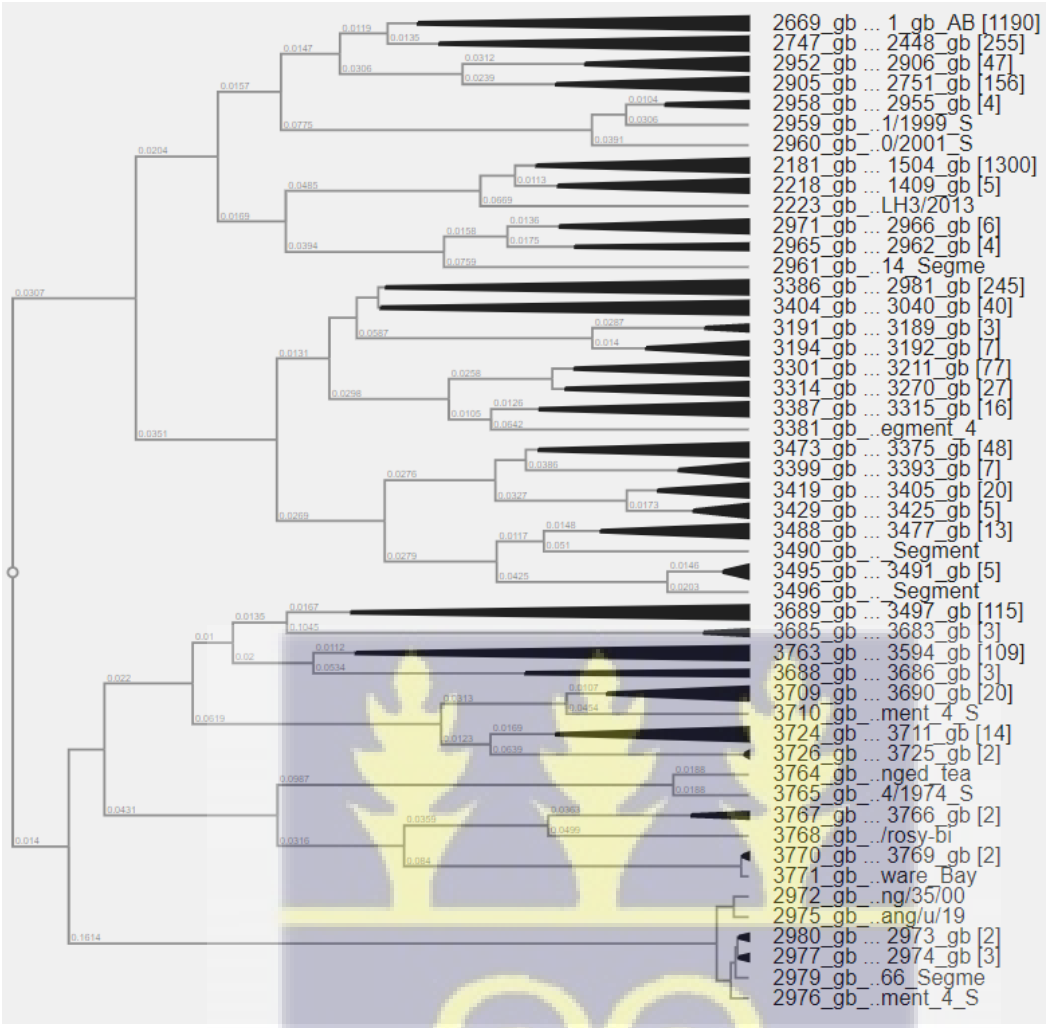
A.



B.



1558 C.



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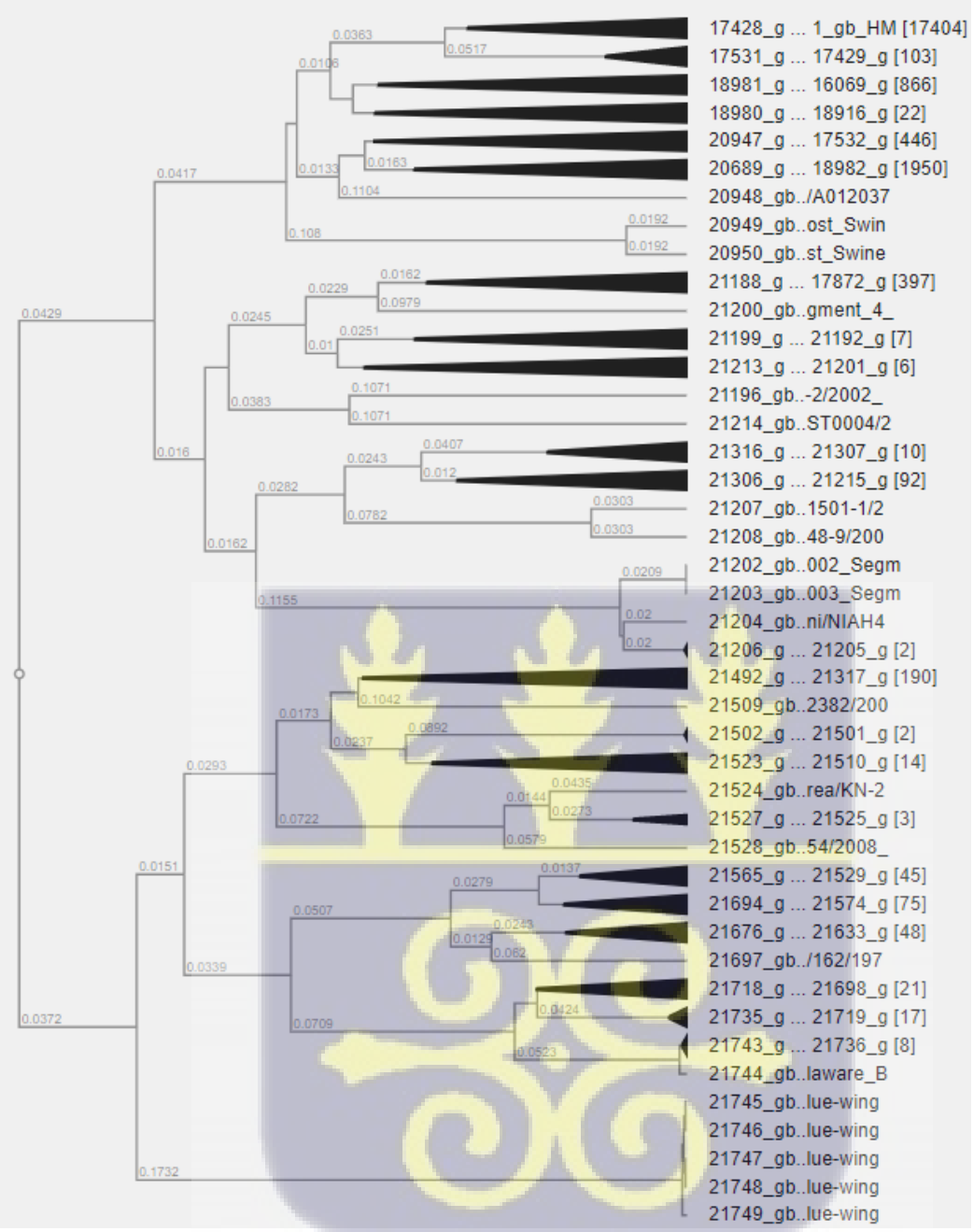
D.



E.

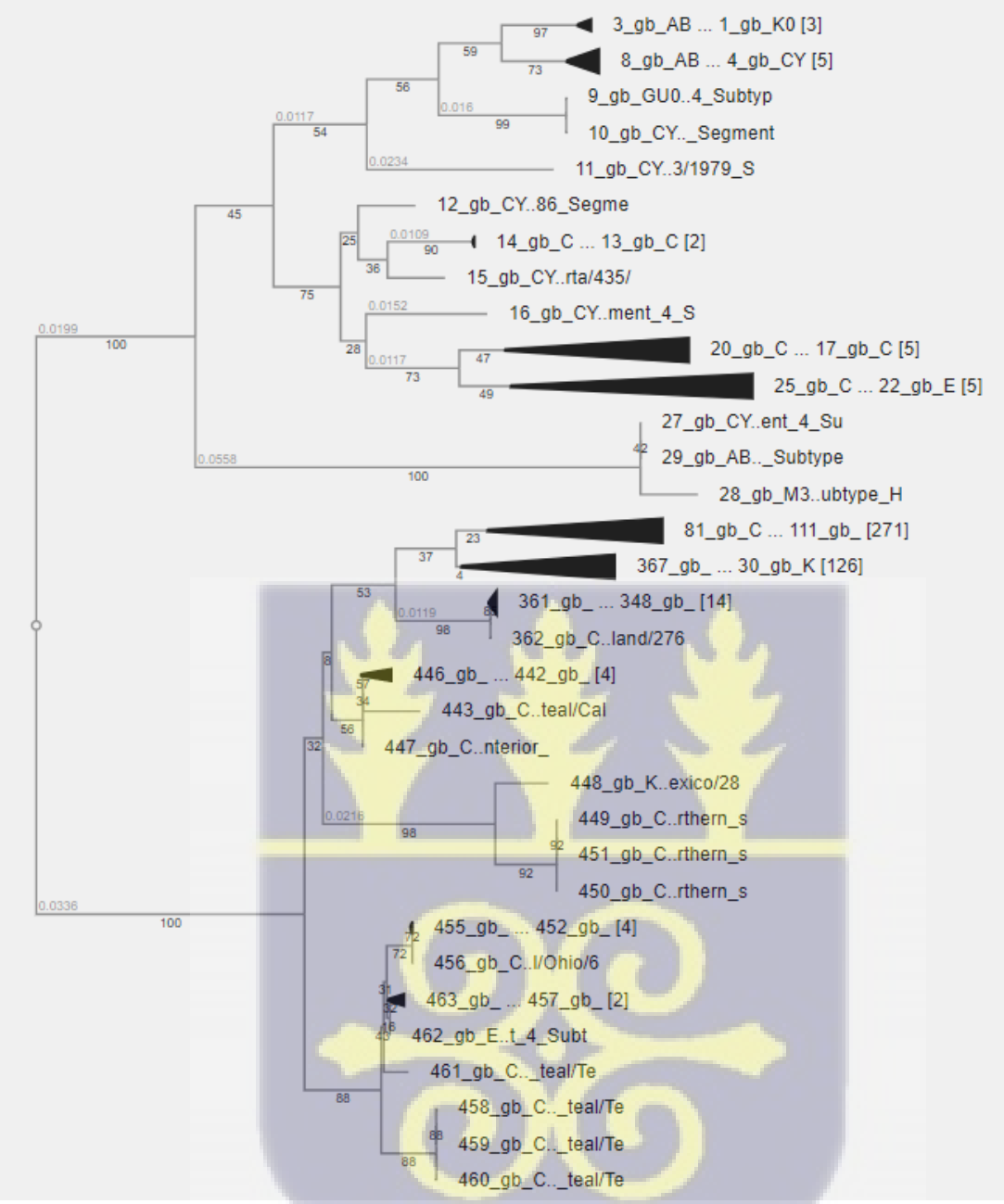
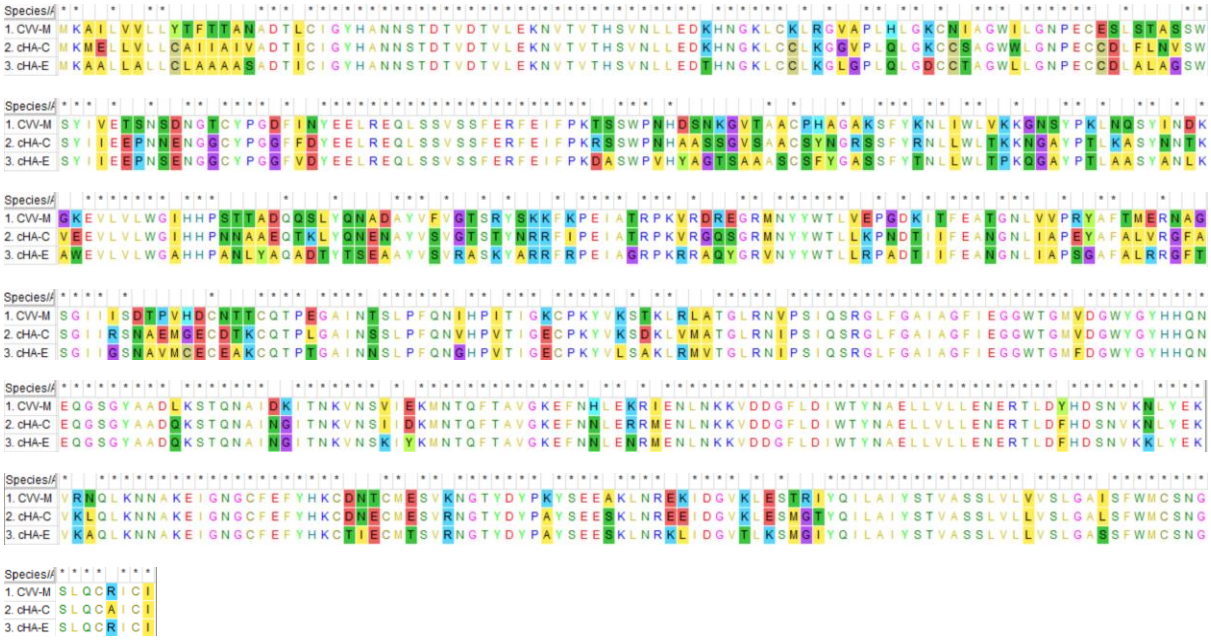


Figure 14: Diversity of selected HA sequences.

Here are phylograms representing the numbers and diversities of HA sequences of H1 (A), H5 (B), H9 (C), H3 (D), and H7 (E)HAs selected at random from the Influenza Research Database (IRD); Sequences span the years 1933-2015 for H1 HAs; 1974-2017 for H3 HAs; 2006-2009 for H5 HAs; 1977-2015 for HAs; and 1966-2017 for H9 HAs. Bootstraps of 100 replicates was applied for H1, H5 and H7 only due to relatively lesser numbers (i.e., <2,000) of sequences that favour such phylogenetic analysis on the online MAFFT platform. Bootstrap assessment could not be conducted for H3 and H9 sequences on the same platform as these were > 2,000. To enable easy capture of the complex phylogenetic trees, they were collapsed to a depth of 6, hence the display of wedges on each phylogram visualized by the online Archaeopteryx phylogenetic tree viewer.

A.



B.

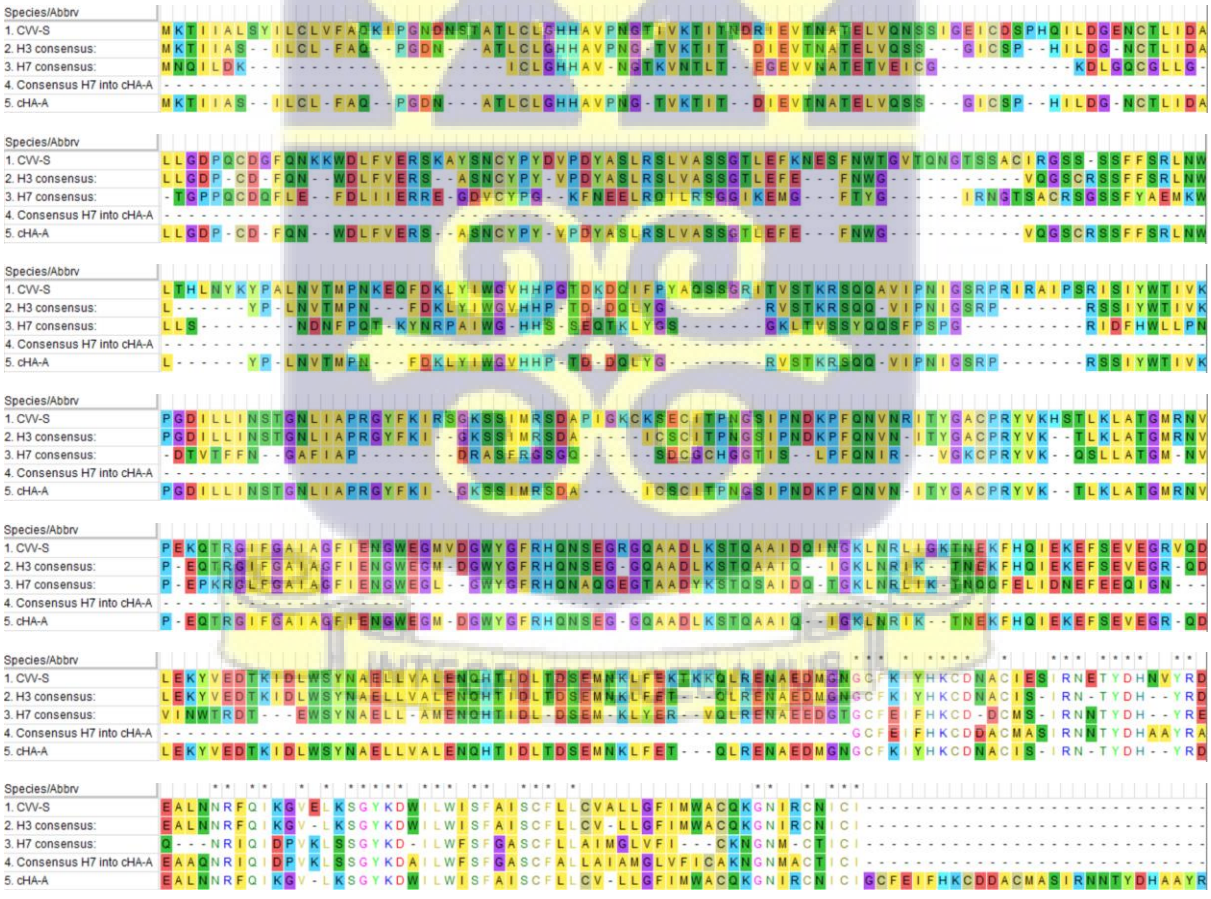


Figure 15: Multiple sequence alignments and consequential generation of cHAs

Here is a summary synthesis of cHAs by blending consensuses of selected HA sequences. Asterix (*) indicate 100% conserved regions based on the sequences analysed. A details comparison among the H1 control sequence Candidate vaccine virus, CVV-M (Michigan strain) and cHA-C and cHA-E detailing clear polymorphic regions within which amino acid sequences from either H5 or H9 have been incorporated. B details the complete design of cHA-A by the adjoining of the H7 consensus to the compressed H3 consensus, and describes the comparison among the H3 control sequence, CVV-S (Singapore strain) and the other H3 and H7 parties.

cHAs are structurally and functionally like a typical HA by in silico prediction.

The structural and functional predictions of the cHAs were assessed using the I-TASSER online platform. Amino acid sequences were deposited on this platform and both structural and functional characteristics were accessible upon the third-week post-submission. Structures of the cHAs were identified to have about 80% and 70% similarity in structures and functions, respectively, by iterative threading against a classical HA (Table 4 and Figure 16). Further, a protein Basic Local Alignment Search Tool (BLASTp) of cHA-A, cHA-C, and cHA-E, generated 80%, 85%, and 82%, respectively, to the hits (HA [Influenza A virus (A/Hong Kong/CUHK 6383/2003(H3N2))]), (HA [Influenza A virus (A/swine/Tianjin/01/2004(H1N1))]) and (HA [Influenza A virus (A/Taiwan/01/1986(H1N1))]), confirming both that the constructs are HAs and predominantly, seasonal HAs.

Table 4: Structural and functional predictions.

cHAs	Structural TM-score	Predicted structural models (PDB codes)	Functional SIA-binding C-score
(cHA-C)	0.831	4f23A	0.77
(cHA-E)	0.836	4f23A	0.7
(cHA-A)	0.8	4wa2A	0.7
CVV-M	Haemagglutinin of the Michigan strain: 2019 Candidate vaccine virus (CVV) with each TM and SIA-binding C-scores assumed 1 (or 100%) [used as H1 HA control]		
CVV-S	Haemagglutinin of the Singapore strain: 2019 CVV with each TM and SIA-binding C-scores assumed 1(or 100%) [used as H3 HA control]		

Notes: SIA= Sialic acid; TM scores are metrics that convey information about the similarity between two proteins. Thus, the TM-scores generated aided in the detection of the closest structure on the Protein Database (PDB) as presented by the codes displayed here. The C-score is a confidence score applicable in grading the quality of predicted models, based on the significance of alignment with several references within the database and concerning the convergent details about their structure and then makes inference to the function of a test molecule. Both CVV-M and CVV-S were assumed to interact with sialic acid (100 %) and possess a structural TM score of 100%.

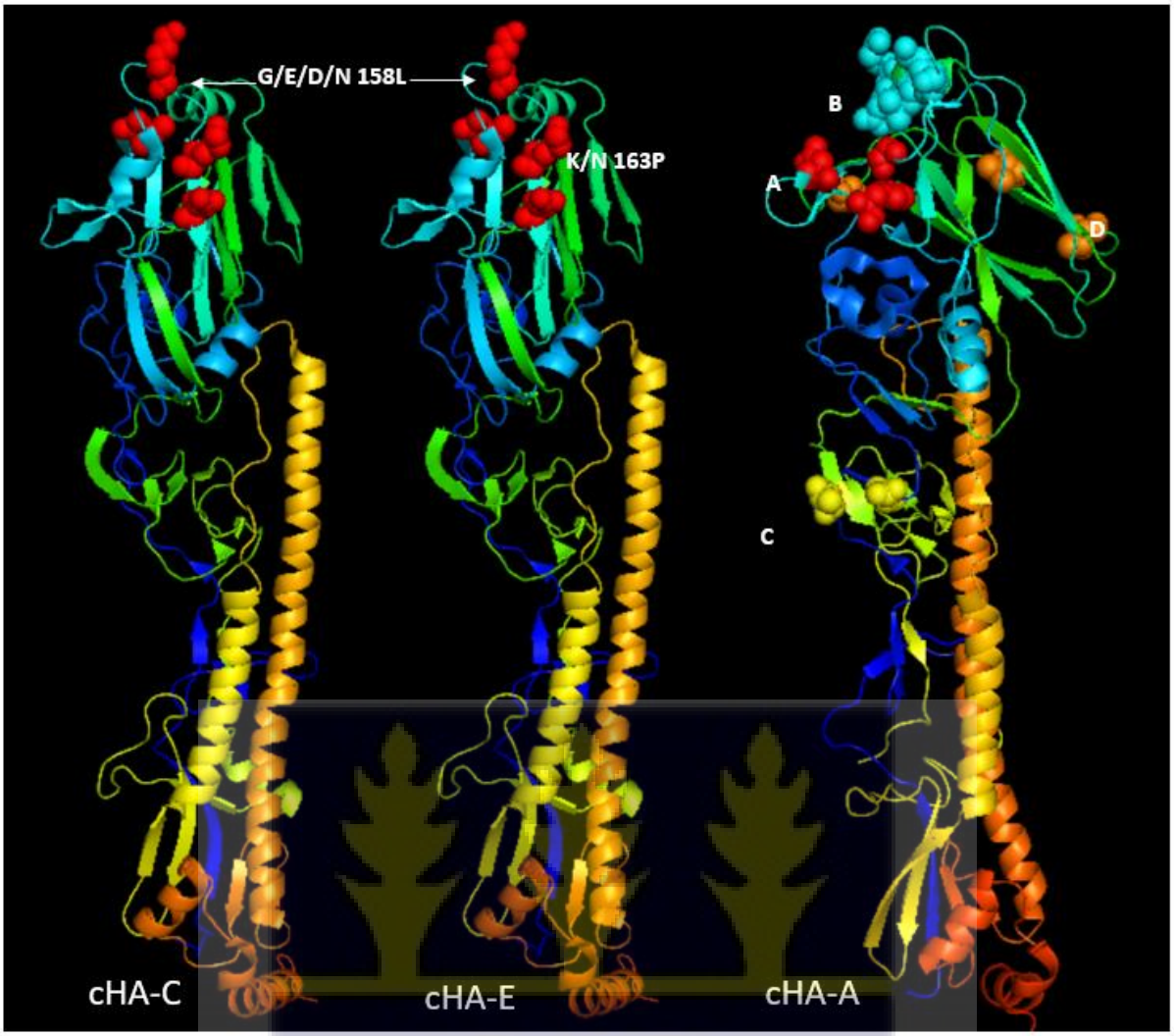


Figure 16: Visualization of predicted cHA structures in Pymol.

These figures are visual descriptions of the predicted structural models of the cHAs when analysed by I-TASSER. Resembling proteins 4f23A (by both cHA-C and cHA-E) and 4wa2A (by cHA-A) accessed from the Protein Database (PDB) were fetched into PyMol to generate the visual representations. All the prevalent secondary structures of the cHAs have been colour coded. The dotted spheres characterized antigenic areas on the predicted structures of the cHAs. Most positions that were substituted on cHA-C and cHA-E were mainly synonymous and are not represented here. Nonetheless, both cHA-C and cHA-E had amino acid substitution with leucine (L) at the polymorphic antigenic position 158. cHA-E recorded a proline (P) at antigenic position 163. Four main antigenic positions are presented for cHA-A (i.e., A, B, C, and D) all of which documented several amino acid substitutions summarized in table 5.

Table 5: Antigenic site substitutions on cHA-A.

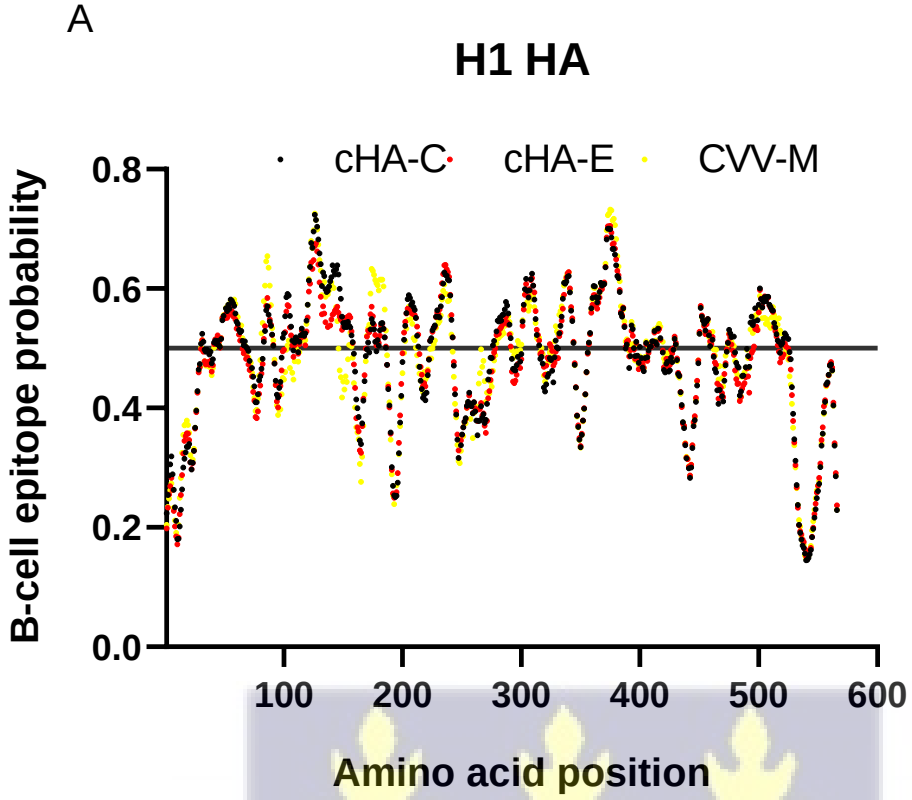
H3 antigenic sites	Positions	Key amino acids	Substitutes
A (red spheres)	133	N	L
	144	G	D
	146	G	L
B (blue spheres)	155	T	T
	188	N	V
	189	G	K
	193	S	I
C (yellow spheres)	54	N	C
	275	D	I
D (orange spheres)	201	R	N
	205	S	P
	220	R	D

Note: Here is a detailed documentation of the degree of substitution on the antigenic sites of cHA-A as displayed on the structure at Figure 15. Of all these sites, only one position (i.e., 155) remained unaffected by the cHA-A generation process.

cHA B-cell epitope and antigenic predictions

Altogether, the cHAs designed have been confirmed to be similar to a classical HA. However, an important factor for the design was for the enrichment of epitopes is enhanced B-cell epitope and immunogenicity; hence, the introduction of conserved sequences of exotic HAs into the consensus conserved sequences of the seasonal influenza A HAs. B-cell epitope predictions returned epitope probabilities that were favourable for cHA-C, cHA-E, and cHA-A versus their control sequences (Figure 17A and B; Figure 18A and B). Though the median epitope probabilities were in favour of cHA-C and cHA-E, there were no significant differences amongst their medians (Figure 17B). A thorough assessment of the antigenicity of the cHAs based on Kolaskar and Tongaonkar algorithms at a threshold of 1.000 returned scores that were comparable between controls and the cHAs. Both cHA-C and cHA-E returned scores with medians relatively higher than the CVV-M; only the median of cHA-E crossed the set threshold. Regardless of these appreciable differences observed amongst the cHAs and controls, they were not statistically significant when their medians were compared by the Kruskal-Wallis test (Figure 17C). Similarly, the B-cell epitopes probabilities favoured cHA-A over the control CVV-S in terms of the returned means of these scores. The one-way ANOVA, however, could not confirm any significant difference between their means. The antigenicity scores predicted, on the other hand, returned scores that favoured the significant difference in the means of cHA-A and CVV-S: cHA-A was significantly more antigenic (Figure 18C). Predicted antigenicity on the cHAs and the controls generated a collection of the antigenic epitopes (Appendices C and D), of which when the common epitopes were crossed out, there were unique epitopes specific to the cHAs: cHA-C had 4 extra epitopes (3, unique and 1, shared with cHA-E); cHA-E had 6 extra epitopes (5 unique, and 1, shared with cHA-C) and CVV-M had 4 extra epitopes (Table 6). cHA-A had 4 unique epitopes, whereas, CVV-S had only 2 unique epitopes (Table 7).

A



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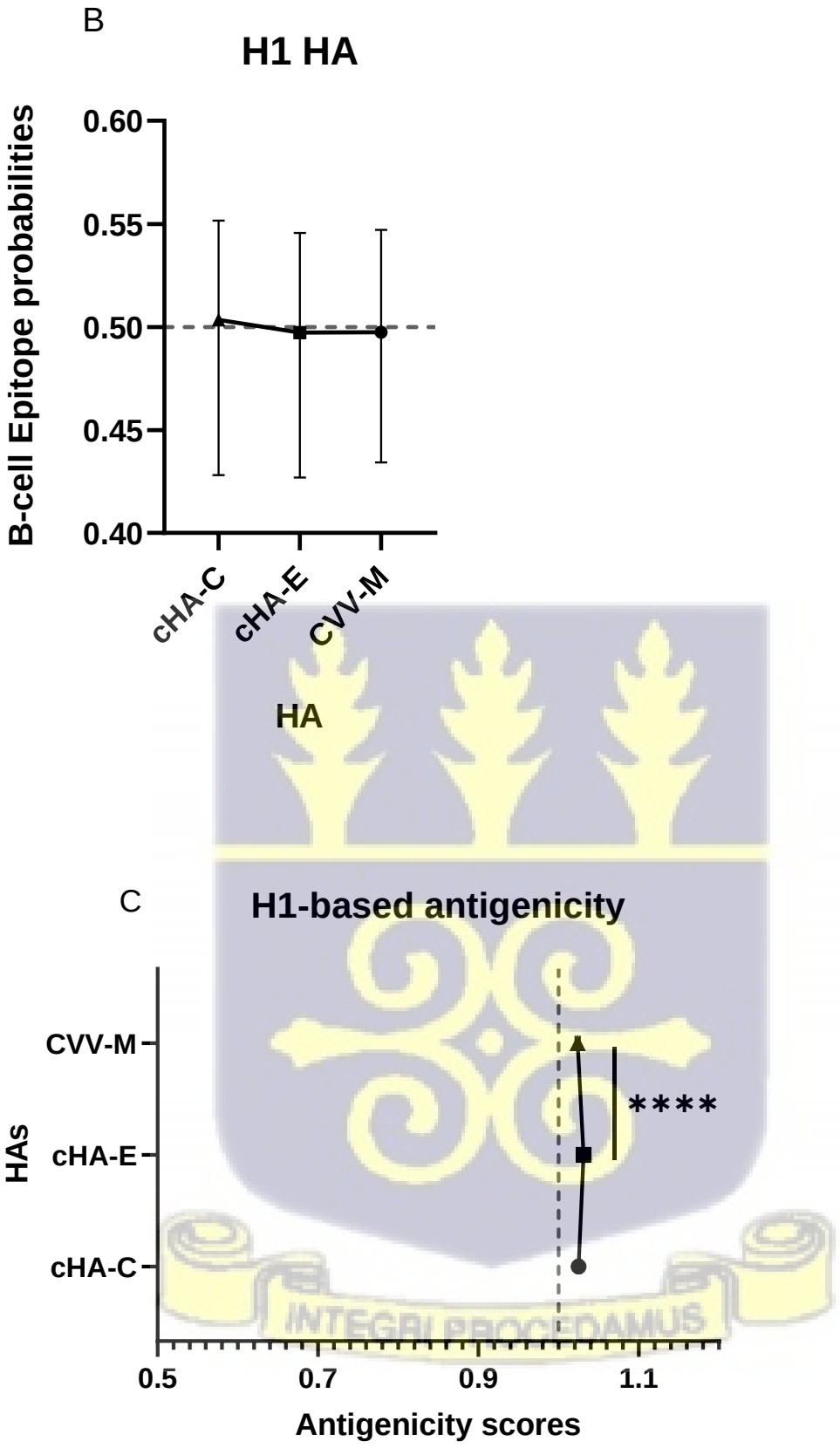

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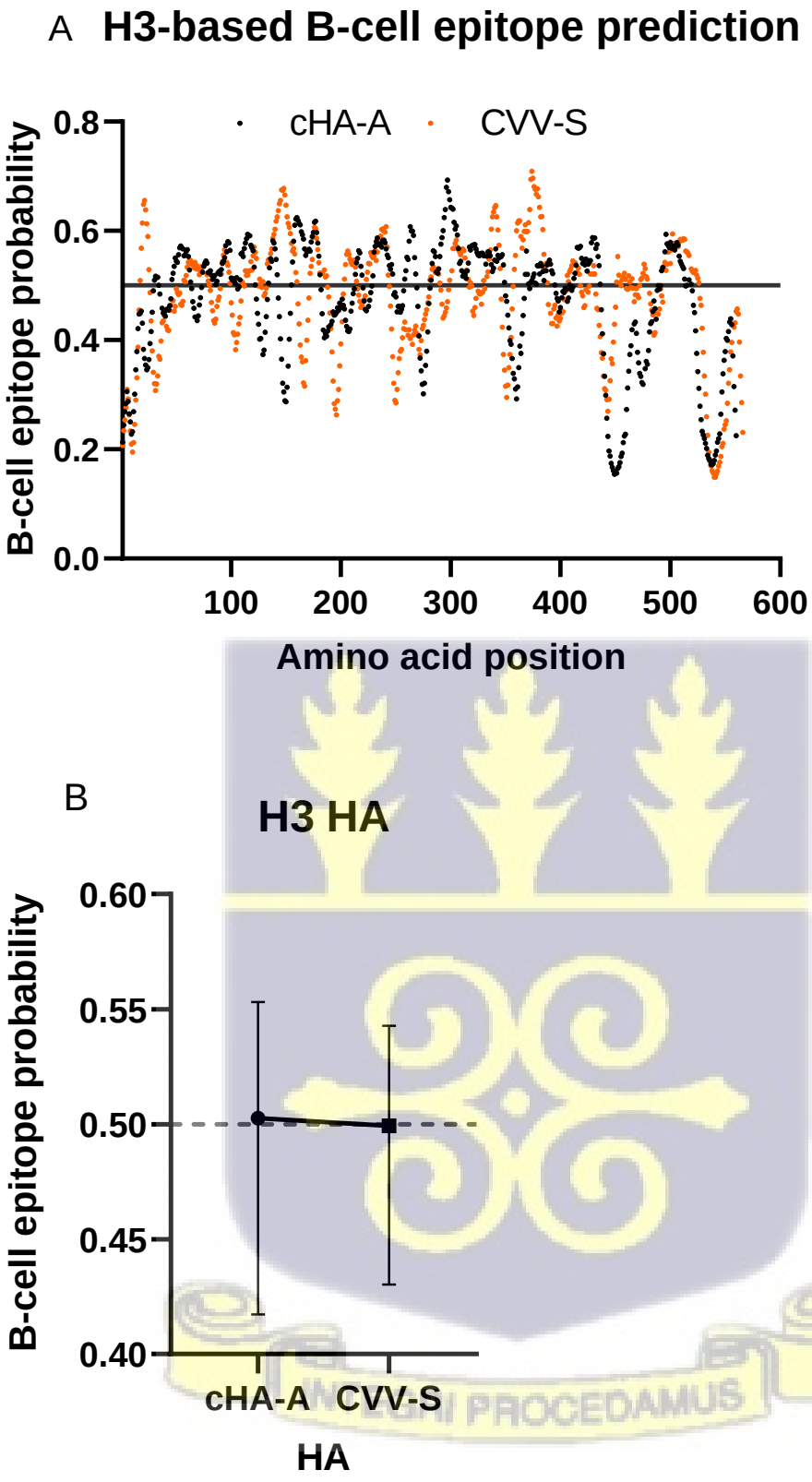
1670

1671 **Figure 17: B-cell epitopes and antigenicity predictions on the H1 HAs.**

Here are charts describing B-cell epitope probabilities and antigenicity scores of the H1 HAs. A, displays the distributions of the probable epitopes at the default threshold of 0.5; B, represents the comparison of the medians of the B-cell epitope probably scores, using Kruskal-Wallis test; C, displays the antigenicity of the HAs given a default threshold of 1.000 antigenicity score: Means of the scores were compared using two-way ANOVA and p-values were corrected using Dunnett's multiple comparisons test. P<0.05: *, P<0.005: **, P<0.005: ***, P<0.0005: ****



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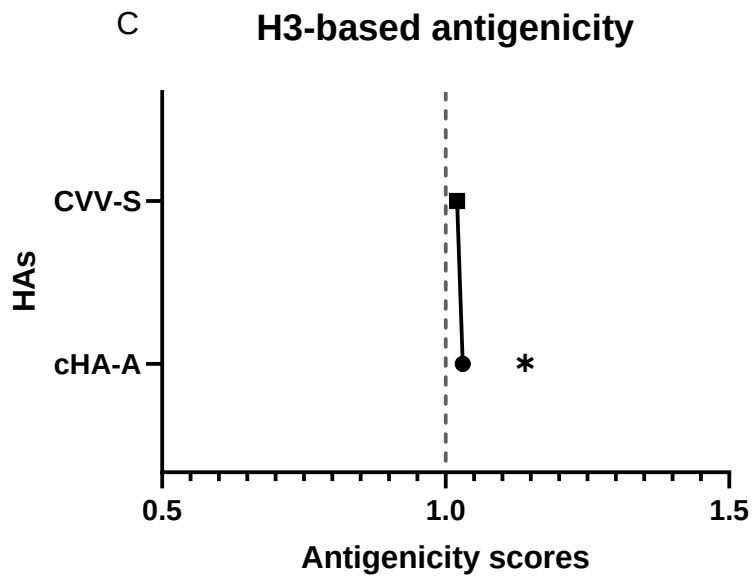


1680

1681 **Figure 18: B-cell epitopes and antigenicity predictions on the H3 HAs.**

1682 Here are charts describing B-cell epitope probabilities and antigenicity scores of the H3 HAs. A, displays the
1683 distributions of the probable epitopes at over the default threshold of 0.5; B, represents the comparison of the
1684 means of the B-cell epitope probably scores, using the unpaired t-test; C, displays the antigenicity of the HAs
1685 given a default threshold of 1.000 antigenicity score: Means were analysed using the unpaired t-test with
1686 Welch's correction. $P<0.05$: *.

1687

Table 6: Unique and shared epitopes in the H1 HAs.

cHA-C				cHA-E				CVV-M			
Start	**End**	**Peptide**	**Length**	**Start**	**End**	**Peptide**	**Length**	**Start**	**End**	**Peptide**	**Length**
146	155	SSGVSAACSY	10	69	76	LGDCCTAG	8	93	98	WSYIVE	6
267	277	APEYAFALVRG	11	139	145	SWPVHYA	7	150	156	TAACPHA	7
347	352	FGAIAG	6	175	184	YPTLAASYAN	10	264	270	NLVVPRY	7
462	469	LYEKVKLQ	8	247	253	YWTLLRP	7	289	295	VHDCNTT	7
				287	297	AVMCECEAKCQ	11				
				460	469	KKLYEKVKAQ	10				

Note: Here are peptides generated based on the antigenicity analysis performed on the H1 HAs: Peach colour represents shared epitopes amongst cHA-C and cHA-E only; blue colour represents unique epitopes on CVV-M, and grey colour represents epitopes that are unique to C. Peptide epitopes were generated following antigenicity analysis based on Kolaskar and Tongaonkar algorithms at the threshold of 1.000.

Table 7:Unique epitopes of the H3 HAs.

cHA-A				CVV-S			
Start	**End**	**Peptide**	**Length**	**Start**	**End**	**Peptide**	**Length**
221	226	AICSCI	6	153	158	SACIRG	6
428	434	IKGVLKS	7	172	182	HLNYKYPALNV	11
470	486	CNICIGCFEIF HKCDDA	17				
512	518	IDPVKLS	7				
523	529	DAILWFS	7				
531	549	GASCFALLAI AMGLVFICA	19				

Note: Here are peptides generated based on the antigenicity analysis performed on the H3 HAs: Green coloured epitopes are unique to cHA-A, whereas the peach-coloured epitopes are those unique to CVV-S. Peptides epitopes were generated based on the Kolaskar and Tongaonkar algorithms at the threshold of 1.000.

Discussion

Influenza viruses are "cunning", and perpetrate massive morbidities and mortalities annually, utilizing a subtle modification of their genetic sequences - mostly via antigenic drift. Drifting is a combinatorial evolutionary factor that has, over the years, maintained the seasonal influenza viruses among the global population (Taubenberger & Kash, 2010). With time, the genetic evolution culminates in antigenic evolution, leading to escape from pre-existing immunity, causing recurring outbreaks. It is therefore important to track the evolutionary patterns of influenza viruses, to harness relatively stable or less evolving viral components or even, hidden domains on the HA that could be used either as a potent vaccine construct or a therapeutic target.

Efforts by Nachbagauer and colleagues have repeatedly shown that immune responses could be focussed on the relatively conserved stalk domain, by replacement of the heads of the seasonal influenza HA heads with exotic head groups (Nachbagauer & Krammer, 2017). Similarly, Krammer and colleagues have repeatedly demonstrated: chimeric HAs – whose antigenic sites have been replaced with those of exotic HAs – have also increased refocussing of increased antibody titres toward both the stalk domain and other conserved areas of the HA (Krammer & Palese, 2019); chimeric HAs whose sequential administration in the form live-attenuated influenza vaccine conferred immune protection transcending stalk-specific antibody to stalk-specific cell-mediated protection against the seasonal influenza p(H1N1)09 and a heterosubtypic influenza virus (H6N1) (Liu et al., 2019). Viral-vectored chimeric H3 HAs formulated with fused internal genes (NP and M1), conferred protection in ferrets based on IFN-γ cellular responses and stalk-specific antibodies to members of the group 2 HAs (McMahon et al., 2019).

How refocussing of antibodies to the less immunodominant conserved stalk-specific domain is achieved, is still not clear. A possible speculation is that some conformational distortions in the head region could influence this mechanism that increases the immunogenicity of the supposedly less immunogenic domain. Therefore, improving these HA-based immunogen designs with retention of the conserved regions across the length of the molecule and replacing polymorphic sequences or sites with sequences from exotic HAs - H5, H9, and H7 might also be useful.

In this regard, multiple sequence alignment on thousands of our select seasonal influenza HA types were performed to ascertain a clear signature of the relatively conserved regions on the HAs of the seasonal influenza A H1 and H3 viruses. Then chimeric constructs were generated by alignment with consensuses of exotic HA types whilst retaining the conserved domains (confirmed by antigenicity analysis in Appendices B and C).

Based on these assessments, the cHA constructs maintained their structure as influenza A HAs and sialic acids were predicted to be most likely their ligands. Predicted structures and functions give a broad view of HAs possessing intact conserved identity, and this was further corroborated by over 80% similarities of cHA amino acid sequences to hits on the NCBI database.

The Bepi-Pred 2.0 has been shown to offer a better predicting power as predictions are based on data derived from crystal structures – which are presumed to be of high quality (Larsen, Lund, & Nielsen, 2006; Singh, Ansari, & Raghava, 2013). So next, cHAs were assessed for B-cell epitopes. Epitope probabilities from here showed relatively comparable indicators to cHAs compared with the controls. For instance, analysis of the medians of these epitope probabilities demonstrated that cHA-C was relatively superior to both cHA-E and CVV-M, as its median surpassed the threshold set at 0.5; there was only a minor difference between cHA-C and CVV-

M, although none of the differences was statistically significant. Also, in terms of antigenicity, cHA-E demonstrated a lead by crossing a set threshold at 1.000, with the others lagging in order of cHA-C and then CVV-M. However, differences in antigenicity were insignificant. On one hand, cHA-C had the highest mean of the B-cell epitope probability score, and this could be justified by the availability of 2 extra epitopes (1, unique and 1, shared with cHA-E) more than cHA-E and CVV-M. However, the assessment of antigenicity is also dependent on the potential of a predicted epitope to be antigenic, meaning that not all epitopes predicted could be potentially antigenic, hence, cHA-E being more favoured, probably due to the presence of limited but more antigenic epitopes. Analysis of the mean B-cell epitope probabilities for cHA-A and CVV-S showed that the epitopes on cHA-A were relatively higher than CVV-S, even though, means of both cHA-A and CVV-S crossed the set threshold, indicating both as molecules enriched with B-cell epitopes. Subsequently, analysis of the means of the antigenicity scores showed that the mean antigenic score of cHA-A was significantly higher than CVV-S, and this was confirmed by the presence of two extra unique epitopes.

Lastly, it is thought that the influenza viral infection occurs by an initial environmentally influenced exposure of some of the conserved HA regions orchestrating its cleavage and fusion to the endosomal membrane of the cell (Stevens et al., 2004). Therefore, alterations done in the generation of the cHAs could play a role in exposing some of these key conserved areas; and that may potentially render the cHAs as better immunogens that could stimulate broad-neutralizing antibody responses against the relatively conserved regions. This is justified, partly, by the presence of extra epitopes on cHA-C, that does not even affect its mean antigenicity scores, though exposed as potential B-cell epitopes.

Materials and Methods

HA sequence selection and consensus sequence cHA construction in silico.

Haemagglutinin (HA) nucleotide sequences (for H1, H3, H5, H7, and H9) were selected at random from the Influenza Research Database (Zhang et al., 2017). Downloaded sequences were viewed using the BioEdit Sequence Alignment Editor software (Hall, 2018). For each set of HA sequences downloaded, multiple alignment analyses were performed by uploading files onto the online MAFFT software (Katoh, Rozewicki, & Yamada, 2019) and ran at default parameters. Each multiple aligned sequence file was exported to the BioEdit software for editing (i.e., trimming of ends of sequences and retaining the start and stop in order of 5' to 3'). Subsequently, each of the nucleotide sequences that comprise the multiply aligned sequences were translated into amino acid sequences and was followed by the generation of a consensus sequence- one sequence representing all multiply aligned sequences. Multiply aligned protein sequences were uploaded again unto the MAFFT server, where the phylogeny analysis was performed: phylogenetic trees were viewed on the java-enabled archaeopteryx software (Han & Zmasek, 2009).

Due to the diversity of sequences for each type of consensus HA, resulting amino acids had gaps that represented polymorphic sites. Considering that chimeric HAs have shown tremendous results in terms of antibody refocussing to the less immunodominant and conserved HA stalk domain (Krammer & Palese, 2015; Nachbagauer, Liu, Choi, Wohlbold, Atlas, Rajendran, Solórzano, Berlanda-Scorza, García-Sastre, Palese, et al., 2017), chimeric HAs were developed by aligning separately different types of group 1 and 2 consensus amino acid sequences, to call amino acids into the polymorphic sites on the seasonal influenza HA amino acid sequence types (i.e. H1 and H3) (Appendix A). In areas where amino acid calls were impossible due to lack of gaps in the template, Alanine (Ala) was the amino acid assigned. In

this design, two group 1 cHAs were designed [H1/H5 HA and H1/H9] and one group 2 cHA was designed [H3/H7 HA]. BLASTp analysis on the NCBI server was performed to confirm that cHAs are HAs (Cock, Chilton, Grüning, Johnson, & Soranzo, 2015).

cHA structural and functional predictions

Based on the encouraging BLASTp results, structure, and/ or functional predictions were made using the I-TASSER platform (Zheng, Zhang, Bell, & Zhang, 2019). cHAs were predicted to preferentially bind to sialic acids and structures were like that of a typical influenza A virus HA monomer (Table 3). The closest models were fetched from the Protein Database (https://www.rcsb.org/) and visualized in PyMol version 4.6.0 (Schrodinger, 2010).

cHA B-cell epitope and antigenic predictions

Noting that cHAs are haemagglutinin-like in both function and structure, further immune parameters, like B-cell epitope prediction (performed on the webserver http://www.cbs.dtu.dk/services/BepiPred-2.0/) and antigenicity prediction (performed on the Immune Epitope Database and analysis resource platform, based on Kolaskar and Tongaonkar algorithms) were done (Dhanda et al., 2019). For the B-cell epitope prediction, FastA formatted amino acid sequences were deposited on the afore-specified web interface and epitope probability scores were recorded at the default threshold of 0.50. Figures 17 and 18 display the predicted epitope scores for the H1 and H3 HAs, respectively. Means of the scores were further assessed for significant differences by one-way ANOVA and unpaired t-test for the H1 and H3 setups, respectively, in comparison to controls [the 2018/ 2019 candidate vaccine viruses' HAs: A/Michigan/45/2015 (H1N1) pdm09-like virus, CVV-M and A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus, CVV-S]. Outcomes of the assessments are presented in Figure 17 A and B and Figure 18 A and B. Further, the predicted antigenic peptides were analysed

based on the HA groups: all common peptides were eliminated, whilst unique peptides present in each of the HA sequences, were documented (Tables 6 and 7).

CHAPTER 5: EXPRESSION OF CHIMERIC HAs IN S2 CELLS

Abstract

The design and advancement of candidates in the search for more promising universal influenza vaccines are ongoing. Diverse approaches for the design, form and administration of immunogens are being explored: the nucleic acid vaccine component versus the conventional protein or whole virus component; the cell systems for expression of a potential immunogen; the most appropriate experimental adjuvant; and the approaches of vaccine delivery. There is a headway with the use of chimeric haemagglutinins (either as proteins or borne on viruses). The cell system for the expression of proteins occasionally plays crucial roles in modifying their antigenicity.

It was, thus, worthy to explore diverse means to enhancing immune responses to the chimeric haemagglutinins that were designed in previous experiments. Insect cells have been associated with minimal carbohydrate moiety modification of expressed proteins, and increased breadth of antibody responses against these proteins.

In this experiment, the in-silico designed chimeric haemagglutinins were successfully expressed to produce higher yields in the Drosophila embryo-derived S2 cells.

Introduction

The conventional propagation of influenza vaccine viruses has faced several challenges: egg-adapted variants are faced with antigenic differences from the parental virus stock, which defeats the purpose for which the vaccines were made; similarly, issues of reactogenicity and potential contamination limit the advancement of egg-based influenza vaccines; and more importantly, scaling up egg production for vaccine virus expansion is not pragmatic (Audsley & Tannock, 2008).

Therefore, should there be any pandemic, advancement of influenza vaccine virus expansion by cell-based approaches would offer a robust platform for more rapid development, large-scale production, and efficient purification (Doroshenko & Halperin, 2009; Tseng et al., 2018). This is because cell culture-enhanced production of viruses or their proteins have been used successfully to induce protective cross-reactive humoral and cell-mediated immunity against influenza virus infection among elderly people -one of the core high-risk groups (Dunkle et al., 2017; Flórido et al., 2018). Also, experimental pieces of evidence indicate that the recombinant influenza virus HA fractions offer a high degree of protection, transcending homotypic viruses, in mice or ferret challenge experiments (Wohlbold et al., 2015; Yamada, Yasuhara, & Kawaoka, 2019).

But, although cells and their cultures remain a relatively better platform for vaccine production, the type of cell system could adversely impact the vaccine formula. For instance, an unfavourable cell type could render an impact on the antigenicity of a vaccine formula due to a varied glycosylation pattern among different cell types, and thereby immensely affecting the antigenicity of proteins or the vaccine viruses (de Vries et al., 2012; Wohlbold et al., 2015; Yamada et al., 2019).

Further, recombinant proteins are more likely to be less immunogenic and may require specific adjuvants to enhance this property; In spite of this shortfall, they remain an important resort in vaccine design due to their massive stable production (Anthony et al., 2016).

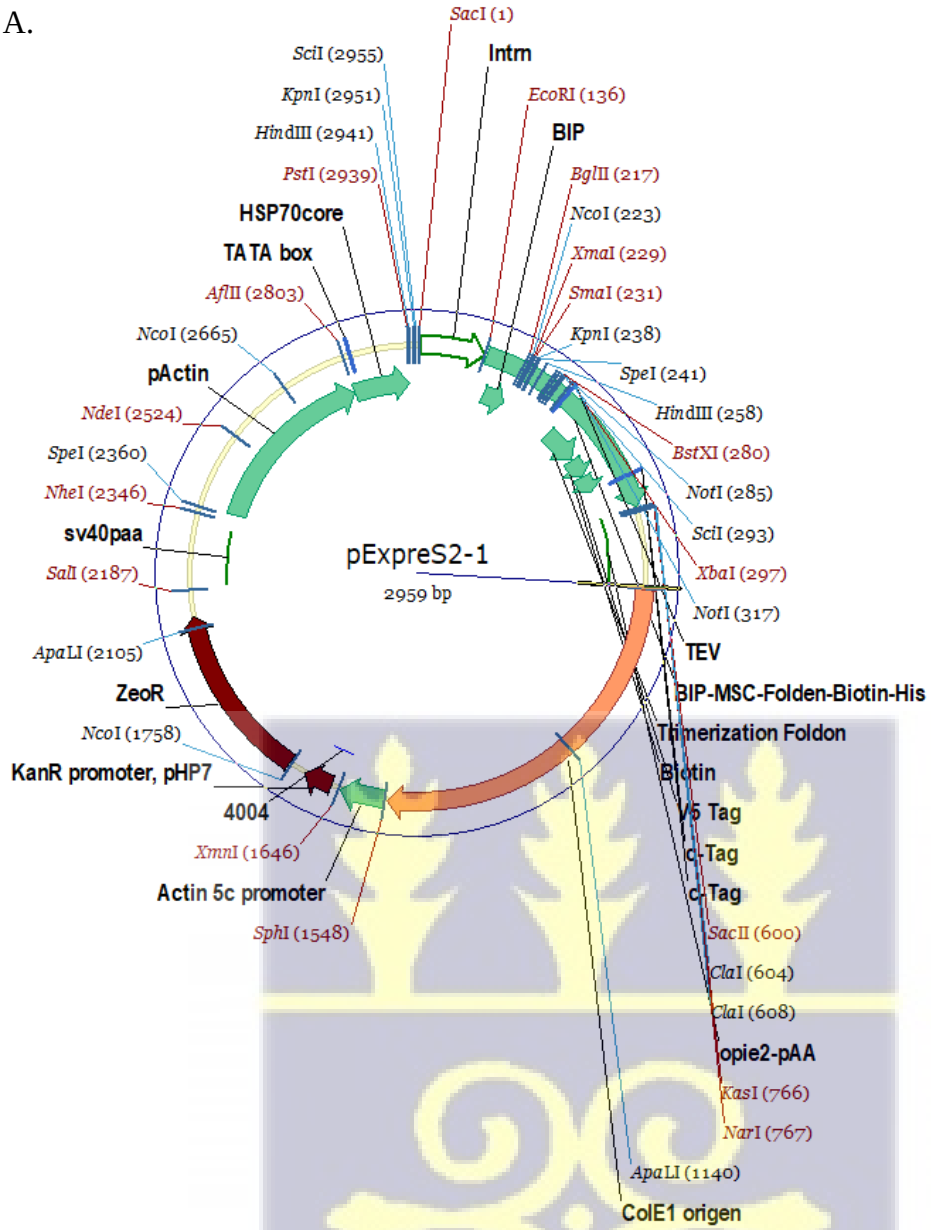
Considering the importance of recombinant proteins in vaccine production, they have the potential to serve as an avenue to explore novel unconventional molecules as is the current interest for the discovery of a universal influenza vaccine candidate. Drosophila S2 cells typically offer an immediate avenue to express several recombinant protein types for both screening and vaccine purposes (de Jongh, Salgueiro, & Dyring, 2013).

cHAs were, therefore, prepared for expression in drosophila S2 cells, with interest in exploring novel post-translationally modified proteins that may be suitable for inducing broadly cross-reactive antibodies.

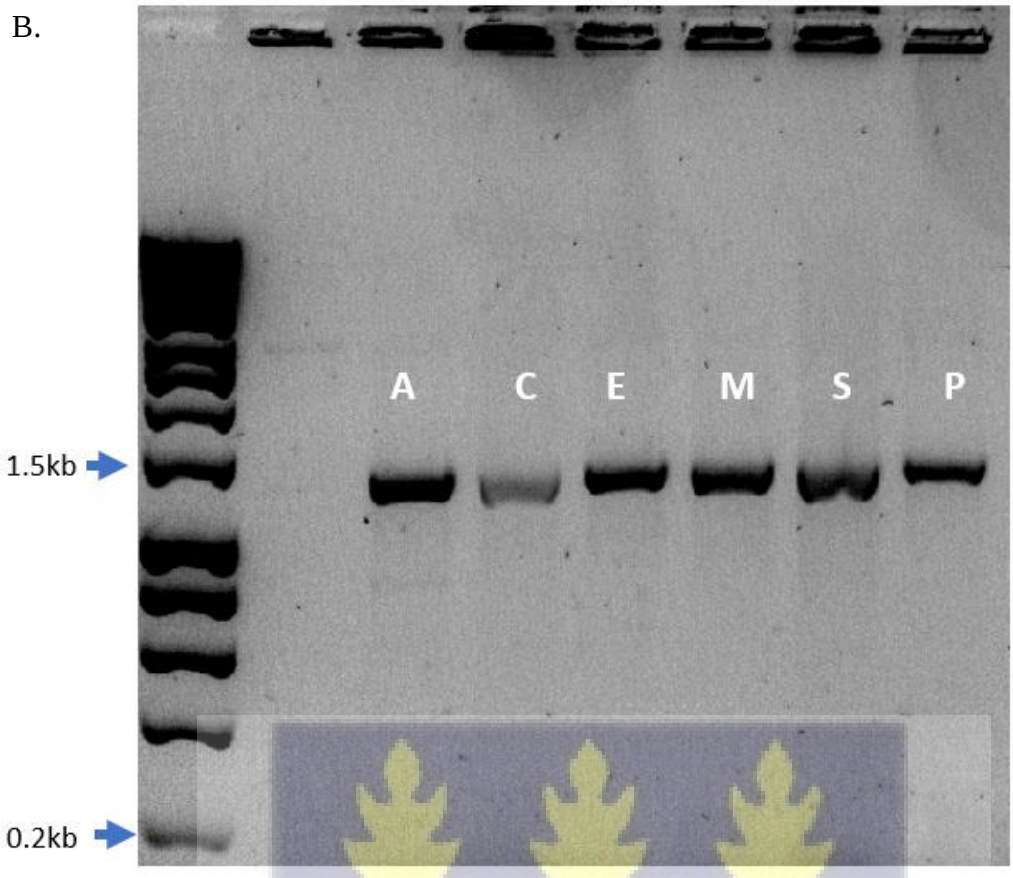
Results

Processed cHA sequences were cloned first on the GeneArt plasmids and then subcloned into the pExpreS2 plasmids. Correct orientations of inserts on plasmids were confirmed by both RFLP and sequencing (Figure 19A, B, C and D).

A.

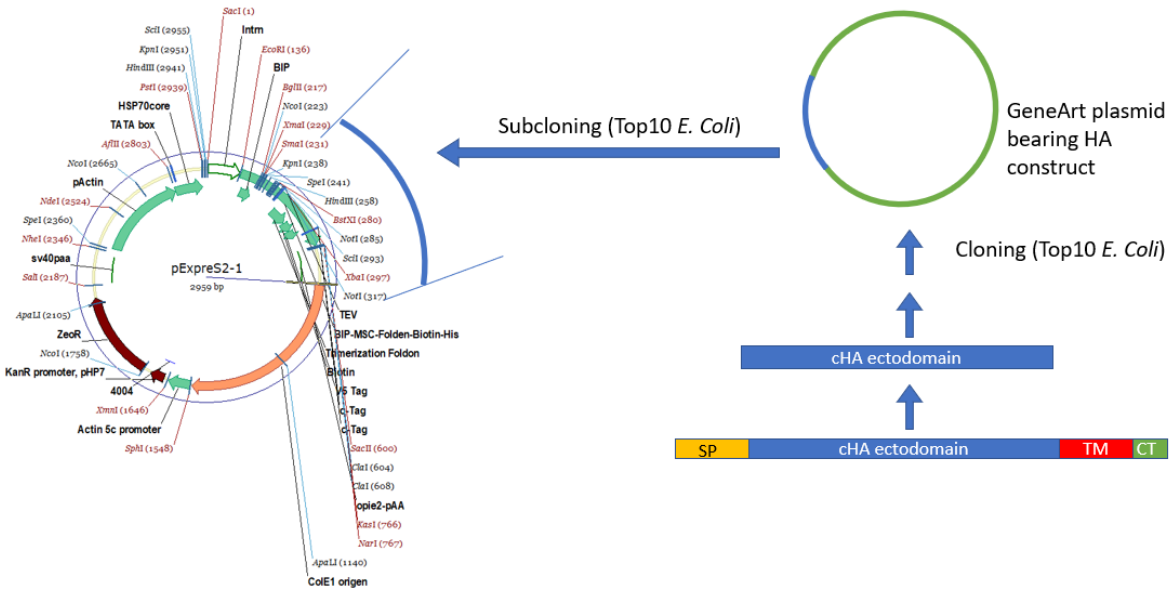


B.



C.

iii



D.

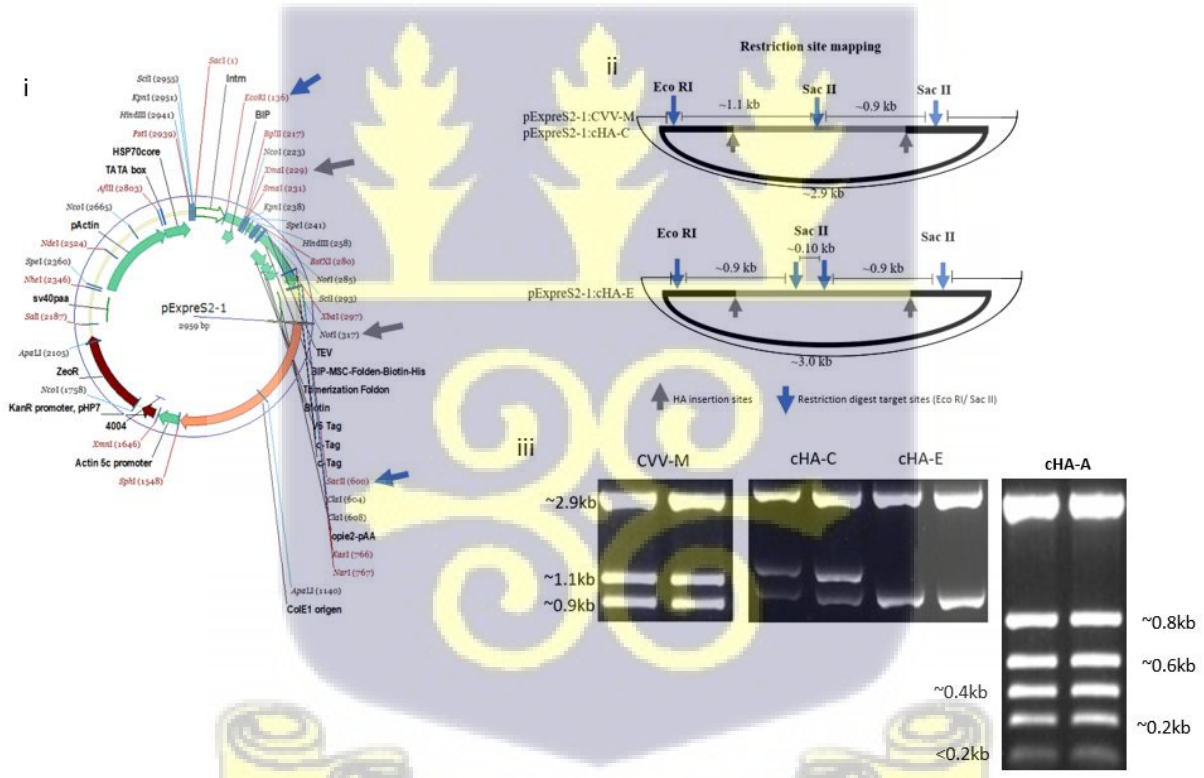


Figure 19: Cloning of cHAs.

A: the features of the pExpreS2-1 cloning/ expression vector. Sequence insertions were performed in between Sma I (231) and Not I (317) sites. Downstream to insertion sites are the V5- and C-tags to aid in the detection and/ or purification of expressed proteins. The presence of a selectable Zeocin resistance marker to aid in cloning and transfection selection processes. The presence of the: TATA box aids the constitutive expression of proteins;

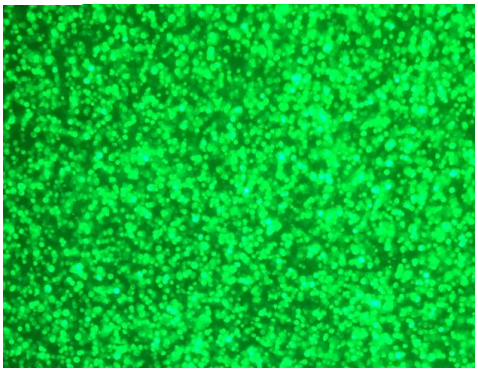
BiP, aids in the secretion of expressed proteins; trimerization foldon aids in folding of the expressed proteins; B: The cHAs (cHA-A, cHA-C, cHA-E) and controls (including CVV-M, CVV-S, and an in-house H5 HA) were PCR assessed on colonies of transformed Top 10 *E. coli* cells grown on solid agar to confirm successful cloning ; C: i, Electrophoretic DNA bands of constructs cHA-A, cHA-C, cHA-E, CVV-M, and CVV-S, each digested with Sma I and Not I, and gel-purified in preparation for ligation with Sma I- and Not I- cut pExpreS2-1 (C ii). Of the 5 constructs, only CVV-S was directly subcloned unto the pExpreS2-1, whilst the others were first corrected from frameshifts; C iii. Schematic of the subcloning of HA constructs into the pExpreS2-1 plasmid. Signal peptides (SP), transmembrane domains, and cytoplasmic tail (CT) were trimmed off to enable cloning of the ectodomains of all the constructs; D: i. Plasmid map detailing HA insertion sites between Not I and Sma I (grey arrows or grey outline in i.) and existing Eco RI and Sac II restriction sites (blue arrows). ii. Representation of Eco RI and Sac II restriction digest sites showing anticipated electrophoretic band patterns in cHA-C, cHA-E, and CVV-M. iii. Restriction digest confirming expected electrophoretic band patterns: Sac II cuts twice the plasmid with the CVV-M and cHA-C at similar sites and Eco RI cuts once, hence the three similar band patterns; cHA-E on the other hand, displays 3 Sac II restriction sites, in addition to one Eco RI site, leaving two major bands. Sac II cuts cHA-A at 3 sites, whilst the Eco RI cuts just once producing 5 prominent bands. Plasmids were also sequenced to confirm the orientation (not shown).

Cloned plasmids for each of the cHA constructs, in addition to the two candidate vaccine viruses HAs (Appendix E) and a green fluorescent protein control was further used to transfect S2 cells to establish stably transfected cells expressing the cHAs. Transfection efficiency was notable by the transfection control GFP-expressing cells (approximately, 90%) during the third-day post-transfection (Figure 20A).

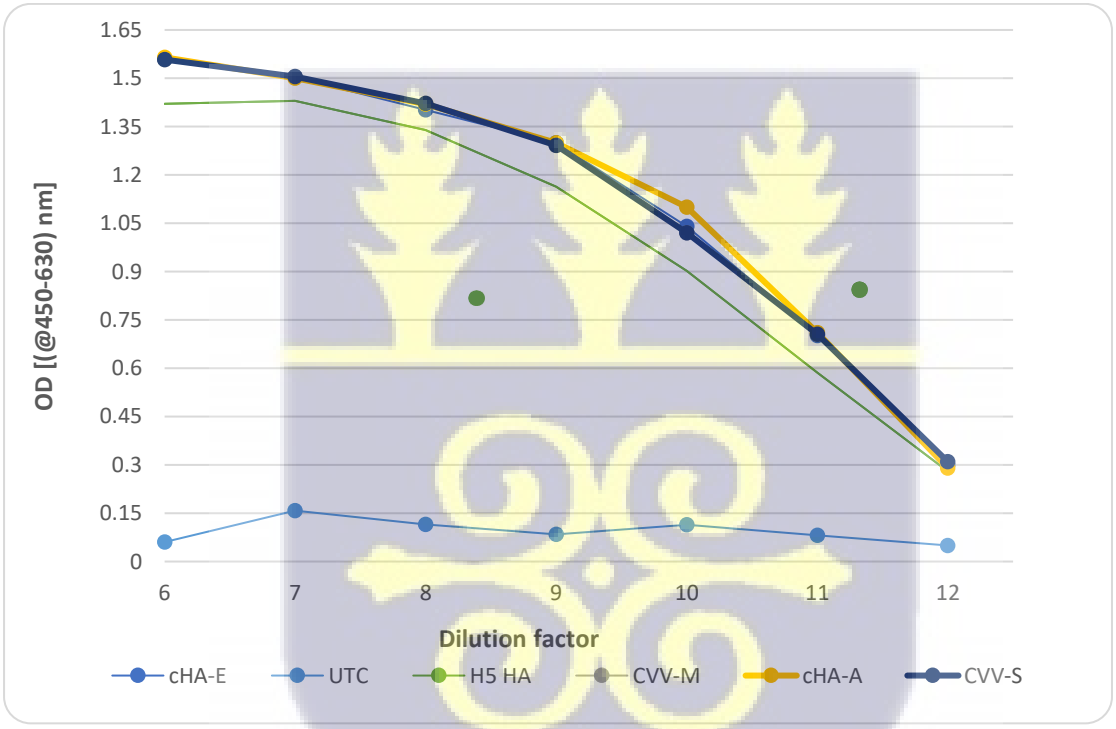
The presence of expressed proteins in both cell lysates and cell-culture supernatant was confirmed by ELISA (Figure 20B) and/ or Western blot (Figure 20C). cHA-A, cHA-C, and cHA-E were better expressed in cell lysates, whereas only supernatant was satisfactory to confirm the expression of CVV-M and CVV-S control constructs by Western blot.

Furthermore, utilizing hemagglutination assays, none of the S2 cell-expressed constructs could show any reaction; only the control (laboratory H5 isolate) recorded consistently titres of 128 on turkey blood.

A



B



C

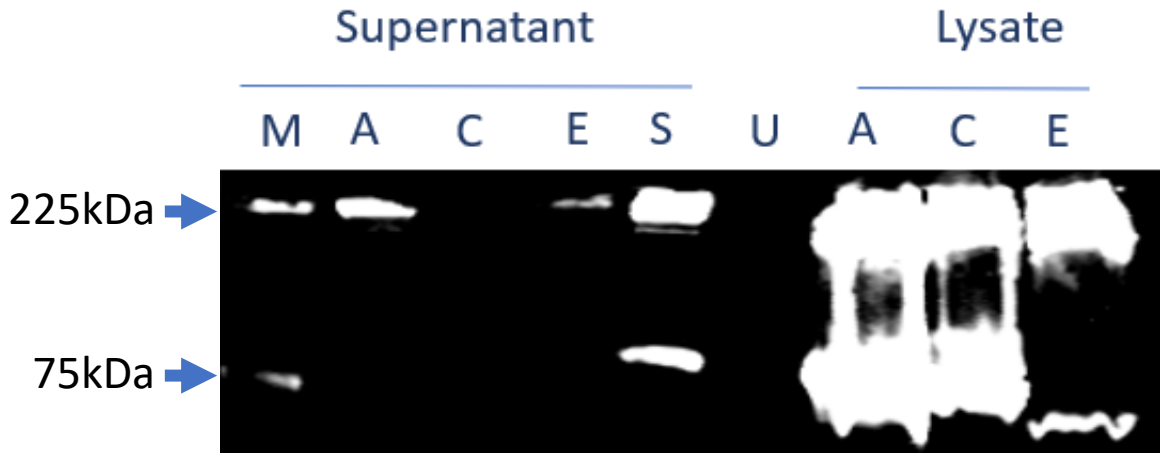


Figure 20: Rapid transfection checks on day 3 post-infection.

A: Here are pExpreS2-1-GFP transfected S2 Drosophila cell lines captured at a magnification of X10, on day 3 post-transfection. B: Supernatants collected over cHA-C-, cHA-A-, CVV-M-, and CVV-S-expressing cells, an in-house H5 HA-expressing cells (as control), and an un-transfected cell control [UTC (ELISA) or U (Western blot)] were harvested first and evaluated by ELISA to validate the expression profiles. Optical densities of each construct at varying dilutions were plotted against their corresponding dilution factors. The focus was placed on dilution factors from 6 to 12 as those exhibited consistency in the decline of optical densities of the constructs with increasing dilution. C: Supernatants and cell lysates of cHA-expressing setups were taken through Western blotting further confirmation of expression, especially in cell lysates as not all constructs were being expressed in the supernatant over the transfected cells.

Discussion

Influenza vaccines are evolving rapidly to match the pace of seasonal influenza viruses. Diverse approaches are being experimented on to meet global demands for both highly effective and universal vaccine candidates. Unconventional vaccine designs have seen more promising experimental outcomes. Thus, this study aimed to design and express atypical cHAs completely possessing their core structure of the conserved amino acids (on H1 and H3 HAs), with preferential insertions of amino acids from consensus sequences of exotic HAs.

In preliminary experiments involving cHAs and their sequential administration regimes, studies showed tremendous induction of stalk-specific antibodies when mice and/ or ferrets were immunized (Nachbagauer, Liu, Choi, Wohlbold, Atlas, Rajendran, Solórzano, Berlanda-Scorza, García-Sastre, Palese, et al., 2017). Subsequent experiments revamping this formulation with viral vector-based administration had met striking induction of specific T-cell responses in addition to the antibody responses (McMahon et al., 2019).

The Drosophila Schneider line 2 cells, derived from late-stage embryos of *Drosophila melanogaster* have been distinguished among other cells as easy-to-transfect, non-adherent, adaptable to grow in serum-free medium, and to also be useful as a source for the syntheses of high levels of a plethora of functional proteins, including exogenous and potentially toxic proteins (Bunch, Grinblat, & Goldstein, 1988; Lim & Cha, 2006; March, Gross, & Long, 2010; Matsumura, Saito, Jackson, Song, & Peterson, 1992; Schneider, 1972).

In this study, attempts were made to express the 3 previously *in silico*-designed cHAs using the Drosophila S2 cells. The in-house modified plasmid termed *pExpreS2*-1was used as the expression plasmid. All 3 cHAs (A, C, and E) including the candidate vaccine virus HA controls (M and S) were successfully cloned on the plasmid. Subsequently, S2 cells were transfected with the plasmid constructs in separate experiments. The transfection process

showed approximately 90% success 3 days post-transfection for all the expressed proteins (as indicated by the GFP transfection control). Supernatants of CVV-M- and CVV-S-expressing cells yielded electrophoretic bands on SDS PAGE at both 50 kDa and 250 kDa, indicating approximately the monomeric and trimeric versions of HAs, as shown elsewhere (Magadán et al., 2013). Also, except for cHA-C, which did not show any expression in the supernatant, cell supernatants of A and E, showed the trimeric HAs only. However, all three cHAs (A, C, and E) had band patterns for both the monomeric and trimeric HAs when their corresponding cells were lysed and electrophoresed on SDS PAGE. We speculate that introduction of exotic amino acids into the core conserved structure of cHA-C might have resulted in the formation of some membrane interacting domains that restricted the expression into the supernatants, or better still, the design process led to the formation of a HA molecule too disordered to be folded properly and hence, their deposit in the inclusion bodies of the cell; Thus, only available for detection in the lysed cell setup. The former explanation partly applies in the case of CHA-A and cHA-E, where the trimeric versions of the HA were present in both supernatants and lysed cell setups, but monomeric HAs were present only in the lysed cell setup. Designs in the two molecules probably favoured the expression of only the monomeric HA in the supernatant, whilst folded trimeric versions were packed as inclusion bodies of the cells.

Supernatant-based proteins as well those from lysed cells were further purified and yields of protein expressed per 500 mL were averagely 2 mg/ mL; Though proteins expressed could not haemagglutinate turkey red cells (Appendix F), their expression in the S2 cells denotes a remarkable system for exogenous recombinant protein expression.

Materials and Methods

cHA processing

To express these proteins, both signal peptide and transmembrane domains were predicted and trimmed off, with the aid of the SignalP-5.0 server (Armenteros et al., 2019) and the ExPASy TMpred tool (Swiss Institute of Bioinformatics, Swiss), respectively. This was done because, secreted proteins were more preferred due to abundance in cell protein expression by several rounds of passage, as opposed to lysing of cells that express membrane-bound proteins (due to the presence of transmembrane domains). Also, due to the presence of a pre-existing signal peptide sequence already engineered onto the expression vector, pExpreS2-1, cHA-signal peptide was needless. Other characteristic features of pExpreS2-1 include the presence of the binding immunoglobulin protein (BiP) chaperone- which has previously been shown to enhance the production of secreted proteins in cell expression systems (Ailor and Betenbaugh, 1998, 1999; Hsu & Betenbaugh, 1997; Whiteley, Hsu, & Betenbaugh, 1997), a TATA promoter- that drives constitutive expression, a zeocin-resistance selectable marker, and V5- and C-tags- both aiding detection and purification of expressed proteins.

Trimmed amino acid sequences were back-translated into nucleotide sequences using the online Emboss back transeq tools (European Bioinformatics Institute, United Kingdom), and this was done to take care of codon usage bias in Drosophila- since the Drosophila S2 cells were used for the cHAs expression. By translating sequences with the ExPASy translate tool (Swiss Institute of Bioinformatics, Swiss) cHA sequences were codon-optimized for expression in S2 cells. Nucleotide sequences were then inserted into pExpreS-1 to check for the right frame of the protein to be expressed on the Vector NTI Advance (R) 11.5.4 platform (Invitrogen). In any case, where there was a frameshift, a nucleotide sequence at the 3' end was either added on or deleted. Resultant nucleotide sequences are as shown in Appendix A, with

an indication of restrictions sites: Sma I and Not I (New England Biolabs, UK) at the 5' and 3' ends of cHAs, respectively.

cHAs Cloning and plasmid purification.

Correct in-frame cHA nucleotide sequences were synthesized by GeneArt (Thermofisher, UK). Synthesized cHAs (borne on GeneArt plasmids – pMK-RQ (KanR) for cHA-E, cHA-A, cHA-C, and CVV-S; and pMA-T for CVV-M) were reconstituted with nuclease-free water to make up a concentration of 5 µg/mL, as directed by the GeneArt manufacturers. Subsequently, 5 µL of each plasmid bearing the cHA was used to transform a 50 µL of competent Top 10 *E. coli* cells (Thermofisher, UK). After SOC medium (Sigma-Aldrich) enhancement of growth of transformed cells at 37^{o}C for an hour, 50 µL of the transformed cells were plated on LB agar (supplemented with either Kanamycin or Ampicillin based on GeneArt parent plasmid bearing each cHA construct) and incubated overnight. The following morning, plates were each assessed for the growth of transformants. Colony PCRs were performed on select colonies from each plate on which transformed *E. coli* were grown (Figure 19 B). Concurrently, the same select colonies used for the PCR were subcultured in designated areas on new plates. Following PCR confirmation of colonies with plasmids bearing each of the cHA, these colonies were each inoculated in 5 mL Kanamycin or Ampicillin-supplemented LB broth (in universal tubes), and then grown overnight at 37 ^{o}C whilst shaking. Cultures were harvested, 1 mL of each, temporarily stored at 4 ^{o}C, and then 4 mL of each is poured into 15 mL falcon tubes and spun at 4, 600 rpm for 10-15 minutes. Supernatants were discarded using the Qiagen miniprep kit and its associated procedures. Cells were lysed; plasmid DNA precipitated; washed and then eluted in 50 uL and quantified using the NanoDrop spectrophotometer. A Sma I and Not I (procured from the New England BioLabs) restriction double digestion was set up with about 1-3 µL of the plasmid and incubated for up to 4 hours for efficient digestion. A 1% agarose gel was used to confirm the expected restriction digest DNA bands sizes that are informative of

successful cloning procedures (Figure 19C). Subsequently, electrophoretic bands indicating the expected size of each cHA insert was cut from the gel under blue light; and extracted using the Qiagen gel extraction kit. Cut DNA was eluted in about 30 uL of the kit-provided elution buffer: which was just enough for ligation onto the pExpreS2-1 plasmid (The Avian Influenza Group, The Pirbright Institute). About 7 uL of the eluted insert was ligated [using T4 DNA ligase and T4 DNA ligase buffer (New England Biolabs, UK)] with 3 uL of Xma I or Sma I- and Not I-cut-pExpreS-1 plasmid (gel extracted and quantified) [Figure 19 C]. Cloning was repeated as demonstrated earlier, using zeocin as the selectable antibiotic supplement in both LB agar and LB broth. Plasmid DNAs were similarly isolated from 4 mL of LB broth-grown transformed E. coli. Aliquots were shipped out for sequencing by Eurofins to confirm the orientation of insert, and a restriction digest was set up to also confirm the successful insertion of each cHAs to the pExpreS-1 vector. Following confirmation of desirable insertion by both RFLP (Figure 19 D) and sequencing, 1 mL remnant of each of the transformed E. coli cells, was partly used in the preparation of glycerol stocks (comprising 500 uL each of glycerol and E. coli culture) and the rest, inoculated in 250 mL LB broth (supplemented with zeocin) and incubated overnight at 37^{o}C whilst shaking. All the overnight cultures were poured in 50 mL falcon tubes and plasmid DNAs were isolated with adherence to the Qiagen Maxiprep protocol. Plasmid DNAs were eluted (500 uL each) and then quantified with the NanoDrop (Appendix E) and stored at -20 ^{o}C, until later use for the transfection of Drosophila S2 cells.

Drosophila S2 cell culture preparation

S2 cells (obtained from The Avian Influenza Group, The Pirbright Institute) were recovered from a -85°C freezer; two vials were rapidly thawed, and each vial cultured in a T25 cm^2 flask containing about 5 ml of Schneider's S2 medium (supplement with 10 % foetal calf serum, FCS), for up to 3 days at 28°C, in the absence of CO_2, until a microscope-aided observation of confluence of about 80-90 %. Each of the flasks containing the cells was then split and grown

in T75 cm^2 flasks at similar conditions for up to 3 days. Cells were then counted using a haemocytometer, with which cell estimates of 1.0E6 cells were made and seeded in T25 cm^2 flasks and allowed for 24 hours' incubation at 28°C before transfection.

cHAs Expression in S2 cells

The transfection process involved the dropwise transfer of a mixture of a calcium chloride treated-plasmid DNA (32 µg), plus water, to bubbling HEPES-buffered saline. The resultant solution was incubated for up to 1 hour and subsequently, added to cells at about 3.0-5.0 E6 cells. Transfection controls involved a known H5 HA-pExpreS2-1 plasmid, a GFP-bearing pExpreS2-1 plasmid, and an un-transfected cell. Transfected cells were incubated at 28°C. FCS-supplemented S2 medium was changed over transfected cells 24 hours post-transfection and was further incubated for 72 hours, until antibiotic selection, that involved the use of 750 µg/ mL zeocin in FCS-supplemented S2 medium. Concurrently, supernatant over the cells was harvested for and stored for protein expression check by either ELISA or Western blot. After 4-5 days through the incubation process, the concentration of zeocin was increased to 1,500 µg/ mL in the S2 medium, which was used to further select transfection-confirmed flasks. This was performed 3-4 times after every 4-5 days until about day 30, to establish stably transfected S2 cells.

Transfection check by fluorescent microscopy or ELISA or Western blotting

Initially, the success of transfection was confirmed to be about 90% by observation of the transfection control cells that were transfected with the GFP-bearing pExpreS2-1 (Figure 20A). The following ELISA procedure required overnight incubation of supernatant-coated 96 wells in ½ serial dilution. Blocking was carried out using 5% BSA (bovine serum albumin) in 0.1 % Tween-20 in PBS (PBST) and incubated at room temperature for 1 hour. A 1/2000 anti-V5 tag

mouse antibody (primary antibody) was applied after discarding the blocking buffer and incubated for up to 1 hour. Subsequently, all wells were washed 4 times with PBST, and then rabbit-anti-mouse polyclonal secondary antibody conjugated to horseradish peroxidase (HRP) [1/4000] was added and plates covered with foil and incubated for up to 1 hour at room temperature. TMB (3, 3', 5, 5' tetramethylbenzidine) substrate was then added to each well and incubated for 15 minutes for colour development and then finally stopped by the addition of 2 M H_2SO_4. Optical densities of each well were read at (450 nm and 630 nm) using the BioTek spectrophotometer. Readings at 630 nm were subtracted from those at 450 nm to produce more precise optical densities for all expressed constructs, including an in-house H5 HA control, and an un-transfected cell control (UTC) [Figure 20B].

All cHAs-expressing supernatants and/ or lysed cells were subsequently electrophoresed on SDS-PAGE. The acrylamide gel was incubated in tris glycine methanol buffer (TGM) for 20 minutes; the nitrocellulose membrane (Amersham) was activated in water and incubated in TGM for 10 minutes; Whatman filter papers were also incubated in TGM. Proteins were blotted at current and voltage of 13 A and 2.5 V, respectively for 10 minutes onto the nitrocellulose membrane and then incubated with a blocking buffer, consisting of 10% milk in PBST for up to an hour. Blocking buffer was discarded and an anti-V5/ anti-C-tag mouse antibody (primary antibody at 1/2000) was applied and incubated for another hour. Washing with PBST was performed 4 times whilst incubating for 10 minutes during each wash step. Goat-anti-mouse secondary antibody (1/4000) conjugated to green infrared dye (800 CW) was used to probe for the cHAs for an hour, whilst setup was covered in foil. Subsequently, the setup was washed 4 times with PBST and finally, blots on nitrocellulose membranes were scanned with the Odyssey Li-Cor (Figure 20C).

Purification of cHAs

For all confirmed expressing S2 cells, supernatants were saved after every 3-4 days, whilst the medium was changed. Stably transfected cells were produced by several rounds of antibiotics selection every 4-5 days. For constructs cHA-A, cHA-C, and cHA-E, proteins were rather detectable in cell lysates, but not supernatant. Cells were subsequently grown from T25 cm^2 flasks through T150 cm^2 flasks to roller bottles, in which proteins were massively produced with FCS-free medium (Excel 420). Supernatants and/ or cell lysates were pooled for each expressing protein and stored at 4^oC before purification.

Protein-containing supernatants were each filtered using 0.22 µm filters (by Millipore); further dialyzed in PBS [with no Ca + or Mg +] (PBS-) overnight at 4^oC; and purified on a column specific to trap proteins with c-tag. In detail, the cHA purification column was mounted on a retort stand and was initially washed with PBS [containing Ca+, Mg+] (PBS+) adjusted to a pH of about 7.05. Dialyzed protein-supernatants were separately purified using a C-tag affinity matrix column [by CaptureSelect $^{(TM)}$], washing the column with about 70 mL PBS+ and bound cHAs were each eluted with about 15 mL of elution buffer (comprising 2M MgCl2 and 20mM Tris). Eluted fractions containing proteins were further dialyzed in PBS. Finally, proteins were concentrated (using the Amicon ultrafilters-3k, Millipore), quantitated using the BCA assay, and stored at -85^oC.

Expressed HA Haemagglutination activity assessment.

Using a 96-well plate, 50 µL of the concentrated cHAs (~ 2 mg/ mL) were serially diluted two-fold and challenged with an equal volume of 1% Turkey red cells. Setup was gently agitated, incubated for 30 minutes, and observed for haemagglutination. Experiments were performed in triplicate, and each was controlled using a lab-isolated influenza virus (Appendix F).

CHAPTER 6: ASSESSING ANTI-cHAs ANTIBODY REACTIVITY AND PROTECTION OF INFLUENZA VIRUS-CHALLENGED MICE

Abstract

In silico predictions of epitopes or antigenicity are necessary to assess molecules, but do not warrant the advancement of candidates for vaccine development. Pre-clinical experiments are required for the initial assessment of mainly effect and safety. Mice have been used extensively for pre-clinical laboratory experiments, mainly due to their genetic resemblance to humans and in other regards, such as prolificity, small size requiring relatively lesser numbers of cages and spaces, including (but not limited to) easy handling.

In this experiment, mice were immunized with S2 cell-expressed chimeric haemagglutinins to allow for the assessment of seroconversion, breadth of serum inhibition to influenza viruses, and ability of recipient mice to be protected against influenza virus challenge.

All the chimeric haemagglutinin-immunized mice had seroconverted by the third week post induction, and seroconverted blood against the H1-based chimeric haemagglutinins conferred inhibition to 2 distinct H1 viruses and an H5N2 virus. A similar observation was made with seroconverted mice that were immunized with H3- based chimeric haemagglutinin, which also inhibited two distinct H3 strains and an H7N7 virus. Mice pre-immunized with these cHAs were better protected post-influenza virus challenge.

Introduction

Influenza viruses have caused several disease burdens across all six continents. Due to their long existence and associated recurring outbreaks and/ or pandemics, there have been tremendous efforts to better influenza management: Treatment with potent antivirals through to using egg-grown candidate vaccine viruses to formulate seasonal vaccines have had a remarkable impact, especially on the degree of mortality (Blanton et al., 2017; Hughes et al., 2019).

However, the conventional egg-grown vaccines have suffered several setbacks including (but not limited to) mismatch, potential contamination, suboptimal efficacy, and scaling up (Audsley and Tannock, 2008). This situation has driven scalable approaches such as the production of candidate vaccine viruses in cell cultures, which could also minimize potential contaminants (Audsley and Tannock, 2008).

Other approaches have looked at bettering the immunogenicity of the viral surface proteins (HA and/ or NA) to enhance the overall efficacy of the vaccine formula produced; With this, two mains arms that are being explored comprise the modification of viral parts on a whole virus, such as mosaic H3N2 viruses designed to confer immunity to mice against divergent strains of H3N2 (Broecker et al., 2019b); and the other, modification of viral proteins. The second approach has gained more attention as it is amenable to a wide array of modifications, including the generation of chimeric HA, giving rise to antibodies refocussed on the less immunodominant, relatively conserved HA stalk domain (Krammer & Palese, 2019; Liu et al., 2019; McMahon et al., 2019; Nachbagauer & Krammer, 2017; Nachbagauer, Liu, et al., 2017a). Along similar lines, “mosaic” HAs displayed on nanoparticles triggered broadly-neutralizing antibodies against diverse ancient to current strains of H1N1 viruses (Masaru Kanekiyo et al., 2019).

Also, immunization of ferrets with viral-vectored chimeric HA and the relatively conserved internal gene products (fused NP and M1) have shown remarkable protection in ferrets (McMahon et al., 2019). Other proposed viral vectors have been extensively reviewed by de Vries and Rimmelzwaan (de Vries and Rimmelzwaan, 2016).

More so, the adjuvants MF59 and AS 03 have been shown to enhance the immunogenicity of the conventional vaccines administered to children (Wilkins et al., 2017). Likewise, the alum adjuvanted B-cell activating factor incorporated a virus-like particle, which was shown to induce broadly-neutralizing antibodies that protected mice against an H5 virus challenge (Hong et al., 2019).

Convalescent plasma and serum have also gained momentum in their potential application as either prophylaxis or treatment for influenza management (Berry et al., 2014). Antibody composition in the blood of recovering or recovered persons has been shown to exhibit a more virus type-specific neutralizing capacity, as they mostly target the receptor-binding sites of the HA of the infecting virus (Ni, Kondrashkina, & Wang, 2013; Tewawong et al., 2015). Therefore, convalescent serum/ plasma could be more applicable to persons infected with the same strain of the virus, or another strain with a similar antigenic property. More broadly neutralizing and cross-reacting antibodies are thus most desired for the management of influenza.

However, as noted earlier, natural infections and/ or conventional vaccination regimens rarely induce stalk-specific antibodies – with the capacity to inhibit a wide array of influenza viruses (Ni et al., 2013; Tewawong et al., 2015). Therefore, this study assessed the protective capacity of induced antibodies when mice immune systems are stimulated with the currently designed chimeric HAs.

Results

Mice selected for immunization and challenge experiments are pristine.

Mice were grouped (n=5) into 7 groups: 2 naïve controls (U and X) and A, C, E, M, and S. Before the first immunization, blood was drawn from each mouse in a group and group-specific blood specimens were pooled together for serological analyses by HI (Figure 20). The HI testing against 2019 WHO serological H1 and H3 antigens and experimental challenge viruses (A/ England/195/2009 and A/ARI-19-361/2019), showed no signs of mice exposed to any of the viruses as all HI titres recorded from the challenge were <20 (Appendix G).

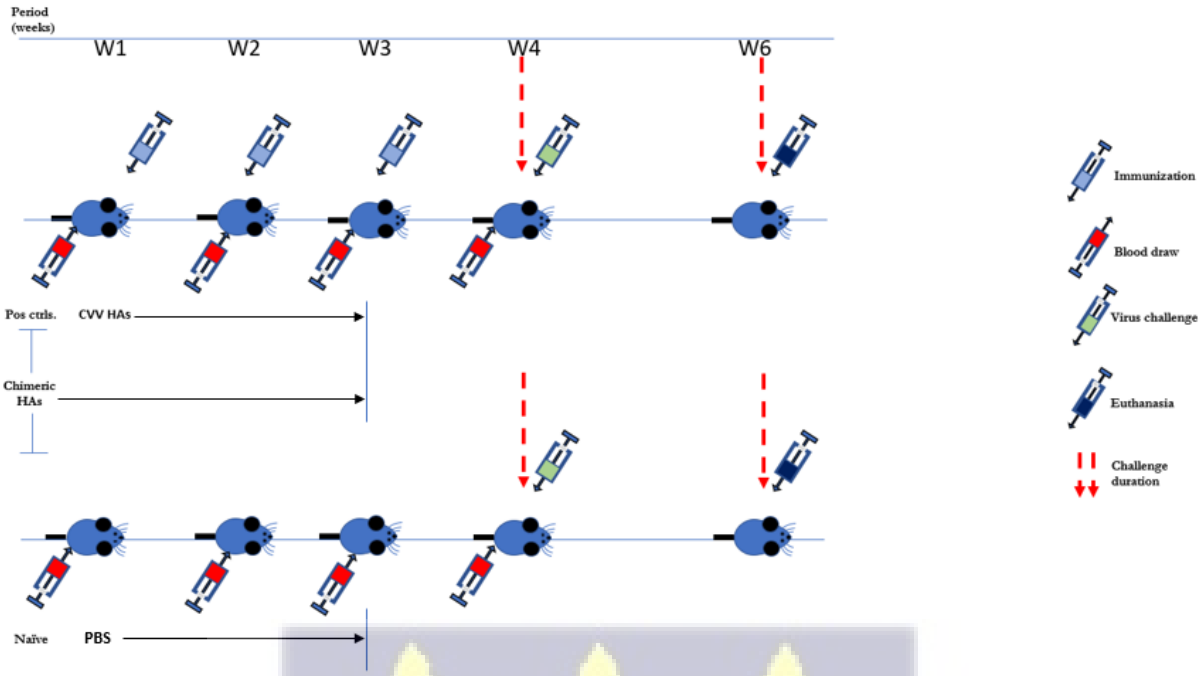


Figure 21: Schematic mice immunization and challenge regimen.

This figure is the summary of the immunization and virus challenge work up. Seven (7) groups, each comprising 5 International Cancer Research (ICR) immunocompetent mice: Groups were A, C, E, M, S, U, and X, where members of the groups were immunized with 30 µg/ mL CHA-A, cHA-C, and cHA-E, Michigan candidate vaccine virus HA (CVV-M), Singapore candidate vaccine virus HA (CVV-S), and the last two (U and X) were naïve controls for both groups 1 and 2 HA, respectively. For weeks 2 and 3, each mouse (excluding the naïve groups) was boosted with 30 µg/ mL per week, of the respective construct. At the start (i.e., from week 1 (W1), blood was drawn from each mouse and was pooled at the group level for exposure-to-influenza virus checking. From W2 to W4, group-specific pooled blood specimens were compared with the baseline blood to estimate seroconversion by haemagglutinin inhibition assay. After the blood draws on W4, each member of groups A, S, and X was challenged with 2.0E7 PFU of laboratory H3N2 influenza virus isolate (A/ARI-19-361/2019). Similarly, each member of groups C, E, M, and U was challenged with 1.0E7 PFU of A/England/195/2009 influenza virus (donated by Prof Wendy Barclay at the Imperial College London). The permit to work on the mice was obtained from the University of Ghana Institutional Animal Care and Use Committee (UG-IACUC) under the license (UG-IACUC 006/19-20) [Appendix H] and the animal immunization workup was performed at the Centre for Plant Medicine Research (CPMR).

Stimulation of mice with cHAs or CVV HAs induced seroconversion.

Sera from mice induced with constructs cHA-C, cHA-E, CVV-M, CVV-S, and cHA- A, as well as their PBS-induced controls, X and U, were assessed further for both seroconversion and broad neutralizability against a heterosubtypic virus isolate. For sera collected mice induced with cHA-C, cHA-E, and CVV-M, including the naïve control (U) were used to challenge A/ England/195/2009 and the WHO H1 antigen. Except for the naïve control that recorded an HI titre of <20 for both WHO H1 antigen and A/England/195/2009, constructs cHA-C, cHA-E, and CVV-M recorded titres of 320, 1280 and 640 for WHO H1 antigen, respectively. Also, each of constructs cHA-C, cHA-E, and CVV-M recorded a titre of 320 for A/England/195/2009. Like U, the naïve control (X) recorded a titre of <20 for all the challenge viruses, leaving each of S and A-induced mice sera to a titre of 640 for the WHO H3 antigen; also, S and A recorded titres of 640 and 320, respectively when challenged with the laboratory H3 isolate (A/ARI-19-361/2019) [Table 8].

Table 8:Assessment of murine seroconversion to cHAs.

Serum	Virus	C	E	M	U
3D	WHO H1	320	1280	640	<20
	A/ England/195/2009	320	320	320	<20
	FRO-H5N2	160	160	160	<20
		A	S	X	
	WHO H3	640	640	<20	
	A/GHANA/ARI-19-361/2019	320	640	<20	
	FRO H7N7	160	320	<20	

Blood specimens were drawn on week 3 before boosting, and these were pooled in each group. Sera harvested from the pooled blood specimens were RDE-treated and used for HI assays to assess for both seroconversion and broad reactivity to heterotypic viruses belonging to specific HA groups. The WHO H1 and H3 antigens, in addition to the experimental viruses (A/England/195/2009 and A/Ghana/ARI/19-361/2019), were used for the assessment of the seroconversion; whereas, the IRR isolates, H5N2 and H7N7 were used for the broad-reactivity assessment. Captured in the table are the HI titres recorded.

Anti-cHAs antibodies cross-react with heterotypic HA viruses.

Broad inhibition sera capacity of the cHA or the CVV HA controls was assessed on heterotypic viruses belonging to their specific HA groups: for instance, the H1-based sera were used to challenge an H5N2 isolate from the IRR, whilst the H3-based sera were used to challenge an H7N7 virus isolate from the IRR. Except for the titres of the naïve controls (X and U) remaining at <20, each of the H1-based sera generated a titre of 160 when used to challenge the H5N2 virus isolate; S and A generated 320 and 640 titres, respectively in the challenge against the H7N7 virus isolate [Table 8].

Anti-cHAs antibodies enabled weight rebound among cHAs-induced mice during virus challenge.

cHAs or CVV HA- immunized mice were challenged either with A/England/195/2009 or A/ARI-19-361/2019 and their weight monitored for 14 days. For the A/ARI-19-361/2019, there was a global drop in the weight of mice, though the weights of the cHA-induced mice rebounded shortly afterwards before 20% decrease. The naïve group, on the other hand, maintained a steady decline in weight until all animals euthanized by day 6 due to drop off in weight below 30% (Figure 23). Viral load assessments by PCR revealed lesser protection -as expected, due to a highly significant increase in the challenge virus, compared with immunized groups that kept the virus in control due to the presence of H3-specific antibodies- translating significantly lesser viral loads (Figure 24). The A/England/195/2009 caused a similar instant on the H1-based setup; though, there was a relatively steeper decline in body weights on the naïve group compared to the C, E, and M groups, perhaps, due to a relatively more virulent virus (Figure 25). Further, this naïve group were euthanized a day earlier (i.e., on day 5) than the naïve control group of the H3-based setup due to increased viral load as detected by PCR (Figure 26). It could be speculated a day earlier euthanization could be due to the H1 challenge

virus being relatively virulent than the H3 challenge strain. Interestingly, in all two setups (H1 and H3-based), there were significant suppressions of viral loads when the cHAs (cHA-C, cHA-E, and cHA-A) were compared with their corresponding CVV HA controls (S and M) (Figure 24 and 26).

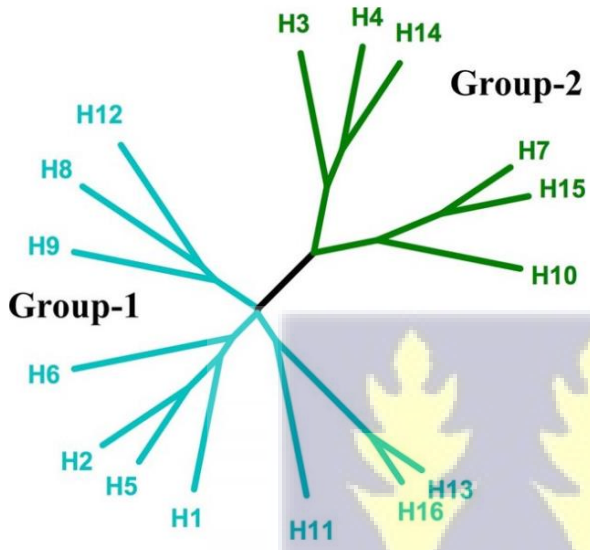


Image adopted from R. J. Russell et al. (2008)

Figure 22: Phylogenetic relationship amongst HAs of influenza A viruses.

This image shows how the HAs of influenza A viruses have evolved in time. Seasonal influenza A H1 HA falls into group 1, just as the H9 and H5 (hetero-subtypes); on the other hand, the seasonal H3 HA categorizes with H7 (hetero-subtype) as group members.

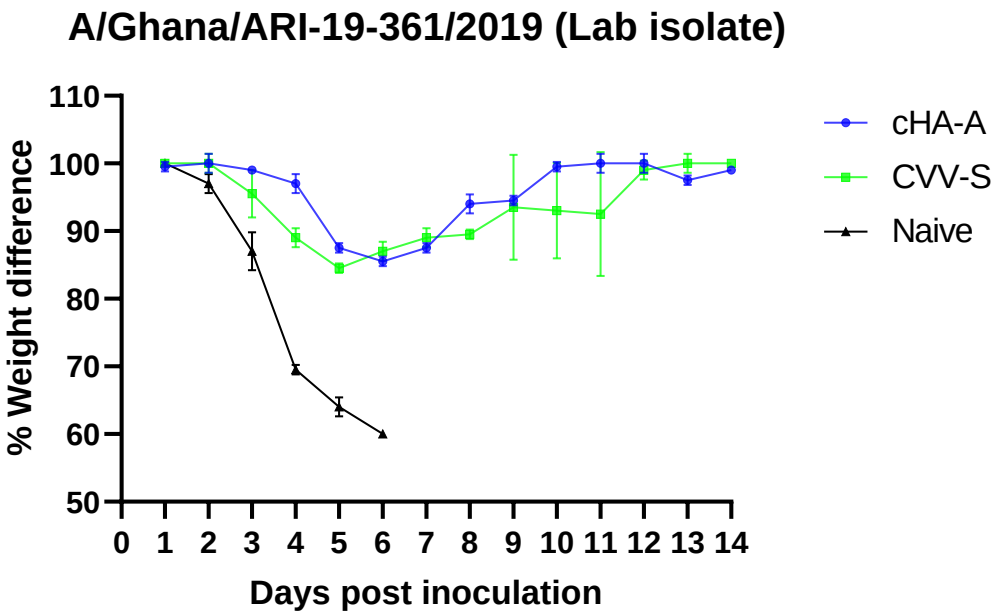


Figure 23: Weight monitoring of mice challenged with A/ARI-19-361/2019.

The chart summarizes the weights of mice recorded over a period of 14 days, with documented protection of mice that were immunized with constructs cHA-A and CVV-S before the challenge with the laboratory H3N2 isolate (A/ARI-19-361/2019). Mice that lost 30% of initial weight were euthanized by day 6.

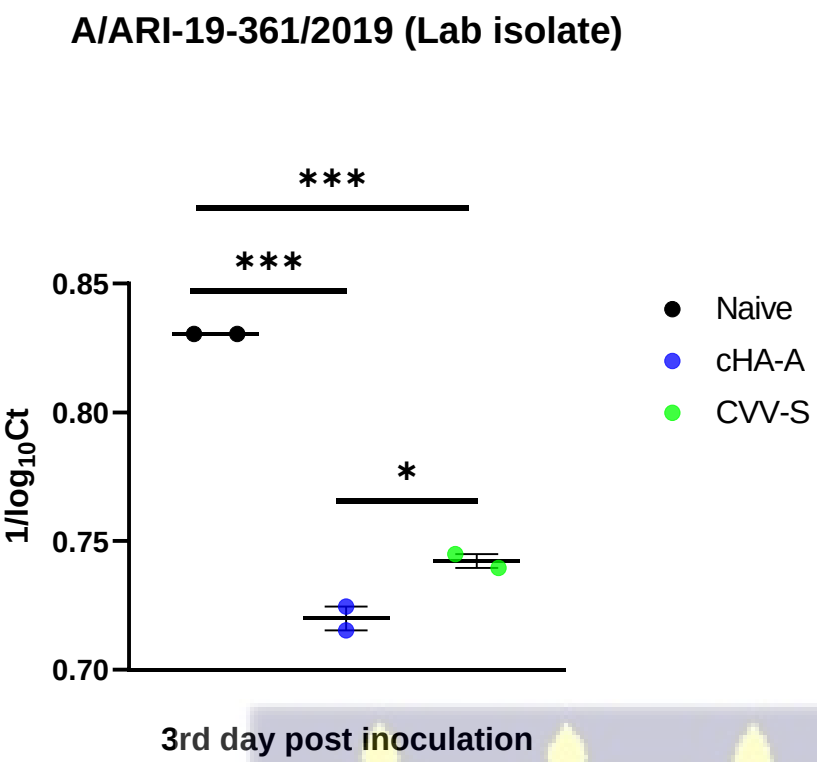


Figure 24: Lung viral load investigation in H3N2-challenged mice.

One mouse each euthanized on the third day due to the dips in body weight. Viral RNA was isolated from lungs and assessed for viral load by real-time PCR. Since Ct values are inversely proportional to the log of viral load, 1/log10 Ct values were used as indices of viral load and were analysed by using the one-way ANOVA followed by Tukey's multiple comparisons test using the GraphPad prism 8.0.1. P<0.05: *; P<0.005: **; P<0.0005: ***.

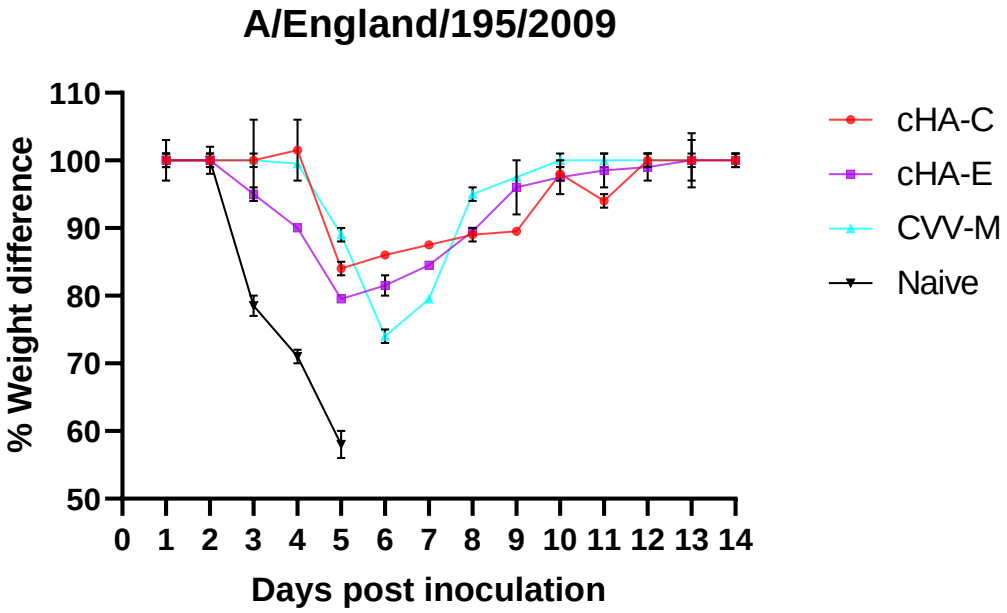


Figure 25: Weight monitoring of mice challenged with A/England/195/2009.

Here is a summary of the weights of mice recorded over a period of 14 days, with documented protection of mice that were immunized with constructs cHA-C, cHA-E, and CVV-M before the challenge with the H1N1 isolate (A/England/195/2009). Mice that lost 30% of initial weight were euthanized by day 5.

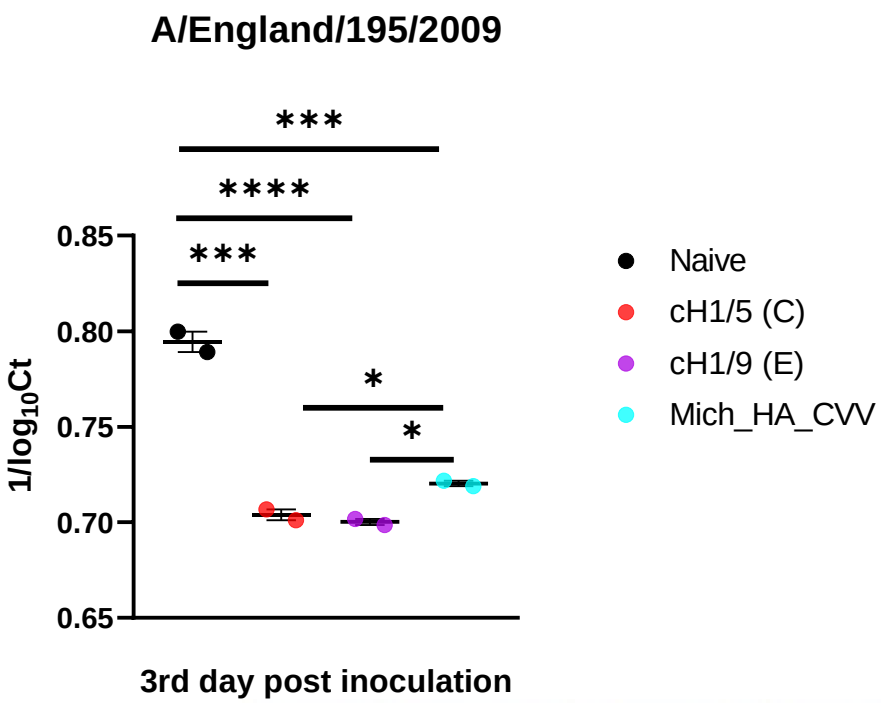


Figure 26: Lung viral load investigation in H1N1-challenged mice.

One mouse each euthanized on the third day due to the dips in body weight. Viral RNA was isolated from lungs and assessed for viral load by real-time PCR. The 1/log10 Ct values were analysed by using the one-way ANOVA followed by Tukey's multiple comparisons test using the GraphPad prism 8.0.1. P<0.05: *; P<0.005: **; P<0.0005: ***; P<0.00005: ****.

Discussion

The use of chimeric HA (cHA) antigens or chimeric antigens borne on VLPs has gained some attention lately, due to their ability to induce high-titre anti-HA stalk antibodies (Crevar et al., 2015; Nachbagauer & Krammer, 2017). For the past 5 years or so, based on some of these crucial findings, there has been hope in generating a universal vaccine candidate that has an equal ability to induce either anti-HA stalk antibodies or others that have the potential to recognize other conserved areas of the influenza virus.

In this regard, the current work here does not only focus on design or the generation of vaccines but, also to identify antibodies that can broadly react with diverse strains of influenza A viruses. To explore the serum capacity of broadly reactive antibodies, chimeric HA designed to possess the intact conserved regions were expressed in an unconventional cell expression system for influenza-related proteins due to the pauci-mannosidic nature – the Drosophila line 2 cells (R. P. de Vries et al., 2012). As has been shown, glycosylation patterns affect host mannose-binding lectins and thus, can define the precision to which fine antibodies are produced as was the result of a glycosylated immunogen in the form of HIV nanoparticle (Tokatlian et al., 2019). Therefore, using constructs generated with the pauci-mannosidic S2 cell system will demonstrate an avenue to characterize the therapeutic relevance of antibodies upon S2 cell-expressed cHA induction. The cHAs expressed in the S2 cell system were, thus, purified, concentrated, and used to immunize mice: of 7 groups (n=5 in each group), 2 were the naïve controls (U and X) that did not receive any prior immunization during the immunization workup; 3 cHAs (cHA-A, cHA-C, and cHA-E) and 2 candidate vaccine virus HAs for the 2018/2019 vaccine as controls – the Michigan (H1N1)pdm09 and Singapore (H3N2) strains [extensively referred here as CVV-M and CVV-S, respectively].

Before the start of the immunization, baseline blood was drawn (by tail snipping) from each mouse pooled together, group-wise both to have an ample amount of serum and to facilitate rapid screening for exposure to any seasonal influenza A virus by HI. The HI titres recorded for the challenge of serum either against H3 viruses (a 2018/2019 WHO H3 antigen and a laboratory H3 isolate, A/GHANA/ARI-19-361/2019) or H1 viruses (a 2018/2019 WHO H1 antigen and an A/England/195/2009) generated titres <20 (Appendix I), depicting no previous mice exposure at least to the study influenza A viruses. This led to the commencement of the immunization workup and that required boosting at weekly interval until week 3 (Figure 20).

But, before week 3 boosting, blood (also referred to as third blood drawn or 3D serum for short) was drawn and pooled as described earlier for seroconversion assessment. Seroconversion was necessary to guide through the subsequent experiments that involved the challenge of experimental mice with a mouse LD50 of viruses. Using HI, blood was challenged again against specific groups that had previously been immunized with specific cHAs or CVV HAs; That is, sera from groups C, E, M and U, were challenged with 2018/2019 WHO H1 antigen and an A/England/195/2009; whereas groups A, S, and X, were challenged with 2018/2019 WHO H3 antigen and a laboratory H3 isolate, A/GHANA/ARI-19-361/2019. HI titres recorded for the H1-based groups, C, E, M, and U were 320, 1280, 640 and <20, respectively, indicating an appreciable induction of H1-specific antibodies. Similarly, there was an appreciable induction of H3-specific antibodies for groups, A and S (each recording an HI titre of 640 versus the naïve group, X that recorded a titre of <20).

At this stage, two main things were of interest: 1, whether the stimulated sera could have inhibitory capacity when challenged with a neighbouring HA-bearing hetero-subtype and 2, whether the stimulated sera could offer real-time protection for mice that are challenged with lethal doses of influenza H1 or H3 virus. To assess the first interest, an H5N2 virus isolate (from the IRR), that shares the same HA group as the H1 HA-bearing viruses, was grown,

titrated and HI performed by challenging it with the H1-based 3D sera. An HI titre of 160 was recorded for each of C, E, and M versus U, that remained <20, indicating the cross-reactive capacity of anti-HA antibodies induced by both the H1-based cHAs and the H1 control (M). Similarly, a challenge of an H7N7 strain (from the IRR) with the H3-based 3D sera, yielded titres 160 and 320, respectively for A and S, versus the naïve control (X) that recorded <20.

Therefore, as good as these cHAs seem to be, the second interest was turned to: here, both the H1 and H3-based groups were challenged with specific viruses – A/England/195/2009 and A/Ghana/ARI-19-361/2019, respectively, to assess if stimulated antibodies would offer some protection as a measure of mice weight and viral load control, given a 14-day timeline. Hence, on week 4, all the H1-based groups were challenged with the A/England/195/2009; whereas, the H3-based group, with the A/Ghana/ARI-19-361/2019. By day 3 post-inoculation, there was a sudden global drop in the weight of all mice in both groups.

For the H3-based challenged mice, there was a sharp rebound through days 4 and 5, whilst mice in the naïve control group steeply lost weight to below 30% of initial weight, and so were euthanized by day 6 post-inoculation. This scenario demonstrates both the lethality of the virus and more importantly, the ability of the challenged mice in the H3-based group to regain weight shortly after the drop. Another critical observation was the fact that construct cHA-A immunized mice rebounded relatively faster than the control, CVV-S, demonstrating some superiority in the protective capacity of cHA-A. Further, viral load analysis was performed by PCR one mouse from each group of the H3-based challenge. This indicated significantly higher viral titres in the lungs of the naive mouse compared with both A and S groups. Viral loads amongst group A were significantly lower than the group S, further conferring superiority to the cHA-A. This corroborates the steeper decline in weights of mice in the naïve group. On another hand, both the cHA-A- and CVV-S-immunized groups controlled the virus challenge better, even though, the A group did significantly better than the S.

The H1-based group also experienced a global decline in weight by day 3, just as the H3-based setup; however, this time, there was a steeper reduction in weight in the naïve group, that lead to a day earlier (day 5 post-inoculation) euthanization. Mice in the H1-based immunized groups shortly rebounded through days 5 and 6 to gradually ascend to normalcy. As with the H3-based setup, the H1-based setup was investigated for viral loads: the naïve group, as expected showed a significantly higher viral load relative to the immunized groups, C, E and M; whereas the C and E were both associated with relatively lower viral loads when each was compared with the control CVV-M). Once again, the two cHAs (cHA-C and cHA-E) were more protective compared to CVV-M, making them, in addition to the cHA-A, very useful immunogens for the induction of protective polyclonal anti-HA antibodies.

The desirable characteristics thus observed makes these cHAs potentially useful to generate therapeutic antibodies that could circumvent some of the pressing challenges associated with active immunization – such as waning of immunity, antigenic sin (directing a more robust immune response to an earlier infecting virus rather than the present one) and immune-pressure induced virus evolution (Andrews et al., 2015; Cobey & Hensley, 2017; McLean et al., 2014).

Materials and Methods

Serological assessment of mice for exposure to influenza A virus by the Haemagglutination inhibition (HI) assay

Seven pools of blood drawn from thirty-five 6-to-8-week's old mice was performed in a serum-separator tube to assess mice exposure to influenza A infection with a currently circulating influenza virus isolate. Haemagglutination assay (HA) was used to titrate the WHO reference antigens (of pdmH1 and H3) as previously described. Recorded titres were used for an 8-HA unit standardization, effecting the dilution of the virus for the performance of the HI. In the HI, serum harvested from each of the pool was treated with 4 parts of receptor destroying enzyme (RDE), incubated on a heated block at 37 °C overnight, and stopped by the addition of 5 parts of physiological saline at 56 °C for 30 minutes. This makes up a dilution of 1:10, which was serially diluted (in two-fold) with PBS in a 96-well plate. Each dilution in the 96-well plate was then challenged with an equal volume of the HA-standardized virus, followed by two equal volumes of 1% Guinea pig (for H3 lab isolate) or 0.75 % turkey red cells (for pdmH1 lab isolate) and tapped gently to mix. HI titres were recorded after 45 minutes.

Immunization regimen

Once all the 7 pools (representing 7 groups) had been confirmed to be unexposed, immunization was performed: 5 µg of each of the 3 cHAs and two positive controls [HAs of 2019 candidate vaccine viruses (CVV)]. The last two groups were negative controls and thus received no antigen. Boosting was performed for both test groups with the same amount of the priming antigen after a week and two. Before the boosting periods, blood was drawn, pooled, and assessed for seroconversion amongst the test groups and positive controls. At the same time, blood was drawn from the naïve negative control mice for continuous monitoring of

exposure. Another round of blood was drawn immediately before the virus challenge also to assess both mice seroconversion and antibody reactivity. Schematic details are shown in figure 21.

The inhibitory capacity of resultant antibodies by Haemagglutination assay (HI)

Sera (3D sera) from pooled blood collected from mice just before week 4 were assessed for the presence of neutralizing antibodies by the HI procedures elaborated earlier.

Challenge and outcomes

During week 4, H1-based cHAs (controlled by the candidate vaccine virus HA) immunized mice were challenged with 50 µL of 1.0E7 PFU of A/England/09/H1N1 intraperitoneally; whereas 2.0E7 PFU laboratory H3N2 virus isolate was used to challenge mice immunized with H3-cHA or the candidate vaccine virus HA. Mice weights were monitored daily through14 days – the end of the experiment. However, mice that suffered weight loss >30 % of their initial, were euthanized.

Also, by day 3, the lungs of a mouse per group (n=1) were harvested, homogenized, RNA extracted, and tested for the presence of an influenza A virus by PCR using the CDC protocol (Shu et al., 2011). This was done to ascertain the active spread of the virus due to a sudden global dip in weights.

CHAPTER 7: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

General Discussion

Just like other viruses, it is thought that vaccination is the best means by which influenza virus infections can be prevented (Budd et al., 2017). Influenza vaccines over several decades have evolved, sticking closely with already drifted viruses. The situation is such that immune pressures, drug pressures, host-ranges, amidst several others, have always kept viruses evolving, and the evolved viruses in circulation are always a step ahead of vaccines that are developed, affecting matching of vaccines to the circulatory strains (Marchi, Lässig, & Mora, 2019; Petrova & Russell, 2018). Thus, the prevention of influenza virus infections has stimulated the need to constantly update vaccine viral composition. But, more importantly, constantly updating vaccines that do not offer efficacies >90% would not be economically viable in the continuous fight against influenza epidemics or an unforeseen pandemic; certainly, the way forward is to devise new strategies to circumvent scientific and technical barriers, such as, challenges in identifying novel conserved viral molecules that are potentially immune correlates of protection, and new immunological methods for screening these molecules: reviewed by (Erbelding et al., 2018; Valkenburg et al., 2018).

Great efforts in the development of novel vaccine designs and platforms are admirable, as they offer new scope for exploration. It is, however, noteworthy that a potential universal influenza vaccine is more desired – vaccines that offer a minimum of one-year-long protection with at least 75% efficacy in the control of symptomatic influenza caused by the two groups of the influenza A viruses (Paules et al., 2017). Nevertheless, the practice of active vaccination against influenza could be a major problem for the selective pressures driving the evolution of influenza viruses because of exposure to induced antibodies (Petrova & Russell, 2018). Since

it is possible for even the best influenza vaccine to induce an antibody-based selective pressure to drive viral evolution, it has become needful to explore unconventional but potentially efficacious approaches of managing influenza. The work presented here reviewed the potential relevance of broadly cross-neutralizing antibodies in the management of influenza viruses. Information presented here is in line with the fact that whilst active vaccination may often induce adverse consequences of influenza virus evolution in the long term, or even be faced with the challenges stemming from the "original antigenic sin" concept (also referred to as, the antigenic imprinting that leads to only a short-term immunity), broadly cross-reactive antibody engagement could arrest viral infection during the initial stages of influenza. Mechanisms of the arrest are discussed as through the ADCC, ADCP, ADCL or direct virus neutralization. In this approach, in the case of an acute infection, the patient's immune system is supported with an exogenous antibody whilst allowed to mature against particles of the live-infecting strain of the virus which might act as a natural booster to provide a more robust immunity, possibly in the nearest future (Kotey et al., 2019).

The plan of the study also sought to engage an interesting platform that deals with the generation of chimeric and mosaic haemagglutinins as have been promising means to inducing broadly reactive antibodies following immunization in animal models (Bernstein et al., 2020; Broecker et al., 2019a). But, whereas immunization with viruses bearing mosaic HA would lead to the induction of HI-active, neutralizing and stalk-reactive antibodies (due to recognition of both conserved globular HA head and stalk regions), immunization with viruses bearing the chimeric HA would induce HI-inactive stalk-directed antibodies (due to the varied globular HA head regions – "stalk focusing"). Therefore, in this chapter, extensive mosaic seasonal H1 and H3 viruses were generated via a consensus generation strategy, where thousands of H1 and H3 sequences were involved. H5 and H9 HA consensus sequences were further aligned with the parental H1 consensus sequences initially generated to form both an intermediate between

chimeric and mosaic HA sequences referred to as cHA-C (comprising H1 and H5 consensuses) and E (comprising H1 and H9 consensuses). The H3 consensus was also aligned with an H7 HA consensus sequence to form the HA referred to as cHA-A. Primarily, these constructs were all designed to retain seasonal influenza HAs (H1 and H3) as the parental structure, and this was confirmed by the BLASTp tool on the NCBI, that yielded about 80% similarities to the HAs type-specific viruses.

More so, the cHAs had the B-cell epitope probabilities and antigenicity scores that were very highly comparable to those of the 2018/2019 candidate vaccine virus HAs (the Michigan H1N1 strain, denoted as CVV-M and the Singapore H3N2 strain, denoted as CVV-S). Additionally, assessments have demonstrated the introduction of unique epitopes into the cHAs designed as was purposed, and these were predicted as epitopes, even though their presence was inconclusive whether they offered any relevance in terms of the nature of antibodies that could be induced. This was a concept to be assessed during subsequent experiments. The interesting findings from the *in-silico* construction and assessments of the cHAs, therefore, drove interests to assess the feasibility of expressing these proteins. As described previously, HAs expressed in the Drosophila line 2 cells (S2 cells) lead to the post-translational modifications involving less complex, paucimannose moieties (de Vries et al., 2012). Though the complexity of the glycosylation is known to have a bearing on the immunogenicity of an influenza virus HA, which may translate into differences in HI titres (i.e., a more complex glycosylation pattern induces higher HI titres and vice-versa), the breadth of the HA-stimulated antibodies is not affected (de Vries et al., 2012).

The experiments, thus, proceeded to the expression of the cHAs (cHA-A, cHA-C, and cHA-E) and the controls (CVV-M and CVV-S) in S2 cells. First, signal peptides and transmembrane domains were predicted and trimmed off since the pEpreS2-1 has been designed with a signal peptide present and the pressing interest was to produce proteins that are secreted into the

supernatant of the transfected cell culture. Secondly, since all *in-silico* assessments performed by far relied on the amino acid sequences, these were later reverse-translated to obtain the nucleotide sequences- the products that were inserted into the plasmid vector (pExpreS2-1) to check and correct for any possible frameshifts. Furthermore, due to interest in expressing constructs in the S2 cells, the nucleic acid sequences generated were translated again (with a Drosophila codon-optimization checked in the ExPasy translate online tool) to obtain optimized codons for S2 cells. These amino acids were now documented as the true sequence for the cHAs (Appendix A). Sequences were outsourced to Eurofins' GeneArt for synthesis. Constructs were subsequently received on commercial plasmids and so by the conventional cloning and subcloning procedures, constructs were inserted on to the pExpreS2-1 vector which was applied in the transfection of the S2 cells. Stably transfected S2 cells were established, but interestingly, instead of all proteins being expressed in the supernatants of the culture system, only the control constructs (CVV-M and CVV-S) were detectable by Western blotting in the supernatants. Trimerized versions of cHA-A and cHA-E were detected, with no detectable versions of cHA-C (whether monomeric or trimeric) in the supernatant. However, both monomeric and trimeric versions of cHA-A, cHA-C, cHA-E were detectable in cell lysates, informing subsequent lysing of the cells to free proteins before purification. By way of inspecting the functionality of these expressed proteins (both cHAs and controls), none was able to haemagglutinate turkey red cells. As described by de Vries et al. (2012), HAs expressed in S2 cells were associated with poor HA and HI titres due to reduced affinity to the receptors on the susceptible cells; also, since these were recombinant proteins, perhaps, higher concentrations were required to see any appreciable HA titres. The fact that decent amounts of proteins have been concentrated and purified induced focus on studying the murine humoral response to the cHAs and to assess if the anti-cHAs antibodies could potentially protect immunized mice and to further investigate the capacity of these antibodies to inhibit

haemagglutination when challenged with a heterosubtypic virus considering the exotic HAs used to the design the cHAs.

An immunization workup commenced shortly after the expression. This was performed on 7 groups of mice, each comprising 5 mice: 2 naïve groups (U and X); 3 groups for the cHAs (cHA-A, cHA-C, and cHA-E) and 2 groups for the controls (CVV-M and CVV-S). But immunization was warranted upon confirmation that all groups of mice were unexposed by the HI assay. The immunization workup was in a prime-double-boost fashion via the intraperitoneal route: priming with the cHAs and the controls were done on week 1, whilst boosting was performed on weeks 2 and 3. Before the booster shots on week 3, blood was drawn from each mouse and processed to obtain serum (3D serum) that was used to assess for seroconversion to the constructs by an indirect HI assay that challenged WHO H1 and H3 serological antigens, in addition to the two challenge viruses [i.e., A/England/195/2009 (H1N1) and A/Ghana/ARI-19-391/2019 (H3N2)]. Appreciation of the titres against these viruses confirmed seroconversion amongst immunized mice. Further, the 3D serum was used to challenge heterosubtypic viruses – H5 against the serum from the H1-based setup and H7 against the serum from the H3-based setup. Also, the appreciable titres recorded demonstrated significant cross-reactivity of the anti-cHA antibodies against heterosubtypic viruses, even higher than those observed for the control groups.

Week 4 was the start of the influenza virus challenge, and mice were inoculated intraperitoneally with mouse LD50 (for groups C, E, M, and U) and (for groups A, S, and X) and weights monitored for 14 days. Altogether, there was a general dip in weights by day 3, but only naïve groups dipped steeply beyond 30% by days 5 to 6, whilst the dips in cHA or control-immunized groups did not go below 20%. Naïve groups were euthanized by day 5 or 6 according to the described protocol. Nevertheless, around day 3, because of the global dip, a mouse each in a group was euthanized and lungs harvested to investigate both the presence of

virus and their respective loads. By influenza A-specific real-time PCR, it was confirmed that the influenza A viruses were present and that loads of the challenge viruses (estimated arbitrarily as the reciprocal of the log10 Ct) were significantly higher among the naïve controls. This implies that the immunized groups in the two experimental setups (i.e., the H1- and H3-based groups) controlled the challenge viruses better, even better than their respective controls.

Of note, the antigenicity scores captured seemed to correspond with the inhibitory capacities of cHAs: in all circumstances of viral load assessment, the antigenicity scores were strongly corroborated by significantly reduced viral loads amongst both the cHAs and the naïve or control groups.

Work done enlightens on one of diverse means by which the conserved HA sequences of the seasonal influenza A viruses could be harnessed to produce antibodies, with which therapeutic application could potentially circumvent many, if not all the challenges facing the development of a universal influenza vaccine candidate.

More importantly, the cHAs developed in this study could be used to generate potentially cross-reactive antibodies of therapeutic value. This is demonstrated in the recent report by Nachbagauer et al. (2020) who conducted the first ever clinical trial with cHA-based vaccines, to both confirm safety and the induction of broadly-reactive stalk-directed antibodies.

Conclusion

1. The conserved domains of seasonal influenza A viruses (H1 and H3) have been obtained via crucial *in silico* approaches that could pave the way for the designing of the next-generation immunogens.
2. An unconventional system in influenza recombinant protein expression system has enabled the assessment of cHAs. This also opens new avenues for the exploration of immunogens for influenza viruses or other pathogens per the breadth of antibodies induced by insect cell culture-expressed proteins.
3. Anti-cHA antibodies conferred protection to mice that were challenged with lethal doses of seasonal influenza viruses. These antibodies also demonstrated cross-reactivity to other distant members of both groups 1 and 2 HA-bearing influenza A viruses. This protection could potentially foster the development of a multi-subtype or a pan-group vaccine candidate or their cognate therapeutic antibodies.

Recommendation

This study has identified both potent seasonal influenza A viruses' HA immunogens which generated broadly cross-reactive antibodies induced by these cHAs. Further work is therefore required to assess the ability of these specific antibodies to cross-react with other diverse strains, as a limited number of strains were used for this study. Further work is also required to ascertain the mechanisms by which these antibodies exert protection against influenza viruses.

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APPENDICES

Appendix A: Full-length cHAs generated by consensus building of seasonal HAs and exotic HAs

cHA-A

MKTIIASILCLFAQPGDNATLCLGHHAVPNGTVKTITDIEVTNATELVQSSGICSPHIL
DGNCTLIDALLGDPCDFQNWDLFVERSASNCYPYVPDYASLRSLVASSGTLEFEFNW
GVQGSCRSSFFSRLNWLYPLNVTMPNFDKLYIWGVHHPTDDQLYGRVSTKRSQQVI
PNIGSRPRSSIYWTIVKPGDILLINSTGNLIAPRGYFKIGKSSIMRSDAICSCITPNGSIPN
DKPFQNVNITYGACPRYVKTLKLATGMRNVPEQTRGIFGAIAGFIENGWEGMDGWY
GFRHQNSEGGQAADLKSTQAAIQIGKLNRIKTNEKFHQIEKEFSEVEGRQDLEKYVE
DTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFETQLRENAEDMGNGCFKIYHKC
DNACISIRNTYDHYRDEALNNRFQIKGVLKSGYKDWILWISFAISCFLLCVLLGFIMW
ACQKGNIRCNICIGCFEIFHKCDDACMASIRNNTYDHAAYRAEAAQNRIQIDPVKLSS
GYKDAILWFSFGASCFALLAIAMGLVFICAKNGNMACTICI

cHA-C

MKMELLVLLCAIIAIVADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNGK

LCCLKGGVPLQLGKCCSAGWWLGNPECCDLFLNVSWSYIIEEPNNENGGCYPGGFF

DYEELREQLSSVSSFERFEIFPKRSSWPNHAASSGVSAACSYNGRSSFYRNLLWLTKK

NGAYPTLKASYNNTKVEEVLVLWGIHHPNNAAEQTKLYQNENAYVSVGTSTYNRR

FIPEIATRPKVRGQSGRMNYYWTLLKPNDTIIFEANGNLIAPEYAFALVRGFASGIIRS

NAEMGECDTKCQTPLGAINSSLPFQNVHPVTIGECPKYVKSDKLVMATGLRNIPSIQS

RGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSI

IDKMNTQFTAVGKEFNNLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH

DSNVKNLYEKVKLQLKNNAKEIGNGCFEFYHKCDNECMESVRNGTYDYPAYSEES

KLNREEIDGVKLESMGTYQILAIYSTVASSLVLLVSLGALSFWMCSNGSLQCRICI

cHA-E

MKAALLALLCLAAAAASADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDTHNG

KLCCLKGLGPLQLGDCCTAGWLLGNPECCDLALAGSWSYIIEEPNSENGGCYPGGFV

DYEELREQLSSVSSFERFEIFPKDASWPVHYAGTSAAASCSFYGASSFYTNLLWLTPK

QGAYPTLAASYANLKAWEVLVLWGAHHPANLYAQADTYTSEAAYVSVRASKYAR

RFRPEIAGRPKRRAQYGRVNYYWTLLRPADTIIFEANGNLIAPSGAFALRRGFTSGIIG

SNAVMCECEAKCQTPTGAINNSLPFQNGHPVTIGECPKYVLSAKLRMVTGLRNIPSIQ

SRGLFGAIAGFIEGGWTGMFDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVN

SKIYKMNTQFTAVGKEFNNLENRMENLNKKVDDGFLDIWTYNAELLVLLENERTLD

FHDSNVKKLYEKVKAQLKNNAKEIGNGCFEFYHKCTIECMTSVRNGTYDYPAYSEE

SKLNRKLIDGVTLKSMGIYQILAIYSTVASSLVLLVSLGASSFWMCSNGSLQCAICI

A/Michigan/45/2015 (H1N1) pdm09-like virus (CVV-M)

MKAILVVLLYTFTTANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNG

KLCKLRGVAPLHLGKCNIAGWILGNPECESLSTASSWSYIVETSNSDNGTCYPGDFIN

YEELREQLSSVSSFERFEIFPKTSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKK

GNSYPKLNQSYINDKGKEVLVLWGIHHPSTTADQQSLYQNADAYVFVGTSRYSKKF

KPEIATRPKVRDREGRMNYYWTLVEPGDKITFEATGNLVVPRYAFTMERNAGSGIIIS

DTPVHDCNTTCQTPEGAINTSLPFQNIHPITIGKCPKYVKSTKLRLATGLRNVPSIQSR

GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADLKSTQNAIDKITNKVNSVI

EKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWTYNAELLVLLENERTLDYHD

SNVKNLYEKVRNQLKNNAKEIGNGCFEFYHKCDNTCMESVKNGTYDYPKYSEEAK

LNREKIDGVKLESTRIYQILAIYSTVASSLVLVVSLGAISFWMCSNGSLQCRICI

A/Singapore/infimh-16-0019/2016(H3N2)-like (CVV-S)

MKTIIALSYILCLVFAQKIPGNDNSTATLCLGHHAVPNGTIVKTITNDRIEVTNATELV
QNSSIGEICDSPHQILDGENCTLIDALLGDPQCDGFQNKKWDLFVERSKAYSNCYPY
DVPDYASLRSLVASSGTLEFKNESFNWTGVTQNGTSSACIRGSSSSFFSRLNWLTHLN
YKYPALNVTMPNKEQFDKLYIWGVHHPGTDKDQIFPYAQSSGRITVSTKRSQQAVIP
NIGSRPRIRAIPSRISIYWTIVKPGDILLINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKC
KSECITPNGSIPNDKPFQNVNRITYGACPRYVKHSTLKLATGMRNVPEKQTRGIFGAI
AGFIENGWEGMVDGWYGFRHQNSEGRGQAADLKSTQAAIDQINGKLNRLIGKTNE
KFHQIEKEFSEVEGRVQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKL
FEKTKKQLRENAEDMGNGCFKIYHKCDNACIESIRNETYDHNVYRDEALNNRFQIK
GVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGNIRCNICI

Appendix B: Trimmed nucleic acid sequences used for the cloning experiment**.**

cHA-C

cccggggacaccatctgcatcggctaccacgccaacaactccaccgacaccgtggacaccgtgctggagaagaacgtgaccg
tgacccactccgtgaacctgctggaggacaagcacaacggcaagctgtgctgcctgaagggcggcgtgcccctgcagctggg
caagtgctgctccgccggctggtggctgggcaaccccgagtgctgcgacctgttcctgaacgtgtcctggtcctacatcatcgag
gagcccaacaacgagaacggcggctgctaccccggcggcttcttcgactacgaggagctgcgcgagcagctgtcctccgtgt
cctccttcgagcgcttcgagatcttccccaagcgctcctcctggcccaaccacgccgcctcctccggcgtgtccgccgcctgctc
ctacaacggccgctcctccttctaccgcaacctgctgtggctgaccaagaagaacggcgcctaccccaccctgaaggcctccta
caacaacaccaaggtggaggaggtgctggtgctgtggggcatccaccaccccaacaacgccgccgagcagaccaagctgta
ccagaacgagaacgcctacgtgtccgtgggcacctccacctacaaccgccgcttcatccccgagatcgccacccgccccaag
gtgcgcggccagtccggccgcatgaactactactggaccctgctgaagcccaacgacaccatcatcttcgaggccaacggcaa
cctgatcgcccccgagtacgccttcgccctggtgcgcggcttcgcctccggcatcatccgctccaacgccgagatgggcgagt
gcgacaccaagtgccagacccccctgggcgccatcaactcctccctgcccttccagaacgtgcaccccgtgaccatcggcgag
tgccccaagtacgtgaagtccgacaagctggtgatggccaccggcctgcgcaacatcccctccatccagtcccgcggcctgttc
ggcgccatcgccggcttcatcgagggcggctggaccggcatggtggacggctggtacggctaccaccaccagaacgagcag
ggctccggctacgccgccgaccagaagtccacccagaacgccatcaacggcatcaccaacaaggtgaactccatcatcgaca
agatgaacacccagttcaccgccgtgggcaaggagttcaacaacctggagcgccgcatggagaacctgaacaagaaggtgg
acgacggcttcctggacatctggacctacaacgccgagctgctggtgctgctggagaacgagcgcaccctggacttccacgac
tccaacgtgaagaacctgtacgagaaggtgaagctgcagctgaagaacaacgccaaggagatcggcaacggctgcttcgagtt
ctaccacaagtgcgacaacgagtgcatggagtccgtgcgcaacggcacctacgactaccccgcctactccgaggagtccaag
ctgaaccgcgaggagatcgacggcgtgaagctggagtccatgggcacctaccagagcggccgc

cHA-E

cccggggacaccatctgcatcggctaccacgccaacaacagcaccgataccgtggataccgtgctggagaagaacgtgaccg
tgacccacagcgtgaacctgctggaggatacccacaacggcaagctgtgctgcctgaagggcctgggccccctgcagctggg
cgattgctgcaccgccggctggctgctgggcaaccccgagtgctgcgatctggccctggccggcagctggagctacatcatcg
aggagcccaacagcgagaacggcggctgctaccccggcggcttcgtggattacgaggagctgcgcgagcagctgagcagc
gtgagcagcttcgagcgcttcgagatcttccccaaggatgccagctggcccgtgcactacgccggcaccagcgccgccgcca
gctgcagcttctacggcgccagcagcttctacaccaacctgctgtggctgacccccaagcagggcgcctaccccaccctggcc
gccagctacgccaacctgaaggcctgggaggtgctggtgctgtggggcgcccaccaccccgccaacctgtacgcccaggcc
gatacctacaccagcgaggccgcctacgtgagcgtgcgcgccagcaagtacgcccgccgcttccgccccgagatcgccggc
cgccccaagcgccgcgcccagtacggccgcgtgaactactactggaccctgctgcgccccgccgataccatcatcttcgaggc
caacggcaacctgatcgccccagcggcgccttcgccctgcgccgcggcttcaccagcggcatcatcggcagcaacgccgtg
atgtgcgagtgcgaggccaagtgccagaccccaccggcgccatcaacaacagcctgcccttccagaacggccaccccgtga
ccatcggcgagtgccccaagtacgtgctgagcgccaagctgcgcatggtgaccggcctgcgcaacatcccagcatccagag
ccgcggcctgttcggcgccatcgccggcttcatcgagggcggctggaccggcatgttcgatggctggtacggctaccaccacc
agaacgagcagggcagcggctacgccgccgatcagaagagcacccagaacgccatcaacggcatcaccaacaaggtgaac
agcaagatctacaagatgaacacccagttcaccgccgtgggcaaggagttcaacaacctggagaaccgcatggagaacctga
acaagaaggtggatgatggcttcctggatatctggacctacaacgccgagctgctggtgctgctggagaacgagcgcaccctg
gatttccacgatagcaacgtgaagaagctgtacgagaaggtgaaggcccagctgaagaacaacgccaaggagatcggcaac
ggctgcttcgagttctaccacaagtgcaccatcgagtgcatgaccagcgtgcgcaacggcacctacgattaccccgcctacagc
gaggagagcaagctgaaccgcaagctgatcgatggcgtgaccctgaagagcatgggcatctaccagagcggccgc

cHA-A

cccggggacctgtgcctgggccaccacgccgtgcccaacggcaccgtgaagaccatcaccgacatcgaggtgaccaacgcc
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cgctccctggtggcctcctccggcaccctggagttcgagttcaactggggcgtgcagggctcctgccgctcctccttcttctcccg
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gctgtcctccggctacaaggacaagcggccgc

A/Singapore/infimh-16-0019/2016(H3N2)-like

cccgggcagaagatccccggcaacgacaactccaccgccaccctgtgcctgggccaccacgccgtgcccaacggcaccatc

gtgaagaccatcaccaacgaccgcatcgaggtgaccaacgccaccgagctggtgcagaactcctccatcggcgagatctgcg

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gaggccctgaacaaccgcttccagatcaagggcgtggagctgaagtccggctacaaggacagcggccgc

A/Michigan/45/2015 (H1N1) pdm09-like virus

cccgggacactgtgcattggataccacgccaacaacagcaccgataccgtggacaccgtgctggaaaagaacgtgaccgtga
cgcacagcgtgaacctgctggaagataagcacaacggcaagctgtgcaagctgcgcggagtggccccactgcatctgggcaa
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caagtgcgacaatacctgcatggaaagcgtgaagaatggcacctacgactacccgaagtacagcgaggaagccaagttgaac
cgcgagaagatcgacggcgtgaagctggaaagcacccgcatctaccagatcctggccatctacagtgcggccgc

Note: Consensuses generated were blended with consensuses from exotic HAs as described earlier, to build up the cHAs. Using the SignalP and TMpred online software, the signal peptide and transmembrane domains were trimmed off, respectively, and then flanked with restriction enzymes cleavage sites -5' Xma I and 3' Not I- to aid in the cloning of the construct in a selected region on the pExpreS2 plasmid.

Appendix C: Common/unique epitopes amongst the H3 HAs

A				S			
Start	**End**	**Peptide**	**Length**	**Start**	**End**	**Peptide**	**Length**
5	14	IASILCLFAQ	10	4	17	IIALSYILCLVFAQ	14
20	28	TLCLGHHAV	9	27	36	ATLCLGHHAV	10
46	58	ELVQSSGICSPHI	13	65	74	GEICDSPHQI	10
60	73	DGNCTLIDALLGDP	14	80	90	CTLIDALLGDP	11

88	107	SNCYPYVPDYASLRSL VASS	20	110	131	YSNCYPYDVPDYASL RSLVASS	22
148	154	IWGVHHP	7	153	158	SACIRG	6
169	174	QQVIPN	6	172	182	HLNYKYPALNV	11
190	197	PGDILLIN	8	195	201	IWGVHHP	7
221	226	AICSCI	6	226	232	QQAVIPN	7
244	256	TYGACPRYVKTLK	13	255	262	PGDILLIN	8
358	365	AELLVALE	8	318	331	YGACPRYVKHSTLK	14
397	410	FKIYHKCDNACISI	14	441	448	AELLVALE	8

428	434	IKGVLKS	7	483	492	FKIYHKCDNA	10
441	458	LWISFAISCFLLCVLLGF	18	532	550	LWISFAISCFLLCVALLGF	19
470	486	CNICIGCFEIFHKCDDA	17				
512	518	IDPVKLS	7				
523	529	DAILWFS	7				
531	549	GASCFALLAIAMGLVFICA	19				

Notes: Light grey highlights denote common epitopes between A and S; Green highlights denote unique epitope to A; and Peach highlights denote unique epitopes to S.

Appendix D: Common/Unique/Shared epitopes on the H1 HAs

C				E				M			
Start	**End**	**Peptide**	**Length**	**Start**	**End**	**Peptide**	**Length**	**Start**	**End**	**Peptide**	**Length**
4	24	ELLVLLCAIIAIVADTICIGY	21	4	14	ALLALLCLAAA	11	4	12	ILVVLLYTF	9
42	50	TVTHSVNLL	9	18	24	DTICIGY	7	18	24	DTLCIGY	7
56	76	GKLCCLKGGVPLQLGKCCSAG	21	42	50	TVTHSVNLL	9	42	50	TVTHSVNLL	9
82	95	PECCDLFLNVSWSY	14	56	67	GKLCCLKGLGPL	12	56	72	GKLCKLRGVAPLHLGKC	17
121	128	QLSSVSSF	8	69	76	LGDCCTAG	8	93	98	WSYIVE	6

146	155	SSGVSAACSY	10	81	90	NPECCDLALA	10	121	128	QLSSVSSF	8
162	168	YRNLLWL	7	121	128	QLSSVSSF	8	150	156	TAACPHA	7
188	196	EEVLVLWGI	9	139	145	SWPVHYA	7	163	169	KNLIWLV	7
215	220	YVSVGT	6	150	159	AASCSFYGAS	10	189	196	EVLVLWGI	8
267	277	APEYAFALVRG	11	162	169	YTNLLWLT	8	213	221	DAYVFVGTS	9
305	333	SSLPFQNVHPVTIGECPKYVKSDKLVMAT	29	175	184	YPTLAASYAN	10	264	270	NLVVPRY	7
347	352	FGAIAG	6	188	196	WEVLVLWGA	9	289	295	VHDCNTT	7
440	447	AELLVLLE	8	212	221	EAAYVSVRAS	10	317	327	IGKCPKYVKST	11

462	469	LYEKVKLQ	8	247	253	YWTLLRP	7	440	447	AELLVLLE	8
482	487	FEFYHK	6	287	297	AVMCECEAKCQ	11	482	487	FEFYHK	6
528	550	YQILAIYSTVASSLVLLVSLGAL	23	320	327	CPKYVLSA	8	527	550	IYQILAIYSTVASSLVLVVSLGAI	24
				440	447	AELLVLLE	8				
				460	469	KKLYEKVKAQ	10				
				482	491	FEFYHKCTIE	10				
				528	549	YQILAIYSTVASSLVLLVSLGA	22				

Note: Gold highlight denote common epitopes amongst C, E and M; Grey highlights denote unique epitopes to C; Green highlights denote unique epitopes to E; Blue highlights denote unique epitopes to M; and Peach highlights denote epitopes common to both C and E.

Appendix E: Quantitation of plasmids

Expressed HA	Plasmid concentration (µg/mL)
CVV-M	1.3
cHA-A	1.4
cHA-C	1.3
cHA-E	1.2
CVV-S	1.4

Note: Plasmids were purified from maxi-prepared broths; about 3µL of each was aliquoted and quantitated on a nanodrop.

Appendix F: Haemagglutination assessment of cHAs and Candidate vaccine virus HAs

Expressed HA/ virus	Haemagglutination titre		
M	<2	<2	<2
A	<2	<2	<2
C	<2	<2	<2
E	<2	<2	<2
S	<2	<2	<2
H5N2 virus	128	128	128
PBS	<2	<2	<2

Concentrated cHAs and CVV HAs were challenged with 0.75% Turkey RBC. Only control H5 virus yielded consistent titres of 128 in three replicates.

Appendix G: Baseline influenza A (H1N1) and (H3N2) exposure assessments.

Serum	Virus	C	E	M	U
Baseline	WHO H1	<20	<20	<20	<20
	A/ England/195/2009	<20	<20	<20	<20
	FRO-H5N2	<20	<20	<20	<20
		A	S	X	
	WHO H3	<20	<20	<20	
	A/GHANA/ARI-19-361/2019	<20	<20	<20	
	FRO H7N7	<20	<20	<20	

Initially, mice were grouped (n=5) into 7 groups: 2 naïve controls (U and X) and A, C, E, M, and S. Blood drawn from all mice were pooled group-specific. Blood specimens were RDE-treated and used for HI assays to assess exposure to influenza A viruses. Captured in the table are the HI titres recorded.

Appendix H: UG-IACUC clearance

UNIVERSITY OF GHANA

University of Ghana Institutional Animal Care and Use Committee (UG-IACUC)

Phone:
Email: UG-IACUC@ug.edu.gh

P.O. Box LG 581
Legon, Accra
Ghana

Office Location: Department of Animal Experimentation Building, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana

28/01/2020

ETHICAL CLEARANCE
(UG-IACUC 006/19-20)

Your protocol for ethical clearance has been reviewed by the University of Ghana Institutional Animal Care and Use Committee and has been approved as follows:

TITLE OF PROTOCOL: Construction of potent immunogenic epitopes of the seasonal influenza A viruses' haemagglutinins

STUDENT INVESTIGATOR: Erasmus Nikoi Kotey

Please note that the final review report must be submitted to the Committee at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to UG-IACUC for review and approval prior to implementation.

Please report all serious adverse events related to this study to UG-IACUC within seven (7) days verbally and in writing within fourteen (14) days.

This certificate is valid till 27th January, 2021. You are to submit annual reports for continuing review.

Signature of Chairperson
Prof. Major (Rtd.) George A. Asare

Appendix I: Primers for cloning experiments

Primer name	Primer sequence	Target cHA/ CVV/ plasmid	Target modification details
#2287_H3_A for	attcccggggacctgtgcctggg	A	Amplification of H3 HA_A on GeneArt plasmid on GeneArt plasmid, 19AA3WEP
#2292_H3_A rev_2	gcggccgctgtccttgtagccggagg		
#2294_H1HA Mich_Ctrl_F 2	CCCGGGgacACACTGTGCATTGGATA	Mich_CVV	Amplification of H1 HA_Mich_CVV on GeneArt plasmid, 19AAVODP
#2295_H1HA Mich_Ctrl_R 2	GCGGCCGCTGTAGATGGCCAGGATCT		
#2289_H3_CVV for	attcccgggCAGAAGATCCCCGG	Sing_CVV	Amplification of H3 HA_CVV on GeneArt plasmid, 19AA3WDP
#2329_H3_CVV(S)_R	gcggccgctgtccttgtagccggac		
#2260_H1HA_C-For	attcccggggacaccatctgcatc	C	Forward primer inserts a D amino acid and Sma I restriction site 5' into H1 HA_C; both primers amplify the
#2328_H1_C_R	attGcggccgctctggtaggtgcccatggactccagctt		

			construct on GeneArt plasmid, 19AARXRP
#2293_H1 _E rev_2	gcggccgctctggtagatgcccat	E	Forward primer inserts a D amino acid and Sma I restriction site 5' into H1 HA_C; both primers amplify the construct on GeneArt plasmid, 19AA3WFP
#2260_H1HA_C-For	attcccggggacaccatctgcatc		
#1998_FW pExpreS2-1	GACTCTTGCGTTTCTGATAGG	pExpreS2-1	To confirm successful construct ligation to the pExpreS2-1vector
#1999_RV pExpreS2-1	GGAGTGTGTAAATGGACAA		