Production of monoclonal antibody based on HA and NP protein of bat H18N11 influenza virus in mice

by

Nirmalendu Deb Nath

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Approved by:

Major Professor
Dr. Wenjun Ma

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Abstract

Influenza A viruses (IAVs) usually circulate in the waterfowl, but some of them with the capability to cross the species-specific boundaries and infect the mammalian host can cause severe zoonotic outbreaks. The sequences of H17N10 and H18N11 subtypes of bat IAVs have been identified, but the resource of these bat IAVs are still limited. Among eight proteins of bat IAVs, the surface glycoprotein HA and internal protein NP are important and can act as immunogenic proteins. They can be a good target for further research to study bat IAVs. Therefore, in this study, we produced a panel of NP and HA protein-specific monoclonal antibodies (mAbs) of bat H18N11 influenza virus. We immunized the BALB/C mice with baculovirus expressed HA and NP protein and then produced hybridoma cells by fusing spleen B cells with myeloma SP2/0 cells. We further tested these mAbs to identify the characteristics by using different immune assays. Six NP protein-derived mAbs were found specific and weakly bound with NP protein of H18N11 in immunofluorescence assay (IFA). These mAbs only reacted with NP protein of H18N11 virus other than those of conventional IAVs in IFA. In contrast, all the HA-specific mAbs failed to be reactive with HA of H18N11 in IFA. Isotyping of the mAbs was characterized by ELISA, and the results showed that all the NP-specific mAbs belong to IgG3. These NP-protein-derived mAbs specifically recognized the possible epitope with the amino acid sequence of position 445-458. To our surprise, all the NP-specific mAbs were not found reactive in the western blotting (WB) assay to detect the NP protein of H18N11 virus. Though the NP-specific mAbs were not specific in WB, they could detect NP of H18N11 by using IFA and ELISA. Therefore, the panel of NP mAbs can be a useful tool to study bat IAVs.

Key words: Bat influenza virus, monoclonal antibody, ELISA, IFA, western blotting, epitope

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Dedication

This dissertation is dedicated to my respectable parents for their everlasting love and support.

Chapter 1 - Literature Review

1.1 Influenza virus (IV)

Influenza is a respiratory disease caused by Influenza viruses (IVs) that are under the family of Orthomyxoviridae. It is an enveloped virus with single-stranded negative senses segmented RNA. There are four types of IVs, types A, B, C, and D based on the antigenic variation of nucleoprotein (NP) and matrix (M1) protein (Palese et al., 1977). Influenza type A (IAVs) and type B (IBVs) contain eight viral RNA (vRNA) segments (McGeoch et al., 1976; Palese et al., 1977). In comparison, the Influenza type C (ICVs) and type D (IDVs) consist of seven RNA segments (Hause et al., 2014). IAVs and IBVs are the most common pathogens for causing respiratory diseases in humans (Cox & Subbarao, 2000; Monto, 2008). In contrast, type C influenza viruses (ICVs) cause mild respiratory illness or even remain asymptomatic in children less than six years of age (Matsuzaki et al., 2006). Cattle is the reservoir for type D influenza viruses (IDVs), and the most susceptible infections are found in young and naïve calves with low immunogenicity due to the dwindling of maternal antibodies (Ferguson et al., 2015).

Influenza A viruses (IAVs) are responsible for infection in various host ranges including human, pig, horse, wild birds, aquatic animals, and waterfowl (Webster RG., 1998, 2002). IAVs are considered as zoonotic viral pathogens causing various symptoms (e.g., fever, chills, myalgia, cerebral pain, discomfort, inefficient hack, sore throat, and rhinitis). The symptoms generally resolve following 3-7 days for most individuals, even though cough and malaise can persevere for >2 weeks, particularly in older individuals and those with ongoing respiratory illness (CDC 2020). Sometimes, it can become more severe, with primary influenza viral pneumonia and secondary bacterial infection leading to acute lung injury, respiratory failure, septic shock, and multi-organ failure. Consistently every year, between 5 % and 10 % of adults and 20 – 30 % of kids have flu symptoms (Lee et al., 2011), and 3 to 5 million people experienced severe disease condition, and approximately 250,000 to 500,000 death (Hayward et al., 2014).

In the twentieth century, there were three influenza pandemics with very high mortality estimated: 1918 A/H1N1 approximately 20–40 million, 1957 A/H2N2 around 4 million, and 1968 A/H3N2 around 2 million deaths (Luk et al., 2001; Nguyen-Van-Tam & Hampson, 2003; Oxford, 2000). In 2009, another pandemic virus, influenza A(H1N1) pdm09, rose in Mexico and spread

universally, caused 20,0000 respiratory death and 83,000 cardiovascular death during the first year circulation (Dawood et al., 2012). Recently, particularly after the 2009 pandemic season, surveillance has been extended, as suggested by the World Health Organization (WHO), to gather epidemiological information (Ortiz et al., 2009).

IAVs are further allocated into numerous subtypes based on the surface glycoprotein Hemagglutinin (HA) and Neuraminidase (NA) (Szewczyk et al., 2014). At present, 16 HA (from H1 to H16) and 9 NA (from N1 to N9) subtypes have been distinguished in IAVs from diverse host ranges, including humans, pigs, birds, horses, and sea mammals. Aquatic birds are the reservoir of IAVs and play paramount role in creating human pandemic influenza strains (Franci et al., 2016). The reason behind IAVs continuing to be one of the most hazardous organisms throughout the world is their unrivaled capacity to escape the host's immune system. The IAVs become more infectious by frequent mutation of the antigenicity of its surface glycoproteins HA and NA. Recently, two novel genomic sequences of IAVs have been found in two bat species, the little yellow shoulder bat (*Sturnira lilium*) and the flat-faced fruit-eating bat (*Artibeus jamaicensis planirostris*), and subtyped as H17N10 and H18N11 (Tong et al., 2012, 2013).

1.2 Virion structure and genome

Based on the observation under electron microscope, the influenza virion exhibits spherical in shape with ~100 nm in diameter or filamentous measuring up to 20um in length (Badham & Rossman 2016). However, some strains may frame broadened threadlike forms, which surpass 1000 nm (Szewczyk et al., 2014). The virion of IAVs is interconnected with glycoprotein spikes of HA and NA, and M2 (ion channel) on the outside of lipid bilayer anticipating from the plasma membrane of host cells, and M1 (matrix protein) underneath the membrane (Nayak et al., 2009). The bilayer is a mosaic structure with cholesterol-rich lipid rafts executed from the host plasma membrane (Scheiffele et al., 1999; Zhang & Pekosz, 2000). From the lipid raft area of the viral envelope, the HA and NA are anchored. HA is the major envelope protein (~80%); furthermore, it structures the trimeric spikes with receptor-binding sites. The cleavage of HA into HA1 and HA2 are essential for viral infection. NA is the second dominant (~17%) envelope protein and structures the tetrameric spikes. The NA eliminates the cell surface receptor (sialic acid) and plays a fundamental function in removing progeny infectious particles from the cell surface and helps in the transmission of infection from host to host. The third envelope protein, M2 acts as an ion

channel (Lear, 2003; Yujie Wu & Voth, 2003). M2 assumes a primary function in the early period of infection, prompting the uncoating and arrival of the viral ribonucleoproteins (vRNPs) from the M1 matrix. The viral nucleus consists of helical vRNPs including viral RNA which is coated by the viral Nucleocapsid (NP) molecules. In addition to it, three polymerases (3P) proteins (PB1, PB2, PA) framing the viral RNA polymerase complex (3P complex) (Lear, 2003; Yujie Wu & Voth, 2003). The genome of the IAVs is divided into eight segments (segment 1-PB2, segment 2-PB1, segment 3-PA, segment 4-HA, segment 5-NP, segment 6-NA, segment 7-M, segment 8-NS) (Szewczyk et al., 2014). These segments of negative-sense single-stranded RNA (ss RNA) molecule spanning contains approximately 13.6 kb (Ghedin et al., 2005). The first 1-3 segments encode three subunits of the RNA polymerase found in viral RNA. Proteins encoded by segments 4–7 and one protein encoded by the segment 8 — NS2/NEP are found in the viral particle (Szewczyk et al., 2014). Electron microscopy examination demonstrates that every RNA segment is wrapped by the NP protein framing helical structures (Szewczyk et al., 2014).

1.3 Virus adsorption, entry, and uncoating

When the IAVs arrive to a potential host cell, the HA receptor-binding site helps to attach the virus to surface glycoprotein containing terminal sialic acid residues (Gamblin & Skehel, 2010; Hamilton et al., 2012; Weis et al., 1988). IAVs utilize the sialic acids on the cell surface as receptors for viral entry to subsequent host bodies (Gamblin & Skehel, 2010). Then the IAVs search the cell surface for sialylated ‘receptor’ through using the activity of NA in the removal of local sialic acid and liberate HA associations (Sakai et al., 2017). HA of IAVs in various species provides some unique features for specific glycosidic linkages of sialic acids. In humans, HA binds to sialic acids by alfa 2,6 glycosidic bond. However, it binds to sialic acids by alfa 2,3 glycosidic bond in avian species (Matrosovich et al., 1997; Wilks et al., 2012). Although these preferences are correlated with sialic acid linkage in a particular host, previous studies demonstrated that the HA receptor binding mechanism is not pivotal for infection; instead, it is essential for transmission (Imai et al., 2012; Herfst et al., 2012; Linster et al., 2014). After attaching to the receptor, virus particles enter into the endosome through endocytosis. The entire endocytosis is directed either clathrin-dependent manner, which involves dynamin and adaptor protein epsin-1 (Chen & Zhuang, 2008; Lakadamyali et al., 2004; Roy et al., 2000), or by the micropinocytosis (Sieczkarski & Whittaker, 2002). After that, the virus is trafficked into endosome inside the cells, where a low PH

environment activates the M2 ion channel (Lakadamyali et al., 2004; Pinto & Lamb, 2006), cause drastically conformational changes in the HA structure exposing the fusion peptides (Bullough et al., 1994; White et al., 1982; Yoshimura & Ohnishi, 1984). Due to the opening of M2 ion channel, the inside of the viral particles become acidified and release the packaged vRNPs, enabling the transferring of vRNPs to host cytoplasm (Bui et al., 1996; Kelsey Martin & Heleniust, 1991). vRNPs cannot diffuse through nuclear pores, so they should depend on the nuclear transport cellular mechanism. Therefore, newly released cytoplasmic vRNPs enter the host nucleus using importin α, importing β nuclear transport pathway by recognizing the nuclear localization signals (NLS) (Cros et al., 2005; K Martin & Helenius, 1991; O'Neill et al., 1995; Whittaker et al., 1995). IAVs are able to deliver their vRNPs from the surface of a host cell to the nucleus around one hour, where entry and fusion require less time (~10 minutes), and the rest of the time needs for nuclear import (Dou et al., 2017).

1.4 mRNA synthesis and replication

Inside the nucleus, the viral RNA-dependent RNA polymerase assist in transcription and replication of viral ribonucleic acids (vRNAs) (Alexander et al., 2017; Fodor, 2013). Generally, IAVs replication involves two steps: transcription of complementary RNA (cRNA) and transcription of newly RNA copies using the cRNA as templates. The cRNAs are produced through the unprimed process depending on the correct complementation of free rNTPs (specially ATP-GTP) in the 3' end of vRNA templates (Newcomb et al., 2009; York et al., 2013). The nucleotide complementation binds with the polymerase active site within the PB1 subunit and results in A-G dinucleotide formation followed by elongation of cRNA transcript (Robb et al., 2016). After that, the cRNA binds with the NP molecules and copies of viral polymerase to promote the cRNP assembly (York et al., 2013). The vRNA transcription occurs in a similar manner to cRNA synthesis using cRNA as a template; however, there is one distinct difference in positioning the longer 3' end of the positive sense cRNA. Finally, multiple NP molecules and viral polymerases bind to the newly transcribed vRNA to form a new vRNP.

Viral mRNA transcription from the vRNA is primed and comparatively more efficient than the cRNA and vRNA transcription (Reich et al., 2014). During mRNA transcription, the viral polymerase obtains the primers by 'cap snatching' mechanism (Bouloy et al., 1979; Plotch et al., 1979), which further interacts with the cellular polymerase II C-terminal domain (Engelhardt et

al., 2005; Lukarska et al., 2017; Martínez-Alonso et al., 2016). The viral polymerase utilizes the PB2 subunit to bind to the 5′ caps of host transcript (Crepin et al., 2008) and the PA subunit endonuclease domain to divide 10–13 nucleotides (Rialdi et al., 2017; Yuan et al., 2009). The PB2 cap-binding domain then rotates to position the newly acquired capped primer into the PB1 catalytic center, where it is extended using the vRNA as a template (Poole et al., 2014). Finally, each transcription is polyadenylated through a reiterative stuttering′ measure, which happens when the polymerase encounters the short poly-U progression at the vRNA 5′ end (Poon et al., 1999; Robertson et al., 1981). This process probably includes different dissociation, repositioning, and reannealing of the mRNA to this template region of the vRNA to accomplish polyadenylation.

1.5 Virus assembly and release

Assemble and budding are the last steps in the IAVs life cycle to survive and release the viral particles from the host. Without these two stages, infected cells will go through abortive processes without delivering the mature infectious viral particles. To complete the assembly of IAV, the infectious molecule can be separated into three parts: (I) Helical RNA nucleocapsids containing vRNA, NP, polymerase proteins (PB1, PB2, PA). These vRNPs are assembled inside the nucleus. In this manner, vRNPs must be exported from the nucleus and moved to the plasma membrane's assembly site. M1 and NS2 proteins seem to be fundamental to export vRNP from the nucleus into the cytoplasm. (II) Matrix protein (M1) assumes an essential function in viral morphogenesis and budding. (Lohmeyer et al., 1979; Rey & Nayak, 1992; Whittaker et al., 1995; Yasuda et al., 1994). Because of the expected juxtaposition of M1 protein between viral envelope and vRNP, M1 is proposed to interface with viral transmembrane proteins on the outer side and viral RNP on the inner side. These connections of M1 with viral glycoproteins are essential in setting off the budding cycle prompting the arrangement and release of viral particles. (III) The viral envelope containing host lipids is enhanced in cholesterol and glycosphingolipid transmembrane proteins, specific HA, NA, and M2.

It has been hypothesized that viral assembly happens mainly in the lipid rafts (Takeda et al., 2003; Zhang & Pekosz, 2000). The lipid rafts are nonionic lipid structures, improved cholesterol, glycosphingolipids, and are relatively resistant to neutral dextran extraction at low temperature, which re-inside the plasma membrane. This site of assembly of IAVs is supported by the consequences of assessment of the lipid substance of purified viral particles (Kundu et al., 1996;

Rossman & Lamb, 2011; Scheiffele et al., 1997). The critical thing is that HA and NA are post-translationally modified in the endoplasmic reticulum (ER) and golgi apparatus before being coordinated into lipid rafts. Furthermore, in the ER, these proteins become effectively folded and glycosylated, and then they are assembled into oligomers where the HA into trimers and NA into tetramers (Veit et al., 1991). On the other side, in the golgi apparatus, the glycan chain modification of these protein HA and NA with their esterification with fatty acids occurs (Veit et al., 1991). The association of the glycoprotein signals with the lipid rafts is situated with their transmembrane domains (Barman et al., 2001). vRNPs are most likely shipped to the region of assembly at the apical plasma membrane of other structural proteins. Through the assistance of endosomal protein Rab 11, the eight viral protein segments assemble in the cytoplasm, demonstrated by Chou and his partners based on the FISH examination (Chou et al., 2013). Packaging eight distinctive RNA segments in the form of RNPs into virion shells is not fully understood; however, numerous models have been proposed (Bancroft & Parslow, 2002; Enami et al., 1991). The two glycoproteins, HA and NA, have an affinity for sialic acids, yet they have an inverse impact on releasing the virus from cells. HA secures the virus to the cell layer because of its association with sialic acid-containing receptors. NA is needed to eliminate sialic acids to permit virus progeny to leave its host cells. In this way, there must be an inflexible harmony between these two HA and NA's functions, and the progeny of the virus can be delivered.

1.6 The Bat flu organism

Aquatic birds, for instance, ducks, gulls, and shorebirds have truly been recognized as the essential reservoir of all the known IAV subtypes (Kuchipudi et al., 2014). Recently, Northwest Atlantic gray seals were found as an endemically infected wild reservoir for IAVs infections (Puryear et al., 2016). Bats are available worldwide and considered the natural reservoirs of many zoonotic pathogens, including Rabies, Ebola, Henipaviruses, and SARS Covid (Calisher et al., 2006; Ge et al., 2013). Recently, nucleic acids obtained from bats demonstrated that bats might be another reservoir of influenza A virus that is phylogenetically different from other IAVs (Tong et al., 2012, 2013). Bat influenza was first recognized in "little yellow-shoulders bats" in Universidad del Valle at Guatemala during an examination led in 2009 and 2010 by specialists from CDC (Tong et al., 2012). Bat IAVs have been recognized in some different bats in Central and South America (Tong et al., 2013). Lab research led at CDC proposed that these viruses would have to go through

massive reassortment in order to infect people (CDC, 2012). The bat species known to harbor bat influenza viruses are not local to the mainland United States but are regular in Central and South America. There were only 16 HA subtypes of IAVs known to present in the world until the disclosure of the two new subtypes in bats. The new bat IAVs found in Central and South America are different from those previously known subtypes that CDC researchers have classified as new subtypes H17 and H18 (Tong et al., 2012, 2013). The other surface protein of bat IAVs, neuraminidase (NA), is exceptionally unique comparing with known IAVs. Therefore, CDC researchers have proposed new subtypes for the NA found in bats: N10 and N11.

1.6.1 Bat influenza virus cannot reassort with conventional IAVs

The discovery of novel bat IAVs raises numerous questions, including the host range of IAVs and bats' role in the evolution. The potential for the new bat IAVs to reassort with conventional IAVs has been investigated. In recent days, the bat influenza viruses can reconstruct through reverse genetic utilizing engineered DNA, opening up a new era to comprehend the bat IAVs (É. A. Moreira et al., 2016). Numerous trials have indicated that reassortment of bat IAVs and ordinary IAVs does not happen under experimental conditions (A. Moreira et al., 2014; Zhou et al., 2014). The surface glycoprotein HA and NA of bat IAVs show a low nucleotide sequencing similarity with those of the regular IAVs (Tong et al., 2012, 2013). Moreover, it has been discovered that the bat IAVs hemagglutinin (HA) do not have a receptor binding site similar to those of traditional IAVs (Q. Li et al., 2012; Tong et al., 2012). HA of bat IAVs does not bind to the traditional avian (SAα2,3-Gal) or human (SAα2,6-Gal) IAVs' receptors featuring unique characteristics (Ying Wu et al., 2014). Thus, bat IAVs start infection at the basolateral layer of cells, not at all like regular IAVs, which particularly start infection on the apical surface of cells (É. A. Moreira et al., 2016). These discoveries reveal that there are significant transformative limitations to bat IAVs to reassort with regular IAVs as well as infect different species including human beings.

1.6.2 Function of the surface glycoprotein of bat IAVs

The surface glycoprotein HA of traditional IAVs binds to sialic acids as receptors on host cells to initiate the host cells' infection. In contrast, H17 and H18 of the New World bat IAVs cannot utilize sialic acid as receptors to infect the host cells (Li et al., 2013; Tong et al., 2013; Zhu

et al., 2013). Alternatively, they use major histocompatibility complex class II (MHC-II) molecules during entry to the host cells (Giotis et al., 2019; Karakus et al., 2019). Even though MHC-II molecules are essential for H17-and H18 cell entry, it is still unknown whether it is used as a binding receptor or as a cofactor (Ciminski et al., 2019). MHC-II proteins of numerous species, including chicken, pigs, mice, and people, can act as receptors, suggesting that bat IAVs might not maintain the species-specific limitation and might have a vast host range (Barclay, 2019). MHC-II molecules are found in the immune cells of the lymphoid tissues, for instance, B cells, macrophages, and dendritic cells (Roche & Furuta, 2015), but can also express on epithelial cells (Wosen et al., 2018). Actual communications and the coupling interface between H17/H18 and MHC-II molecules are yet to be illustrated. The New World bat IAVs encode a NA protein without noticeable sialidase activity (Q. Li et al., 2012; Tong et al., 2012, 2013; Zhu et al., 2012), as it's genuine activity is still unknown. A preliminary study recommends that bat N11 down-regulates MHC-II molecule expression, but the real mechanism is yet to be known (Ciminski, et al., 2019).

1.6.3 H18N11 replicates prominently in bats

A previous study of serosurvey revealed that South American bat species could be infected with bat IAVs (Tong et al., 2013). However, because of the lack of acutely infected animals, the exact organ tropism and bat-to-bat transmission are still unclear. Neotropical Jamaican fruit bat (*Artibeus jamaicensis*), a nearby relative of the bat species *Artibeus jamaicensis,* which was used to test replication and transmissibility of bat IAVs. Results showed that Jamaican fruit bats are susceptible to bat influenza wild type (WT) H18N11 virus by using intranasal infection and shed high loads of virus through the rectal route, resulting in horizontal transmission to naïve contact bats (Ciminski et a., 2019). Furthermore, in these infected bats, H18N11 RNA and antigen were found in the follicle-associated epithelium (FAE) of jejunal Peyer's patches, a part of gut-associated lymphoid tissue, and the squamous epithelium of the palatine tonsils. Interestingly, histopathological examination revealed that no organs demonstrated necrotizing or inflammatory lesions resulting in the asymptomatic infection (Mandl et al., 2015; Schountz et al., 2017). The finding that viral replication occurred in the FAE of the gut-associated lymphoid tissue suggested that the virus may be taken up from the gastrointestinal lumen like low pathogenic avian IAVs that navigate the stomach of ducks and replicate in the intestine before being discharged at high concentration in defecation (Webster et al., 1978). Inoculation of mutant virus rP11 (which has

lacking N11 ectodomain and acquired mutation in H18 of WT H18N11 bat influenza virus) in Jamaican fruit bats can also shed infectious virus particles in feces (Ciminski et al., 2019). Sequencing of the isolated viral particles from the index animals uncovered that the two mutations at K170R and N250S in H18 HA were maintained. However, restoration of the N11 open reading frame was found (Ciminski et al., 2019). Furthermore, the way that virus encoding a full-length N11 NA was transmitted to naive contact bats recommends a functional N11 NA during viral replication in lymphoid tissues and spread among bats.

1.6.4 Bat influenza virus has restricted cell tropism

A previous study demonstrated that recombinant vesicular stomatitis viruses (VSV) encoding the hemagglutinin-like envelope glycoproteins HL17 or HL18 instead of the VSV glycoprotein were able to infect MDCK II and Vero cell lines that initiate the virus replication (Moreira et al., 2016). Additionally, to allow the virus entry in the cells without the presence of trypsin in infection medium, the study generated two other VSV recombinant viruses designated by VSV*ΔG-HL17pb or VSV*ΔG-HL18pb in which HL protein was expressed in the polybasic proteolytic cleavage site (Moreira et al., 2016). Then, several cell lines of human, monkey, bat, canine, porcine, and avian source were inoculated with VSV*ΔG-HL17pb or VSV*ΔG-HL18pb (Moreira et al., 2016). Results showed that recombinant VSV viruses were able to infect MDCK II cell lines. Most cell lines, including MDCK I cells related to MDCK II cells (Dukes et al., 2011), were not susceptible to recombinant VSV infection, demonstrating that they are resistant to infection (Moreira et al., 2016). The experimental study also used other cell lines including the human cell lines U-87 MG (malignment glioma cells), SK-Mel-28 (melanoma cells), and A549 (lung carcinoma cells) aside from MDCK II and RIE 1495 cells. The result revealed that VSV*ΔG-HL18pb-sNLuc propagated in MDCK II and RIE 1495 cells but less effectively in U-87 MG and SK-Mel-28 cells (Moreire et al., 2016). Generally, infection of VSV*ΔG-HL17pb and VSV*ΔG-HL18pb in MDCK II and RIE 1495 cell lines displayed a productive cell tropism (Moreire et al., 2016). In addition, Moreire et al. (2016), also reported that the WT H18N11 bat influenza virus is able to infect MDCK II cell lines prior sialidase treatment to virus infection.

1.6.5 H18N11 influenza virus utilizes the basolateral site to enter the host cells

Conventional IAVs regularly enter into the cells through the apical plasma layer route of polarized epithelial cells (Slepushkin et al., 2001), following the viruses' airborne transmission. It was astonishing to note that VSV*ΔG-HL18pb and recombinant H18N11 viruses especially entered into the MDCK II cells from the basolateral side (Ciminski et al., 2019). A few other viruses such as VSV (Fuller et al., 1984), hepatitis B virus (Okuyama-Dobashi et al., 2015; Schulze et al., 2012), hepatitis C virus (Harris et al., 2010), adenovirus type 2 and 5 (Walters et al., 1999), vaccinia virus (Vermeer et al., 2007), and measles virus (Ludlow et al., 2010) are known to utilize basolateral receptors to entry the epithelial cells. Most importantly, the junctional and adhesion proteins that are pivotal for the host cells are also used by some viruses for entry and attachment (Torres-Flores & Arias, 2015). Regardless of basolateral entry, synthesized H18 was discovered to be transported to the apical plasma membrane of polarized MDCK II cells, where budding and the virus's release took place. In such a manner, H18N11 (and likely H17N10) follow the traditional IAVs, which additionally bud from the apical space, specifically from cholesterol-rich plasma membrane areas where the envelope glycoproteins HA and NA amass (Rossman & Lamb, 2011). The opposite sites of virus entry and release may clarify why infection in MDCK II cell monolayer with H18N11 was less powerful than infection with regular IAVs. Following release from the apical site, viral particles may have restricted access to the basolateral site to start another infection round.

1.6.6 H18N11 poorly adapts in non-bat species individuals

The conventional IAVs originated from the aquatic birds are now circulating in a myriad host species, which drives the host adaptation evolutionary mechanism and is essential for its maintenance. Conversely, the evolution of bat IAVs host spectrum is yet in its beginning phases, and it is not clear whether different animals other than bats are typically infected. Importantly, studies to decide H17/H18 HA-specific antibodies prevalence in non-bat species in Central and South America are absent. Experimental infection studies of bat IAVs revealed that the bat IAVs could also replicate in other animals, for example, mice and ferrets (Ciminski et al., 2019). Following intranasal inoculation of mice with either WT H18N11 or the mutant rP11, viral replication was found in the olfactory epithelium of the upper respiratory tract (URT) without causing any clinical signs (Ciminski et al., 2019). The rP11 is a recombinant virus selected from

11 passages of H18N11 in RIE 1495 cells and had mutation in H18 and N11, which encodes $H18_{K170R}$, $_{N250S}$ and $N11_{G107X}$ (Ciminski et al., 2019). Besides viral replication in the URT, no transmission among immune-deficient mice that lack interferon (IFN) I and III expression was noticed. The rP11 showed genetically stable in mice and maintained the mutations at $H1_{8K170R}$, $_{N250S}$, and $N11_{G107X}$. Intranasal inoculation of WT H18N11 in ferrets showed no differences in body weight and temperature comparing with the uninfected ferrets, though there was seroconversion with H18 HA-specific neutralizing antibodies (Ciminski et al., 2019). In contrast, following inoculation with rP11, moderate RNA was observed in different organs such as URT, lung, brains. The histopathological investigation of the infected ferrets uncovered moderate pneumonia and rhinitis with multifocal H18 RNA. Like mice, infected ferrets are clinically healthy and cannot transmit to naïve contact animals, suggesting that bat IAVs are poorly adapted to non-bat species (Ciminski et al., 2019).

1.6.7 Zoonotic potential of bat influenza virus

The danger for zoonotic transmission and the related pandemic potential that initiate from IAVs mainly relies upon human preadaptation and the capacity to beat host restriction. Though avian IAVs convey the gene segments with species-specific determinants that permit effective replication in avian species (Long et al., 2019), the New and Old World bat IAVs seem to transmit into humans because of their mammalian origin (Ciminski et al., 2019). New World bat IAVs may infect humans because their HAs can use the MHC-II homolog human leukocyte antigen-DR isotype (HLA-DR) during cell entry (Giotis et al., 2019.; Karakus et al., 2019). However, the previous animal study of H18N11 and rP11 indicated that the low replication properties of WT H18N11 and the rP11 variation and the viral transmission between ferrets might recommend low zoonotic capability of bat IAVs (Maher & DeStefano, 2004). Besides this, H18N11 is also not ready to defeat the intracellular restriction by major host defense factors, such as the human type I and III interferon-mediated antiviral factor Mx (in people, MxA) (Ciminski, et al., 2019). The bat H9N2, which uses of α2,3-rather than α2,6-sialic acid, along with the absence of MxA mutation in NP (Kandeil et al., 2018), proposes that the Old World bat infection has a low level of human preadaptation. Therefore, based on the available preliminary data, there is a reasonable but low risk for the zoonotic overflow of different bat IAVs. Considering their human-adapted internal

gene segments and the capacity to gain new mutations, future potential transmissions to humans cannot be completely ignored (Ciminski et al., 2019).

1.7 Antibody and it's overall structure

An antibody (Ab) is a protein component of the host body's immune system that circulates in the blood. It recognizes foreign substances, such as bacteria, virus and ultimately neutralizes them. Ab has two different capabilities: I) bind specifically with the antigen (Ag); II) another is to provide an immune response against the bound Ag through recruiting different cells and molecules (Sela-Culang et al., 2013). The relationship between the Ab and Ag includes cooperation between epitope (part of an antigen which is recognized by antibody) and paratope (part of an antibody which is recognized by antigen). Most of the Abs molecules belong to the IgG family, are homodimers roughly Y-shaped, having two identical polypeptide heavy chains (HC) of 450 amino acids and two identical light chains (LC) of 250 amino acids (Maynard & Georgiou, 2000). To form an intact immunoglobulin, one LC pair with one HC associates with another identical heterodimer. The four HC and two LC domains' structure has the immunoglobulin folds, which consist of two antiparallel β-sheets with an intramolecular disulfide bond (Maynard & Georgiou, 2000). There are two types (kappa-κ and lambda-λ) of LC are found in an antibody. There should be either λ or κ chain in an antibody, but never be the combination of these two. In contrast, different types of HC can be present in an Ab. Therefore, based on the structural differences in the HC, immunoglobulins are classified as IgG, IgM, IgA, IgD, and IgE (Kuby J., 1997). Among the five distinct classes of immunoglobulin, IgG is the most important in the biological perspective, and to a lesser extent IgM and IgA. Two different regions, variable (V) and constant (C) are seen in the antibody. The amino-terminal of V domains of the HC and LC together to make V region which helps to bind with specific antigen. In contrast, the constant domains of HC and LC make up the constant C region (Maynard & Georgiou, 2000). The proteolytic enzyme cleaves the polypeptide sequences and ultimately dissect the antibody structure to form three fragments; two identical Fab portion which is used to bind with the antigen, and other is Fc portion with no antigen binding activity (Kuby J., 1997). The Fab and Fc fragments are interconnected to each other by hinge region allowing the independent movement of the Fab portion. Different isotypes contain difference in the Fc fragment and hinge, thus determining their functional properties. However, the overall organization of the domains is similar in all isotypes.

1.7.1 Monoclonal antibody

Monoclonal antibody (mAb) possesses a unique antibody characteristic produced from the cloning of white blood cells through immunizing by a specific protein or antigen. Monoclonal antibodies have the monovalent affinity to bind with the same epitope. The primary standard in producing mAb is B cells, delivered by the immunized animals fused with the myeloma cell, which brings about hybridoma cell. This hybridoma strategy is supposed to be the primary mAb production method found by Kohler and Milstein in 1975 (Kohler & Milstein, 1975). The B cells do not possess long-term life expectancy; therefore, the myeloma cells' fusion helps for a long-lasting lifespan. Each mAb is said to have extraordinary features; likewise, it has fundamental applications other than therapies. They incorporate their function as abzymes and drug improvement like cancer immunizations. Although mAb is considered a vital tool in the current analysis; however, it has numerous moral issues regarding the utilization of animals in the advanced strategies for mAb production techniques. The mAb market had an effective development after the revelation of the primary mAb was affirmed. The mAb is said to have an incredible effect in treatments as the FDA endorsed 30 mAbs to treat numerous infected conditions incorporating transplant rejection in humans (J. Li & Zhu, 2010). Nowadays, mAb is playing a pivotal role in the field of biomedical research as well as in molecular immunological investigations. mAb strongly affects research diagnostics as it is utilized to treat infections; for example, disease incorporates hematological malignancy and tumor distinguishing proof. Different methods, including in vitro and in vivo methods, can be used for the rapid production of mAb to use various purposes.

1.7.2 Hybridoma method to produce mAbs

The hybridoma technique was first used to produce mAb in 1975 (Kohler & Milstein, 1975). Hybridoma production includes inoculating a protein that will have target region of an antigen to acquire the B-lymphocytes from the spleen cells of immunized animal. After that, the B lymphocytes from spleen cells are fused with myeloma cell lines lacking hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) along with immunoglobulins producing cells. These hybridoma cells are then cultured in vitro in a specific medium such as medium containing hypoxanthine aminopterin thymidine (HAT) (J.K.H.Liu., 2014). In this medium, only the hybridomas (fused B-lymphocytes and myeloma cells) can survive as they have acquired

immortality from the myeloma cells (Little et al., 2000). The hybridoma culture contains antibodies from a wide range of primary B-lymphocyte clones, each emitting its specific antibody into the medium. Every individual clone can be isolated by diluting it into various culture wells. The cell culture medium would then be screened from many wells for the particular antibody required. The ideal B lymphocytes developed from the positive wells were afterward re-cloned and retested for specific activity (F. Li et al., 2013). The positive hybridomas could be store in a liquid nitrogen for an extended period.

1.7.3 Phage display method

Another strategy to produce the mAb is the phage display method, an advanced and modern technology used as an alternative method to the hybridoma method developed in 1985. This technique permitted the expression of clones outside of the phage particles as a combination protein by cloning foreign DNA into filamentous bacteriophage (Rülker et al., 2012). It incorporates separating B-lymphocytes from individuals' blood, restricting the mRNA, and changing it into cDNA using PCR to amplify all VH and VL portions. These portions would then be able to be cloned into a vector (generally as scFv) close to the PIII protein of a bacteriophage before being utilized to infect *E. coli* to produce a library containing around 10^{10} cells by inoculating the library with a different phage (Schofield et al., 2007). The *E. coli* would then emit the bacteriophage containing the VH and VL fragments as a feature on the bacteriophage's surface. Unequivocal VH and VL pieces against the antigen are picked and used to re-inoculate *E. coli* with the bacteriophage. Cells containing the plasmid would then have the option to be isolated and sequenced (Schirrmann et al., 2011). The beneficial points include: when the library is made, a comparative library can be used to create new antibodies, no immunizations are required, as the entire cycle is done in vitro, antibodies can be procured significantly more quickly than the ordinary hybridoma strategy and the library can be used to deliver antibodies (Roncolato et al., 2015). Biopanning the phage is considered to be helpful in the identification of specific antibody fragments with good affinity. The purpose behind this is to increase the feasibility of antigen-specific scFv, in the solicitation to redesign the affinity of scFV for antigens with extended specificity (Pal et al., 2013).

1.7.4 Single B cell antibody technology

The way to deal with produce mAbs from single human B cells depends on examining the immunoglobulin gene repertoire and reactivity at the single-cell level using reverse transcriptase-polymerase chain reaction (RT-PCR) as well as the manifestation of vector cloning. By acknowledging selected cell surface markers, mouse or human B cells are isolated (e.g., by fluorescence-activated cell sorting), and gene coding with VL and VH is independently intensified by RT-PCR and consolidated by PCR. For the last production of human mAbs in vitro, H and L chain gene transcripts from every cell are enhanced by RT-PCR before cloning and articulating a mammalian framework. This strategy has pivotal importance in the rapid production of mAb in a short period.

1.7.5 Transgenic mice method

The transgenic mice method is the most advanced method to produce human mAb using trans-chromosome mice. In this technique, the human immunoglobulin encoding genes present in HC and LC are inserted into the transgenic mice. Therefore, mice can express immune response as human, which is performed by genetic engineering methods was first revealed to produce human mAbs utilizing traditional cell culture strategy (Wang, 2011). Transgenes encoding human immunoglobulin HC and LC are usually entered into the mice. Progress has been accomplished to communicate various V gene fragments by transgenic mice in recent years, with increment in the quantity of possible repertoire of the recovered mAbs (Chadd & Chamow, 2001). An age of transgenic mice generating human mAbs with different substantial chain isotopes was accomplished. However, immune reactions are found high in transgenic mice contrasted with those seen in ordinary mouse strains utilized in producing mouse mAbs. Around six specific human mAbs by transgenesis have been endorsed for marketing, and more than 50 human mAbs in clinical preliminaries (Wang, 2011). This insertion of human immunoglobulin in transgenic mice helps to reduce the undesirable human anti-mouse antibody responses. It keeps up the benefits of murine regular cell culture method for the potential therapeutics reagents (Liu, 2014).

1.8 Objective of the thesis

Identifying the sequence of two new influenza-like viruses from the bat had brought a new area to study more IAVs as it is known that the bat species harbor different zoonotic pathogens.

The surface glycoprotein HA of the newly identified bat influenza virus H18N11 has a distinct relationship with the other HA subtypes. The previous study characterized that the amino acid sequences of HA of H18N11 showed 49.1% identities with group 1 HA subtype (Tong et al., 2013). Furthermore, another critical protein segment NP H18N11, whose primary function is to encapsidate the virus genome for RNA transcription, replication, and packaging, has 83% amino acid similarities with the other NP gene from H1-H16 IAVs (Tong et al., 2013). The biology about the H18N11 bat IAVs is still limited. To the best of our knowledge, there are no such articles published with monoclonal antibodies based on the HA and NP protein of H18N11. Therefore, this study's specific objective was to produce the mAbs against HA and NP protein through the hybridoma technique in order to study and understand the biology of the bat H18N11 virus.

Chapter 2 - Materials and Methods

2.1 Viruses and cells

In this study, the reverse genetic generated WT bat influenza virus H18N11 (A/falt-faced bat/Peru/033/2010) and a chimeric bat virus were used for further experiments. The chimeric virus consists of six internal gene segments (PB2, PB1, PA, NP, M, and NS) from H18N11 and the surface HA and NA gene open reading frames from the conventional IAV H1N1(PR8) strain (PR8 A/Puerto Rico/8/1934). The WT H18N11 virus was grown in low passaged RIE 1495 cell lines, maintained in the Dulbecco's modified Eagle Medium (DMEM) incorporated with 10% Fetal Bovine Serum (FBS), 1% antibiotic-antimycotic, 1% vitamin, and 1% L-glutamine. Besides this, the Madin-Darby Canine Kidney (MDCK) II mammalian cell lines were used for WT H18N11 virus infection. Furthermore, the chimeric H1N1 and other three viruses, traditional IAV PR8 A/Puerto Rico/8/1934 (H1N1), A/swine/Kansas/10-83533/2010 (H3N2), and A/quail/Hong Kong/G1/1997 (H9N2), were also used in this study and grown in MDCK cell lines in Modified Eagle Medium (MEM) in presence of TPCK trypsin which support the viral replication.

2.2 Mouse inoculation with HA and NP antigen of WT H18N11 virus

To produce mAb, ten 6-8 weeks of aged female BALB/C mice were purchased and closely monitored for five days before immunization with the baculovirus expressed HA and NP protein of WT H18N11 virus. Our lab expressed both HA and NP proteins using the baculovirus expression system on the insect cells sf9. The mice were divided into two groups, each of which contained five mice, and among them four were for immunization and another one for control. HA (1.04 ug/ul) and NP (2.8 ug/ul) proteins were mixed with the Complete Freund's Adjuvant (CFA) according to a ratio of 1:1. After that, the mice were handled carefully, cleaned the immunization site with disinfectant, and gently clipped the hair according to Institute for Laboratory Animal Research (ILAR, 2011). 100ul of mixed protein (NP- 2.8 ug/ul, and HA- 1.04 ug/ul) with CFA were then inoculated subcutaneously to the four mice in chosen four sites (25ul in each site) except for the control mice. Then they were monitored twice daily to observe if there was any adverse reaction. The first booster with HA and NP was performed 14 days after the initial immunization and this time CFA was replaced with incomplete Freund's adjuvant. The dose (25ul in 4 sites) and concentration (NP- 2.8ug/ul, and HA- 1.04 ug/ul) of protein were the same as previous

immunization. During four weeks of the first immunization, the blood sample was collected from the sub-mandibular vein with proper care. The booster was continued for NP and HA immunized group mice until an adequate response was achieved with a serum titer 1:20,000 using indirect Enzyme Linked Immunosorbent Assay (ELISA). The 96 well plates were coated (1ug/100ul) with purified baculovirus expressed HA and NP protein. The following day the serum was diluted into different dilution (1:100, 1:1000, 1:10000, 1:20000) and used as first antibody. Rabbit anti mouse conjugated with HRP was used as second antibody with 1:4000 dilution. After that the final booster was done only with the protein (25 ul in 4 sites with NP- 2.8ug/ul, and HA- 1.04 ug/ul) intra-peritoneally. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Kansas State University.

2.3 Fusion of spleen cells with myeloma cells

The immunized mice were euthanized to collect the spleen under aseptic condition and put into the petri dish without copper mesh. The fatty tissue surrounding the spleen was removed carefully using scissors and tweezers. After that, the spleen was transferred to a copper net on a petri dish, and 5ml of DMEM fresh medium was used to rinse the spleen. The splenic cells were then transferred to a 50 ml centrifuge tube containing DMEM and centrifuged at 1000 rpm for 10 minutes and re-suspended the spleen cells with 20ml of DMEM. In the meantime, the recovered myeloma cell line sp2/0 also centrifuged and re-suspended the cell pellet. The re-suspended myeloma cells and the freshly harvested spleen cells from the immunized mice were fused through repeatedly pipetting in the presence of polyethylene glycol. This substance helps the cell membrane be fused and then re-suspended the fused cells with hypoxanthine-aminopterin-thymidine (HAT) medium after centrifugation. The cells were then seeded to 24 wells plates containing feeder cells from saline peritoneal washes of mice. Feeder cells are accepted to supply development factors that advance the development of the hybridoma cells (Quinlan & O'Kennedy, 1994). Finally, we put the 24 wells plate in 37^{o} C for 10-12 days to produce spleen and myeloma cells' hybridoma.

2.4 Screening of mAb by ELISA

When there was sufficient hybridoma produced in the HAT medium, the supernatants were collected for screening. Briefly, the day before testing, the purified baculovirus-expressed HA and

NP protein were diluted into 1ug/100ul in Phosphate Buffer Saline (PBS), and 100ul was used to coat the 96 wells microtiter plate at 4^{o} C overnight. The next day, the plate was brought to room temperature and washed three times with 100ul of PBS. The excess drops of washing buffer were removed by tapping the plate on a paper towel. After that, blocked the plate using a 200ul commercial blocking buffer in each well and incubated at room temperature for 2 hours; the blocking buffer was used to improve the assay's sensitivity by reducing the background signal and enhancing the noise-to-signal ratio. After 2 hours, the plate was washed vigorously by PBST (1X PBS containing 0.05% tween 20) three times with 5 minutes incubation time of each and then tapped on a paper towel to soak the plate thoroughly. The hybridoma supernatants were used to screen as primary antibodies diluted into PBS by 1:10 dilution. The 100ul of diluted hybridoma supernatants were added into each well of the plate and incubate at room temperature for 2 hours. For the positive control, the commercial rabbit NP polyclonal antibody and rabbit HA polyclonal antibody of H18 were used. On the other hand, only PBS was used to set up the negative control. Then the plate was washed three times in PBST followed by the addition of secondary antibody 100ul rabbit anti-mouse IgG HRP (1:4,000). For the positive control, an anti-rabbit IgG HRP conjugated secondary antibody with 1:4,000 dilution in PBS was used. The HRP response was estimated utilizing an TMB Substrate. The reaction was stopped by adding a stop solution, and the absorbance was calculated at 450 nm. The optical density (OD) value twice or greater than double of the negative control was considered a positive reaction against the specific protein of interest (Classen et al., 1987).

2.5 Single cell cloning by limiting dilution

After fusion, a wide range of hybridoma cells were produced in a single well. The specific antibody-producing colony is prone to be mixed in with other cells that are either non-secreting or delivering an immune response of undesired interest. Therefore, cloning by limiting dilution is a pivotal step of the mAb production technique, ensuring that most of the well contains a single cell hybridoma. For this purpose, the antigen-specific hybridoma cells from the 24 wells plate were collected through scraping and mixed with fresh HAT medium followed by 10,000 rpm for 10 minutes centrifugation. The hybridoma pellet was then re-suspended with 5ml of HAT medium and performed viability count by utilizing the trypan blue exclusion strategy. After counting the cells under the hemocytometer, the limiting dilution of hybridoma cells was comprehended

through 10-fold dilution and continued until there was single-cell hybridoma followed by adding 100ul of HAT medium in 96 wells plates. The plate was then incubated at 37° C to grow a single cell hybridoma. Finally, we expanded the positive clones and transferred them into the T25 flask for further production of mAbs.

2.6 Immunofluorescence assay (IFA)

To determine the specificity of mAbs against WT H18N11, and chimeric H1N1 virus, the MDCK and MDCK-II cells were seeded in 96 well plates and incubated at 37° C. When there was 75-80% cell confluency, the plates were washed thoroughly by PBS and infected with MOI 1 of chimeric H1N1 virus in MDCK cells and WT H18N11 bat influenza virus in MDCKII cell line in the presence of TPCK-trypsin in the infection medium. We also prepared the plates for PR8, H3N2, and H9N2 conventional influenza A viruses following the same procedure in MDCK cells. After 48 hpi, the plates were brought to room temperature, fixed the cells with methanol for 15 minutes, and then washed with PBS three times with 5 minutes of each incubation time. Blocking of the cells was done by 5% FBS in PBS with 100ul/well for 2 hours at room temperature. Then washed 3 times with Phosphate-Buffered Saline with Tween 20 (PBST) in which 0.1% tween detergent mixed with 1X Phosphate Buffer Saline. Before using the mAb as primary Abs, they were ultracentrifuged to enrich the immunoglobulin concentration. The hybridoma supernatants of NP and HA were used as 50ul/well as the first antibody, added at 1:10 dilution. In contrast, rabbit polyclonal NP-specific antibodies (1:1000) as well as rabbit polyclonal H18 (HA) specific antibodies (1:750) were used as positive controls and incubated at 4° C overnight. The following day, prior and after adding the secondary antibodies goat-anti-mouse IgG conjugated with FITC (1:300) and goat-anti-rabbit IgG conjugated with FITC (Thermofisher Scientific, Waltham, MA, USA) (1:300) 50ul/well at 37° C for 2 hours, the plate was washed thoroughly by PBST thrice for five minutes each. The plates were then observed under the EVOS FL fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA).

2.7 Western Blotting (WB)

Western blotting (WB) is an antibody-based diagnostic assay used to recognize and evaluate target proteins separated from cells or tissue. Briefly, the MDCK cells were infected with chimeric H1N1, PR8 A/Puerto Rico/8/1934 (H1N1), A/swine/Kansas/10-83533/2010 (H3N2), and

A/quail/Hong Kong/G1/1997 (H9N2) at an MOI of 1. On the other hand, MDCK II cells were used for infecting with the A/flat-faced bat/Peru/033/2010 H18N11 viruses at an MOI of 1 and incubated at 37° C for 48 hours. After that, the infected cells were washed by PBS. Cells were lysed with RIPA (QIAGEN) buffer followed by SDS-PAGE to isolate the protein band by setting up positive and negative control. The isolated protein bands were then transferred into the PVDF membrane after detachment on SDS-PAGE and then washed by PBST three times. Next, the membrane was blocked by 5% non-fat skim milk and incubated at room temperature for 2 hours, followed by the addition of diluted (1:10) mAbs at 4° C overnight. For the positive control, NP and HA protein-specific rabbit-polyclonal antibodies were added in the membrane with 1:750 dilution and incubated overnight. The following day the membrane was washed thrice by PBST and incubated for 2 hours at room temperature with anti-mouse antibody (1:5,000) (Thermo Fisher Scientific, Waltham, MA, USA) and goat-anti-rabbit (1: 2,000) secondary antibody conjugated with HRP (Thermo Fisher Scientific, Waltham, MA, USA) to interact with the previously used the first antibody. Eventually, PVDF membrane was kept in SuperEnhanced ECL (GBCBIO Technologies), and the chemiluminescence signals were then captured by a FluorChem E imaging framework (ProteinSimple, Silicon Valley, CA, USA).

Chapter 3 - Results

3.1 Generation of monoclonal antibody

Monoclonal antibodies that are targeting to the HA and NP protein of bat influenza H18N11 virus were produced by immunizing the female BALB/C mice with previously baculovirus-expressed HA or NP protein (Fig. 1). The conventional immunization procedure was carried out in this study for the two groups of mice. We collected blood from the mice every four weeks of immunization and therefore processed to have the serum. The serum sample was then further used for ELISA to determine whether there was the desired serum titer 1:20,000 (Fig. 2A). The result revealed that the NP required three boosters to obtain optimal serum titer, whereas it took five boosters for the HA immunized group mice to get the high serum titer (Fig. 2B). When the antibody titer was sufficiently high, mice were boosted intraperitoneally by protein without adjuvant. After the final booster, the mice were euthanized, and the spleen was collected aseptically to obtain the immune B cells, followed by fusion of the immortal myeloma cells SP2/0. The fusion of the B cells and the SP2/0 cells were then allowed to grow in the HAT medium to raise the hybridoma cells. HAT is a choice of medium that works as a combination of aminopterin. For the hybridoma (Fig. 3) production in this selective medium, it took 12 days. Therefore, the hybridoma supernatants were collected and further screened by ELISA to identify which supernatants react against the HA and NP antigen of bat influenza H18N11 virus. The ELISA screening results showed that 17 wells out of 84 were found to secrete anti-NP antibodies against the NP coated protein (Fig. 4). However, 22 wells of hybridoma supernatants revealed a positive reaction for the HA out of 92 (Fig. 5).

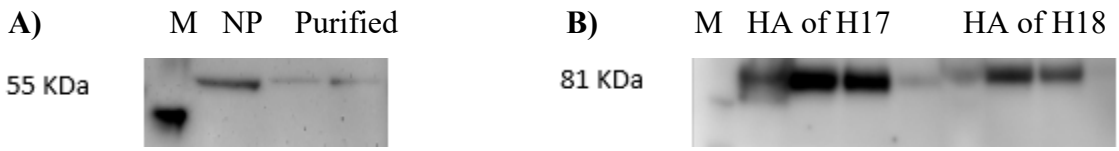

Figure 1: Western blotting detection of baculovirus expressed NP and HA proteins. A) Western blotting data showed NP protein expression of H18N11 bat influenza virus B) HA protein expression of H18N11 bat influenza virus by baculovirus protein expression system in sf9 cells. 6X His-HRP tag was used with a dilution of 1:1000.

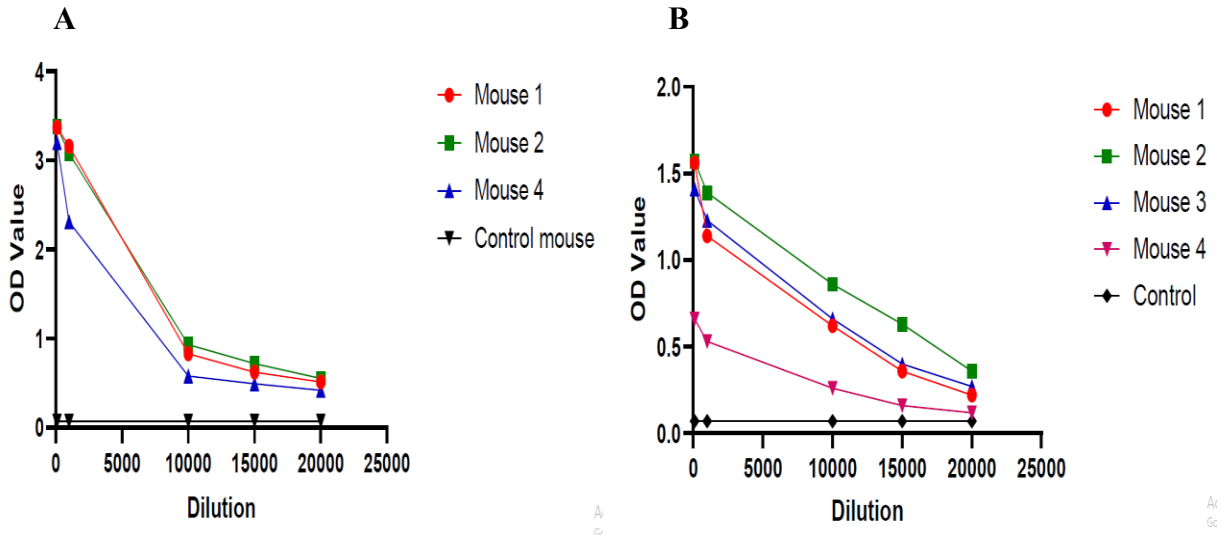

Figure 2: ELISA assay to detect serum titer of HA and NP protein immunized mice. A) Higher serum titer reacted by ELISA against NP. The serum sample of mice 1 and 2 revealed the highest titer with an OD value of 0.51 and 0.55, respectively, whereas mouse 4 had a slightly lower OD of 0.42 at 1:20,000 dilutions. B) Serum titer at different dilutions against HA. The mice 1, 2, and 3 had the highest titer of 0.22, 0.36, and 0.27. In contrast, mouse 4 revealed the lowest titer of 0.14 at 1:20,000 dilutions.

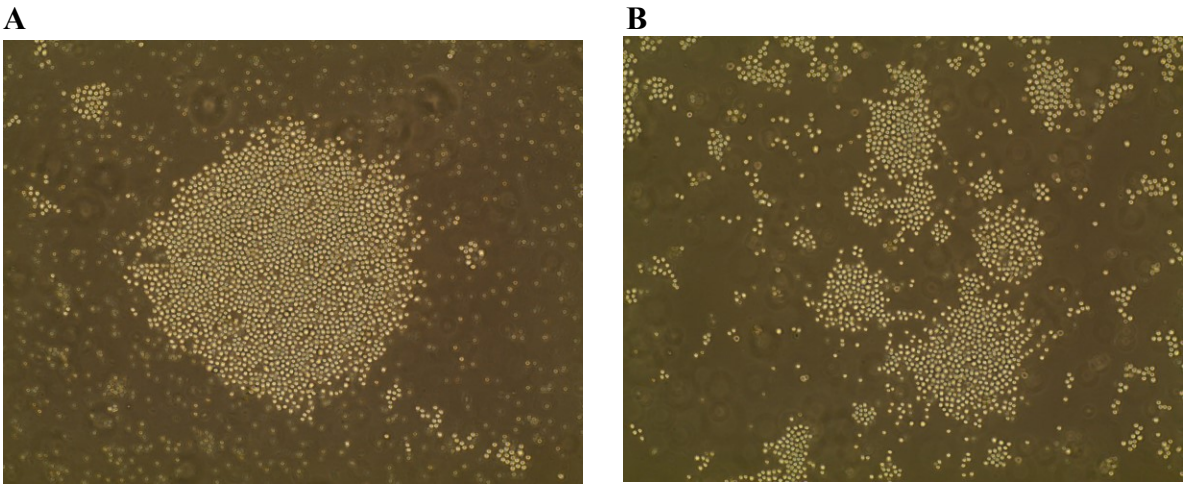

Figure 3: Hybridoma of NP and HA immunized mice produced through the fusion of myeloma cells and B cells from spleen. A) Hybridoma of NP immunized mice grown in HAT medium in 10 days. B) Hybridoma of HA immunized mice grown in HAT medium in 10 days.

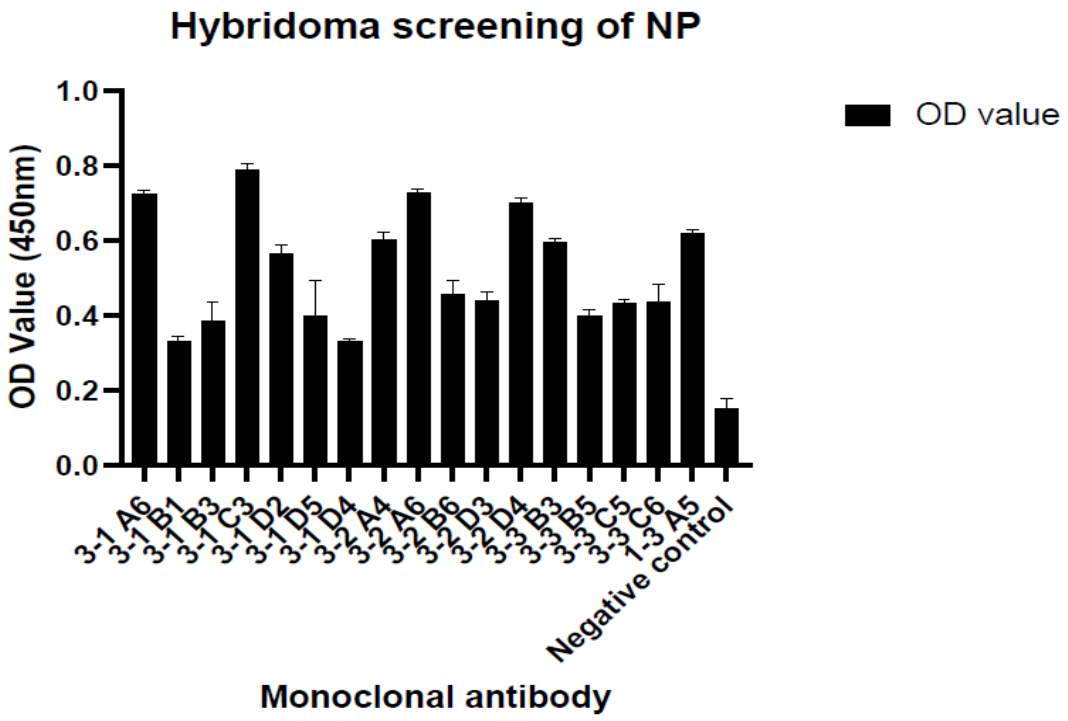

Figure 4: Screening of the NP hybridoma supernatants through ELISA. The OD value of negative control is 0.17. Then the OD value greater than 0.34 was considered as a positive reaction. There are 17 hybridomas showed positive reaction against NP protein.

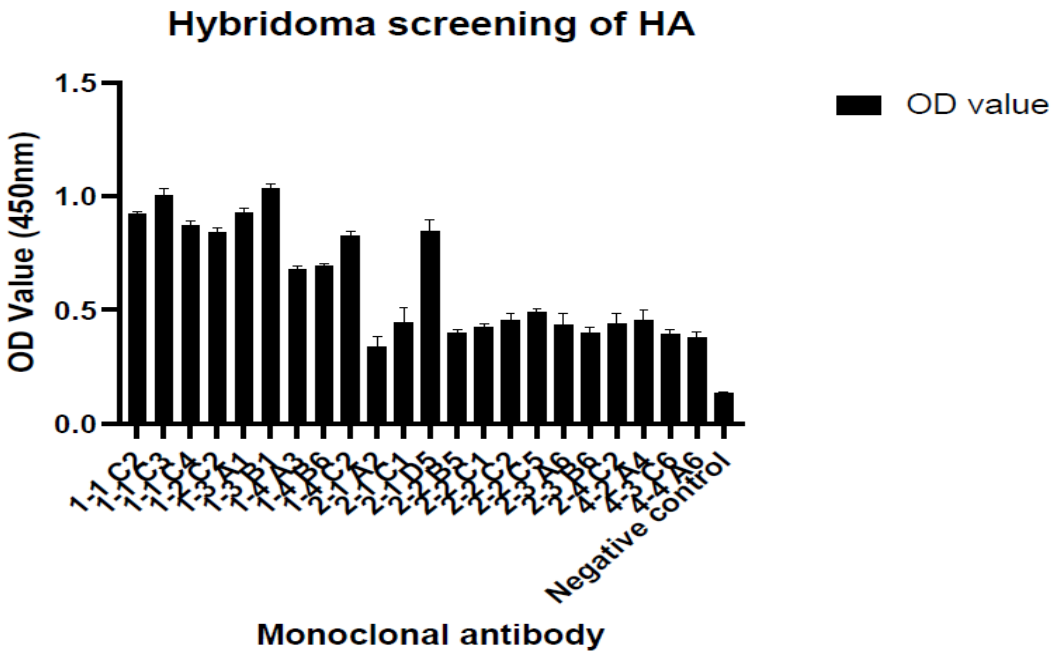

Figure 5: Screening of the HA hybridoma supernatants through ELISA. The OD value of negative control is 0.14. Then the OD value greater than 0.28 was considered as a positive reaction. There are 22 hybridomas showed positive reaction against HA protein.

3.2 Reactivity of anti-NP and HA monoclonal antibodies detected by IFA

To limit the number of false positives of mAbs responsive to HA and NP protein, IFA was performed. IFA was done for the chimeric H1N1, PR8, H3N2, H9N2, and WT H18N11 viruses. The chimeric virus was used to infect the MDCK cells with an MOI of 1. After 48 hpi, the cytopathic effect (CPE) was observed, and necessary steps were followed for IFA which was described in methodology. As shown in figure 6, six anti-NP mAbs reacted weakly against the MDCK cells infected with the chimeric virus. The mAbs 3-1D2-C2, 3-1D2-D6, and 1-3A5-D8 could have the capability to bind somewhat stronger than the mAbs 3-2A62B8, 3-2A62F8, and 3-1D2-E9 which showed a weak signal for the fluorescence activity. We also used other influenza viruses including H3N2, H9N2, and PR8 virus, for IFA to identify whether the produced NP mAbs can even react with other IAVs. The results depicted that NP mAbs showed specificty to react only with chimeric H1N1 virus and did not positively respond to the conventional IAVs (Fig. 7).

Next, we tested the anti-HA specific mAbs collected from hybridoma supernatants to identify the reactivity by dectecing the WT H18N11 virus in infected MDCK-II cells for 48 hours at an MOI of 1. Interestingly, no hybridoma supernatants showed positive reaction as anti-HA

reactivity on the fluorescence signals (Fig. 8) compared to the control positive and negative antibodies.

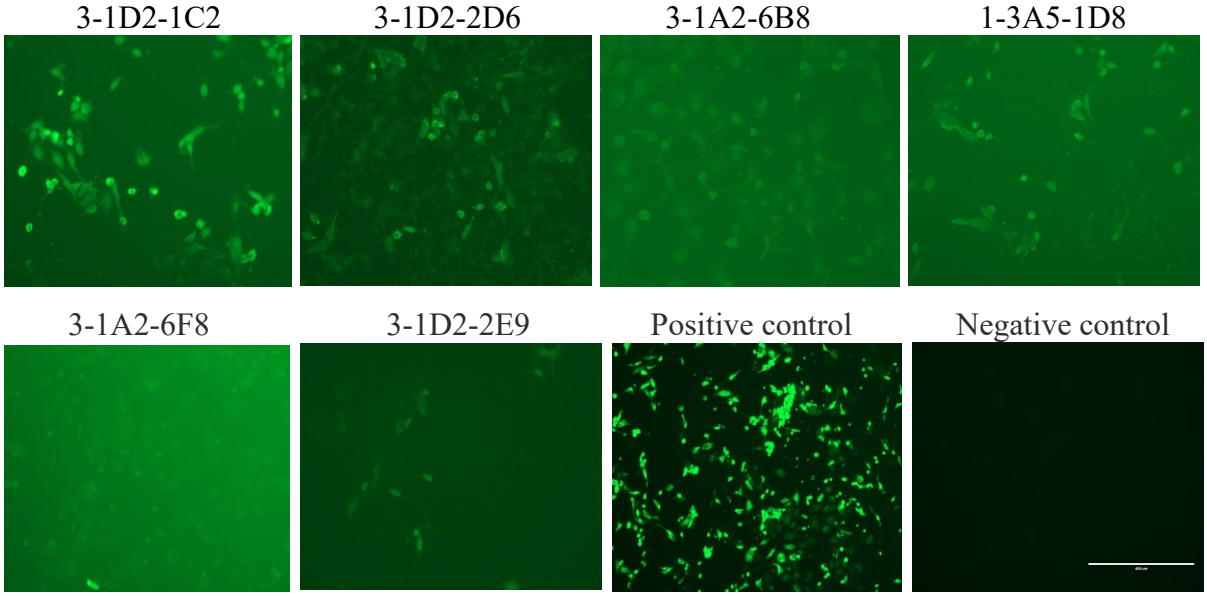

Figure 6: Immunofluorescence assay detection of NP mAbs. MDCK cells were infected with the chimeric virus and for the negative control mock infected cells were used. Cells were fixed and incubated with tested NP-specific mAbs, and finally stained with FITC. Rabbit anti-NP polyclonal antibodies were used for the positive control.

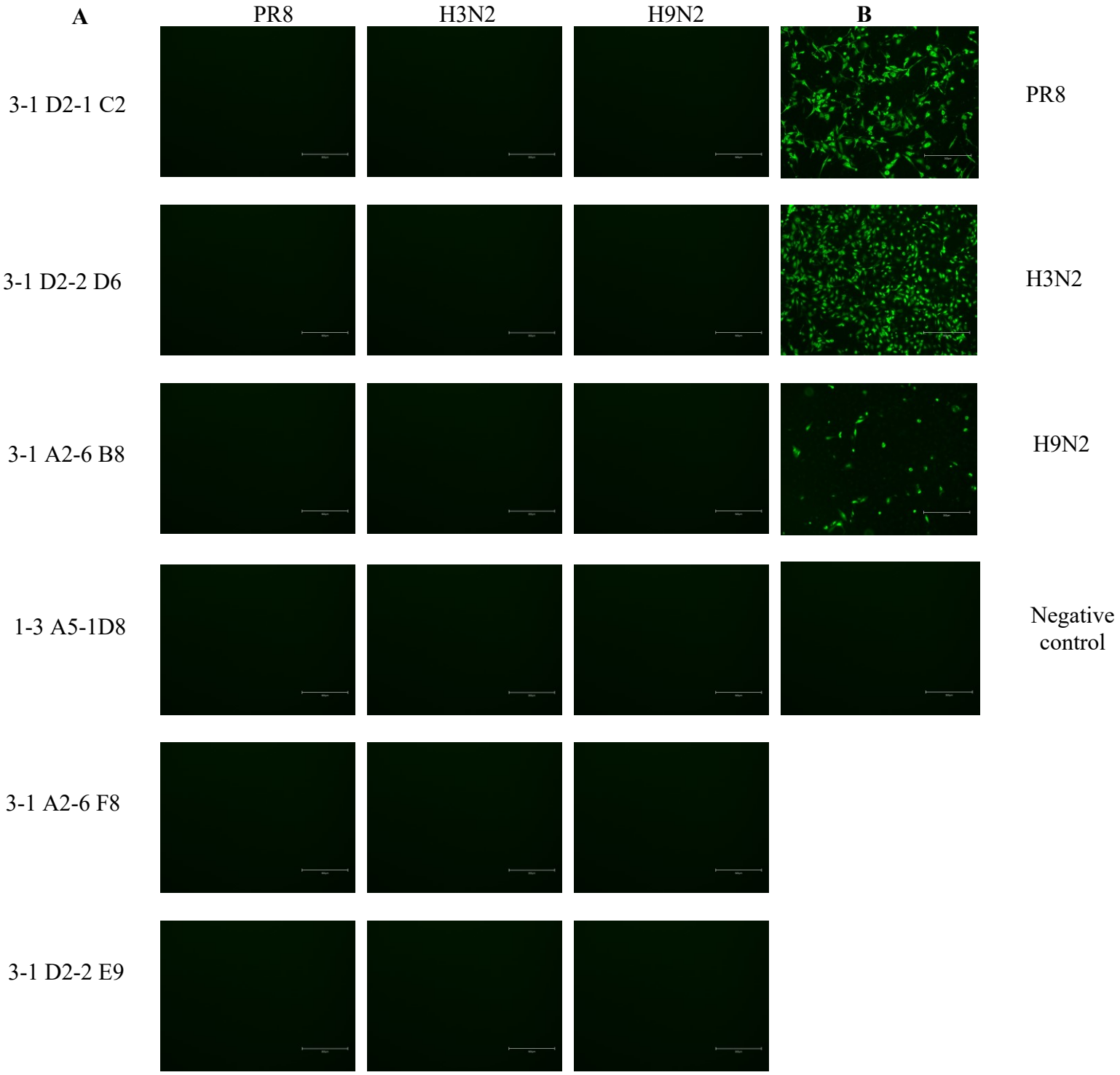

Figure 7: Specificity of NP mAbs by detecting conventional and bat NP through IFA. A) PR8, H3N2, and H9N2 virus was used to infect MDCK cells. Infected cells were fixed with methanol and incubated with tested NP mAbs. Finally, anti-mouse conjugated with FITC used as a second antibody to stain. B) A commercial rabbit anti-NP pAb was used as the control to detect NP in PR8, H3N2, or H9N2 virus infected MDCK cells, and finally, anti-rabbit conjugated with FITC used as the second antibody to stain. Mock-infected cells were used for negative control.

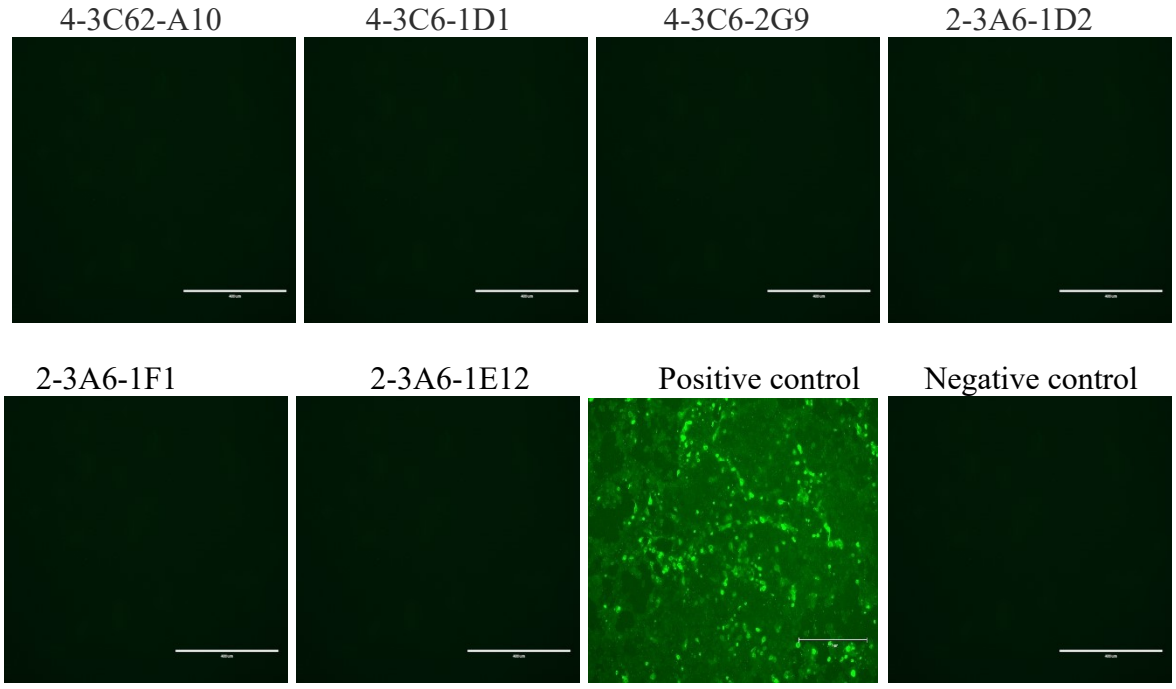

Figure 8: Immunofluorescence assay detection of HA mAbs. WT H18N11 virus-infected in MDCK II cells and mock-infected MDCK II cells were used as negative control. Rabbit anti-HA polyclonal antibodies were used for positive control. Cells were fixed and incubated with HA specific mAbs, and finally stained with FITC. All the HA mAbs showed negative reaction in IFA.

3.3 Isotyping of monoclonal antibody

The hybridoma supernatants produced from single-cell cloning have the capability of monovalent reactivity. Cloning is required to ensure that poly-specificity problems are avoided, and the risk of non-specific cells are minimized. The mAbs that showed a positive reaction in IFA were further used for isotyping by capture ELISA. Briefly, the 96 wells ELISA plates were coated with commercially available immunoglobulin IgG1, IgG2a, IgG2b, and IgG3 with 100ng/100ul overnight at 4ºC. The following day plates were washed thoroughly by PBS and blocked by the blocking buffer for 2 hours at room temperature. After that, the plates were washed by PBST three times with 5 minutes incubation period for each. The produced mAbs were then added as the first antibody with a dilution of 1:10 for 2 hours at room temperature. On the other hand, a negative control was set up by adding PBS. The second antibody, rabbit-anti-mouse conjugated with HRP with 1:4,000 dilution, was used as the second antibody after washing the plate with PBST and incubated for 2 hours. The TMB substrate was then added for 20 minutes, followed by the addition of stop solution. Finally, the reaction was read under 450nm wavelength, and the OD value was recorded. The result showed that all the mAbs were reactive against IgG3 coated wells, which means the IgG3 immunoglobulin was reactive in anti-NP monoclonal antibodies (Fig. 9).

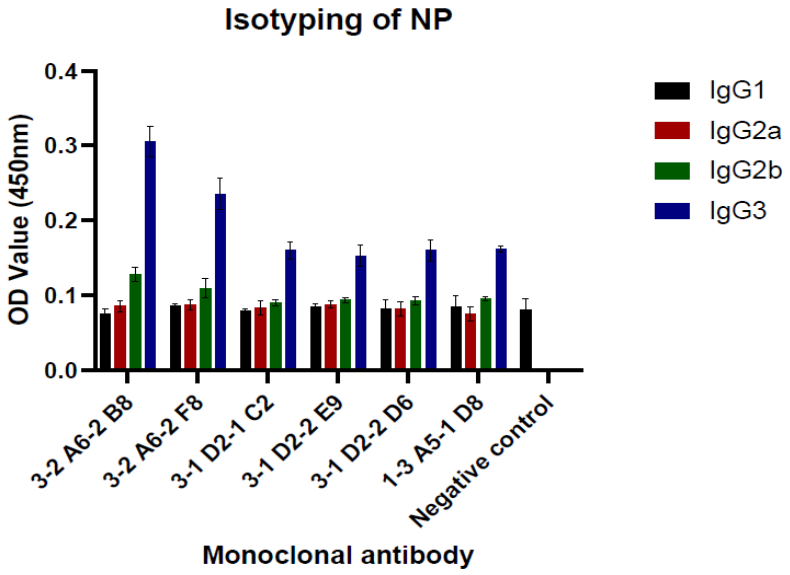

Figure 9: Isotyping of mAbs through ELISA. Six mAbs showed a positive reaction with IgG3 immunoglobulin as compared to the other IgG.

3.4 Epitope mapping

Regarding the epitope mapping, the unique characteristic for a mAb is to recognize surface protein whether it binds to its target's intra or extracellular location (Mancia et al., 2007). We did the epitope mapping for the NP protein of the H18N11 virus. Therefore, we aligned the NP protein sequences, and the highest 83% amino acid was found to similar alignment with other IAVs. We used online epitope predictor tools immunomedicine, which provided a group of predicted epitopes. Then we designed the predicted epitope based on the Kolaskar & Tongaonkar, 1990 method depending on the epitope's physical and chemical properties (flexibility, hydrophibility, accessibility). We picked the predicted epitope amino acid sequence KLMENSDPKDKVFL (coded as bNP2) for H18N11 with the amino acid position of 445-458 aa. We also selected two other epitopes. One from the H17N10 bat influenza virus was coded as bNP3, and it's sequence was LMESSDTKEKVFVG located at positions of 446-459 aa, and another LDEILVISLDLKSHYW (coded as bNP1) assumed to predict both H18N11 and H17N10, which is located in 370-385 aa position. After that, to visualize and determine the area where these epitopes were located, used the software PyMOL 2. Figure 10 revealed that all the epitopes are on the surface area assumed to interact with produced mAbs. Then the peptide synthesize was done from the Genscript. We hypothesized that the only predicted epitope from the H18N11 (denoted as bNP 2) would be reactive with the anti-NP mAbs. To test our hypothesis, we performed indirect ELISA by using these synthesized polypeptides coated the 96-well plates (100ng/100ul per well). The result showed that the bNP2 polypeptide, from the bat influenza virus H18N11, was reactive with produced anti-NP mAbs compared to the negative control and the other two polypeptides (Fig. 11). In another sense, it can be said that the mAbs that were produced from the mice were able to recognize the epitope of synthesized polypeptide bNP2 from the H18N11 virus.

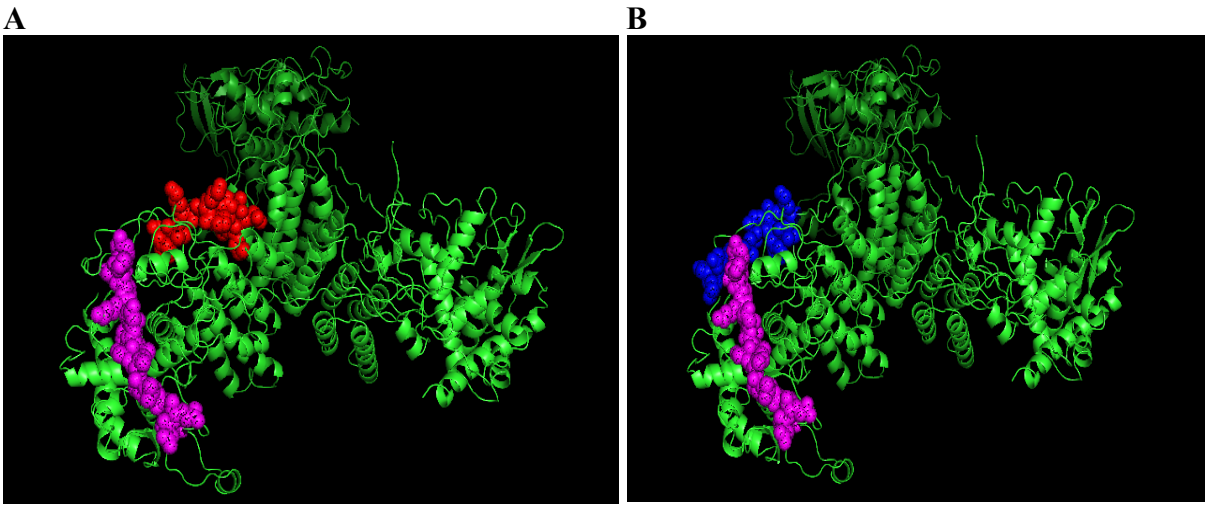

Figure 10: Visualization of the location of synthesized polypeptides on the NP protein. A) The red dot color revealed the bNP2 polypeptide on the surface area of H18N11 and the purple dot color for bNP1. B) The bNP1 polypeptide showed as blue dots, and bNP3 polypeptide showed as purple dots on the surface are of H17N10.

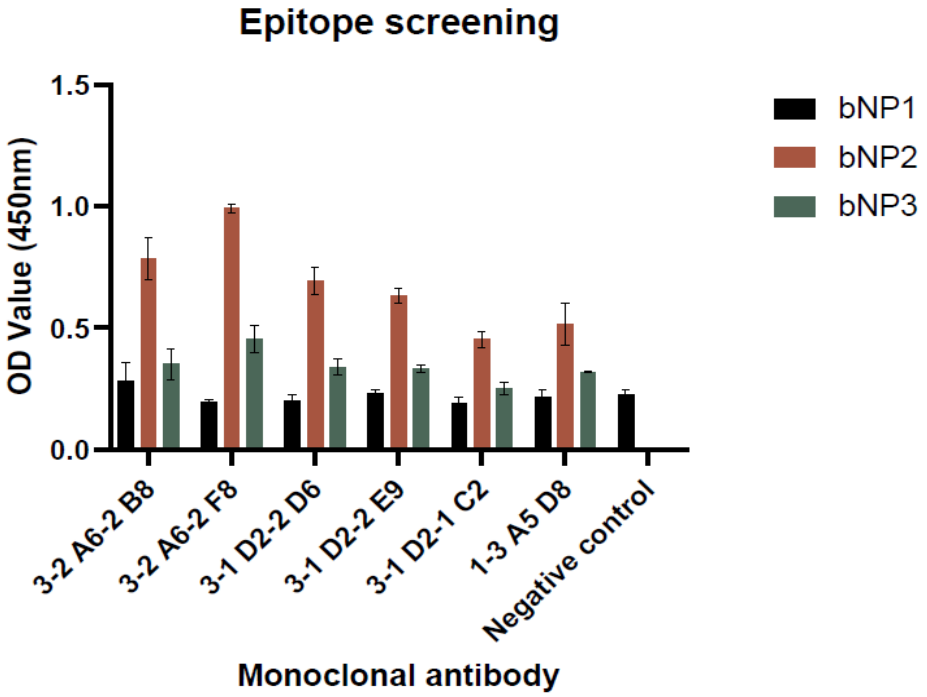

Figure 11: Determination of epitopes recognized by NP mAbs. The 96 wells plate was coated with three different synthesized protein bNP1, bNP2 and bNP3. The six positive tested NP mAbs were used as the first antibody. Anti-mouse IgG conjugate with HRP was used as the second antibody. All the anti-NP mAbs recognized the epitope bNP2 of the H18N11 bat influenza virus compared to the negative control. For the negative control, only PBS was used to coat the plate.

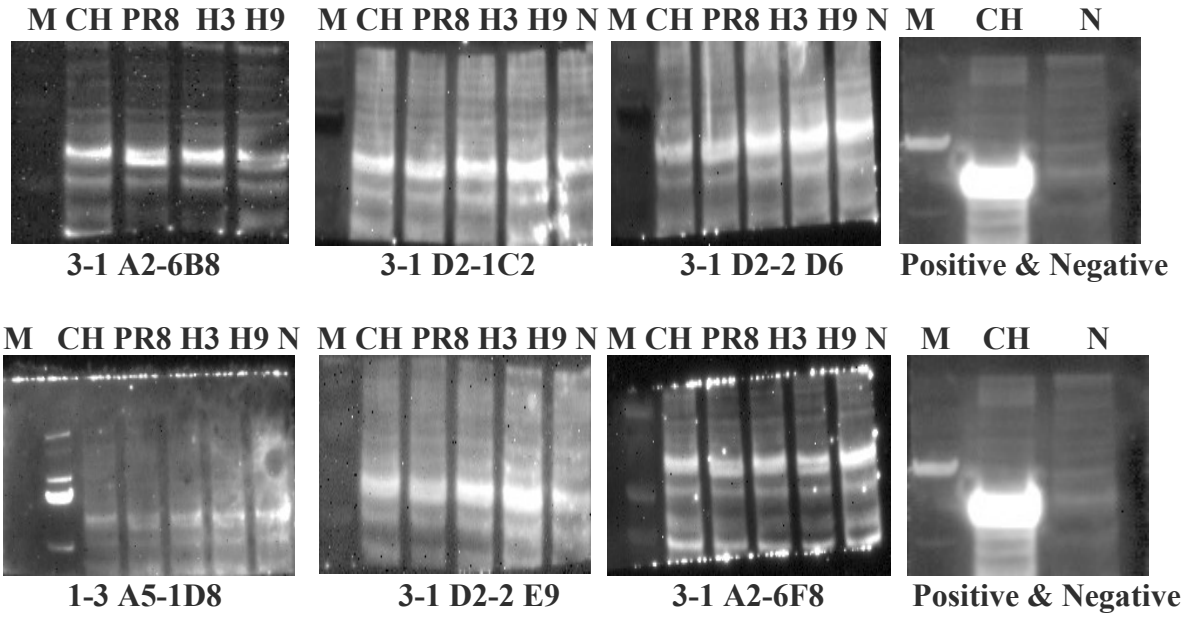

Figure 12: Western blotting data of the NP protein specific mAbs. MDCK cells were infected at an MOI of 1 with chimeric H1N1, PR8, H3N2, or H9N2 IAVs. The cell lysate was collected using RIPA buffer. All the NP-specific mAbs were used as the first antibody with 1:10 dilution, and anti-mouse IgG conjugated with HRP (1:5,000) was used as the second antibody. Rabbit anti-NP pAbs were used as a positive control with 1:500 dilution and anti-rabbit IgG leveled with HRP (1:2,000) used as the second antibody. As compared to the negative control, all the mAbs could not detect the NP protein of H18N11 virus. Only the positive control showed a strong band with 55kDa.

Chapter 4 - Discussion

Emerging infectious diseases (EID) with the ability to produce a severe epidemic are highly prevalent worldwide. More than 60% of EIDs, including influenza virus (category C), can be a zoonotic disease (NIAID, 2015) and have the ability to exhibit negative social and economic impacts (Jones et al., 2008). It is considered a challenge to prevent EIDs, but possible control measures can be installed to mitigate its effect on animals and humans. Targeted surveillance, disease management, and an adaptive control program are required to reduce EIDs spread (Bonneux & Van Damme, 2011).

The introduction of IAVs into the human populations from other animals can cause a severe impact on health worldwide. During the most recent decade, IAVs research has increased significantly and is considered a pivotal microbe in virological research. IAVs are important zoonotic organisms that cause outbreaks in poultry, wild birds, pigs, and different warm-blooded animals. Aquatic birds are the reservoir of IAVs, and 16 HA and 9 NA viral subtypes of IAVs have been isolated from them to date. IAVs are found worldwide and occasionally can transmit in different hosts (including domestic animals, aquatic animals, and human beings) and produce new genetically modified organisms, which can be more dangerous. Recently identified H17N10 and H18N11 subtypes of bat influenza viruses (Tong et al., 2012, 2013) have brought up the scope for more biological research on IAVs. The new subtypes of bat influenza virus H17N10 and H18N11 do not have similar surface glycoprotein HA and NA of traditional IAVs (Ying Wu et al., 2014). Therefore, the entry mechanism into host cells is quite different and uses MHC II molecules instead of sialic acid (Giotis et al., 2020; Karakus et al., 2019). Genomic sequences of the H18N11 revealed some differences among the amino acids of other conventional IAVs. The resources to study the bat influenza viruses are still limited. To best of our knowledge, only polyclonal antibodies targeting the NP protein of IAVs are found commercially to react with bat influenza NPs. However, mAbs against bat influenza viruses are still not available or have not been produced for research and diagnostic purposes. Therefore, the project in this thesis was designed to produce mAbs targeting the NP and HA proteins of the H18N11 virus and to further characterize these produced mAbs.

Monoclonal antibodies are monovalent antibodies that bind to similar epitope and are produced from a single B cell clone (Little et al., 2000). The mAbs were first developed in the

mice through the hybridoma technique, which had made a significant revolutionary change in the biological world (Köhler G. & Milstein C., 1975). With the advancement of sciences, different methods have been applied and modified in order to produce mAbs with the best quality. The mAbs comprises many applications from radioimmunotherapy to virus disease treatment, even can play a pivotal role in developing diagnostic tools in the biological area. In this study, we produced a panel of mAbs against the NP and HA protein of bat influenza H18N11 virus through the hybridoma technique described previously by Köhler G and Milstein C. in 1975. The fusion of spleen cells with immortal myeloma SP2/0 cells were allowed to grow in the HAT medium. HAT is a choice of medium that works as a combination of aminopterin. In the presence of aminopterin drug in the medium, nucleotide's de novo pathway is blocked (Olsson & Kaplan, 1980). Therefore, the myeloma cells die as they cannot maintain the de novo or the salvage pathway for the nucleotide production and ultimately die. On the other hand, unfused B cells from the immunized mice cannot survive for long days as they have a short life span. Hence the fused myeloma and B cells hybridoma only survive in HAT medium. Indirect ELISA was then used to screen the hybridoma supernatants to find out how many wells were able to react with NP and HA-coated 96 well plates. We found that 17 and 22 hybridoma supernatants showed interaction against NP and HA antigen-coated ELISA plates. Then, the ELISA positive reacted wells were used to grow as single cell hybridoma to produce monoclonal antibody, and further characterization and validation were done.

IFA was one of the preliminary techniques for rapid viral diagnosis analysis (Madeley & Peiris, 2002). The previous study already reported IFA identifying the influenza A and B virus for further diagnostic tests (Mcquillin et al., 1985). The use of mAb in IFA can ensure that the detection is specific to the targeted viral antigen. We tested the IFA for produced monoclonal antibodies to identify the reactivity against NP and HA antigenic protein of chimeric H1N1 and WT H18N11 bat influenza virus. We supposed the mAbs would react vividly with the NP and HA protein. The result showed that all the NP-specific mAbs were found to have interacted weakly with NP protein. Surprisingly, the mAbs raised against the HA protein were failed to recognize or bind with HA antigen in IFA. The IFA can make it possible to visualize the virus's distribution and localization with antibodies inside a cell or tissue. Some mAbs respond with just conformational epitopes, so the native structure of a protein should remain stable due to mild chemical reaction (Yang et al., 2008). Therefore, the possible reason the mAbs either weakly bind or could not react

with the specific protein was that chemical treatment with methanol to fix the cell lines would alter the epitope's structure and ultimately could not bind.

The western blot or western blotting is a scientific method in science and used to distinguish specific proteins in tissue homogenate or tissue lysate. It uses basic standards to isolate proteins as indicated by their molecular weight and bind them to permit their detection with a particular antibody. The heat treatment of the cell lysate makes the protein into a denatured form, alters the protein's complex conformation into a linearized structure, and facilitates the binding with the appropriate antibody of interest. To further validate the NP-specific mAbs, we performed the western blotting to identify the reactivity with NP protein. A similar approach was applied in the previous study to determine the NP protein-specific mAbs activity of the avian influenza virus (Yang et al., 2008) and found that the mAbs can bind with the epitope. In contrast, to our surprise, all the six mAbs in this study could not detect the NP antigen through western blotting (Fig. 12). In this research project, as a positive control, we used the commercially available polyclonal antibody, which provided a strong band with specificity. It is challenging to understand what the mechanism is behind not binding with the specific protein. The possible explanation would be the polyclonal antibody contains some features that are more responsive to the native and the denatured protein. The characteristics of mAbs are pretty different, as some mAbs only react with the native protein compared with the denatured protein or vice versa (Yang et al., 2008). Another reason is that the immunogenicity of mAbs depends not only on the amino acid sequences but also on the correct conformation of the epitope structure (Van Regenmortel et al., 1988). Therefore, we assumed that the produced mAbs could only bind weakly with the native protein other than no reaction with the denatured protein by heat treatment.

Chapter 5 - Conclusion and Recommendation

This study produced a panel of mAbs (six for both HA and NP) through the hybridoma technique targeting HA and NP-specific protein of bat influenza H18N11 virus. We further showed that all NP-specific mAbs belong to IgG3 and can target the possible epitope with amino acid position 445-458aa. The NP mAbs showed a weak reaction in IFA to specifically detect the bat influenza H18N11 virus. Whereas none of the HA-specific mAbs were able to detect HA of the H18N11 virus. All the mAbs of HA and NP were failed to react with specific respective proteins of bat influenza H18N11 virus in the western blotting assay. Therefore, more research should be needed to determine why the mAbs weakly bind with NP in IFA, and no reaction with the specific protein NP in western blotting.

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