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Potential strategies and biosafety protocols used for dual-use research on highly pathogenic influenza viruses

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Summary

Influenza A viruses (IAVs), particularly the highly pathogenic avian influenza (HPAI) H5N1, have posed a substantial threat to public health worldwide. Although the laboratory generation of the mutant influenza virus H5N1 with airborne transmissibility among mammals, which has been considered as a dual-use research, may benefit the development of effective vaccines and therapeutics against the emerging infectious agents, it may also pose threats to national biosecurity, laboratory biosafety, and/or public health. This review introduces the classification and characterization of IAVs, pinpoints historic pandemics and epidemics caused by IAVs, emphasizes the significance and necessity of biosafety, summarizes currently established biosafety-related protocols for IAV research, and provides potential strategies to improve biosafety protocols for dual-use research on the highly pathogenic avian influenza viruses and other emerging infectious agents.

INTRODUCTION

"Dual-use research" is defined as a biological research with legitimate scientific purpose that may be misused to pose a biologic threat to public health and/or national security, which has been monitored and evaluated by the National Science Advisory Board for Biosecurity (NSABB) of the United States (http://oba.od.nih.gov/biosecurity/biosecurity.html).

Recently, Fouchier and colleagues at Erasmus Medical Center in Rotterdam, the Netherlands and Kawaoka's group at the School of Veterinary Medicine, the University of Wisconsin–Madison have generated several avian influenza A virus (IAV) H5N1 variants with mutations in hemagglutinin (HA) protein and/or polymerase basic protein 2 (PB2). They found that these H5N1 mutants became more transmissible among ferrets [1–4]. According to Kawaoka's study, a reassortant H5HA/H1N1 virus, which comprised four mutations of H5 HA at N158D, N224K, Q226L and T318I, and the remaining seven gene segments from 2009 pandemic H1N1 virus, was identified to replicate efficiently among ferrets with the ability to droplet transmission in a ferret model [3]. In Fouchier's case, they generated a

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CONFLICT OF INTEREST

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modified A/H5N1 virus, which is able to acquire mutations during passage in ferrets, ultimately obtaining the ability to airborne transmission among these mammals. They showed that four substitutions at H103Y, T156A, Q222L and G224S of the host receptor-binding protein HA and one mutation at E627K of PB2 were able to consistently present in the airborne-transmitted viruses [2].

The transmissibility of these mutant H5N1 viruses between mammals constitutes a significant risk for influenza pandemic in humans [5–7]. Thus, these studies related to mutant influenza viruses were considered as a typical dual-use research by NSABB, a U.S. government advisory panel. Accordingly, a variety of discussions and controversies occurred surrounding the due-use research and the necessity for publication of the due-use research-related findings [8–13]. Therefore, the submitted papers by Fouchier and Kawaoka's groups have been thoroughly reviewed and evaluated by NSABB for the benefits and potential negative consequences before these manuscripts were approved for publications in *Nature* and *Science* [4,14]. After a months-long debate on the controversy of the mammalian-transmissible mutant H5N1 influenza, these scientific findings representing the due-use research were eventually published in the two top journals [4,15,16], with the respective title of "Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets" [3] and "Airborne transmission of influenza A/H5N1 virus between ferrets" [2]. There is no doubt that the findings from these studies will help understanding the mechanism of viral transmission, improving international surveillance and identifying pandemic flu threats since the flu surveillance is currently lacking [17,18]. However, the laboratory-created viral mutants with increased airborne transmissibility may be misused to pose threats to national biosecurity, laboratory biosafety, and/or public health [19–22].

Currently, a number of broadly neutralizing antibodies (nAbs) have been discovered by several research groups on their effectivity against divergent strains of IAVs in group 1 and/or group 2 [23–26], in which nAb F10 and CR6261 having been shown to target all group 1 IAVs tested [27,28], while nAb CR8020 and F16 being able to respectively target most group 2, or neutralize both group 1 and 2 IAVs [29,30]. Thus, based on the discovery of these nAbs and their recognized epitopes, tremendous breakthrough has been made in the efforts to develop cross-reactive vaccines against multiple strains of IAVs [31–34]. Studies have shown that cross-clade nAbs could be induced against various clades of H5N1 viruses by priming mice with hyperglycosylated HA DNA particularly with 83NNT and 127NSS mutants and boosting with virus-like particles [35]. Other reports have also revealed that a triclade DNA vaccine encoding HAs of clades 0, 2.3.2.1, and 7.2 induced broadly nAbs against all H5 clades and subclades that protected vaccinated mice against high-dose lethal challenge of H5N1 virus [36]. In addition, cross-neutralizing antibodies could be elicited against pandemic 2009 H1N1 and seasonal H1N1 IAVs by a point-mutation in HA2 subunit [37]. Nevertheless, although the currently developed vaccines demonstrated effectiveness against identified IAV strains, their cross-reactive efficacy against laboratory-mutated airborne-transmissible viruses as well as other IAVs potentially causing future influenza pandemics is still needed to be elucidated. Therefore, future antiviral therapeutics (including nAbs) and vaccines should be developed and evaluated for the potential efficacy against the laboratory-mutated H5N1 virus and naturally-occurring IAVs in addition to the currently-existing influenza viruses. Accordingly, further understanding of IAVs and their pandemic risks would be of great importance to help executing and reinforcing biosafety-related protocols for the prevention of influenza pandemic, which will significantly promote the development of effective influenza vaccines and antiviral agents.

In the rest sections of the review, we initiated with the introduction of IAVs in terms of their classification and characterization, pinpointed the historic pandemics and epidemics caused

by IAVs as well as their potential threat to public health, emphasizes the significance and necessity of biosafety, further summarized currently established biosafety protocols for IAV studies, and provided suggestions and potential strategies to improve influenza biosafety protocols for dual-use research on the highly pathogenic influenza viruses and other emerging and re-emerging infectious agents.

CLASSIFICATION AND CHARACTERIZATION OF INFLUENZA A VIRUSES

Influenza, commonly known as “the Flu”, is an emerging infectious disease caused by influenza virus. This is a group of negative sense, single-stranded RNA viruses belonging to the family of orthomyxoviridae. Almost all of the A, B and C serotypes can affect birds and mammals, but only IAVs cause human pandemics and epidemics [38]. The genome of IAVs contains eight single RNA strands encoding several important proteins, including surface HA, neuraminidase (NA), and M2 transmembrane protein, as well as nucleoprotein (NP), M1 matrix protein, nonstructural proteins (NS1, NS2), polymerase heterotrimeric complexes (PA, PB1 and PB2), and PB1F2, an additional protein encoded by some IAVs [39].

Among all of the proteins encoded by IAVs, HA and NA are the two major antigenic determinants, and, correspondingly, HA and NA play the most important role in virus infection. These are two large glycoproteins located on the outside of viral particles. HA is an antigenic glycoprotein responsible for the attachment of the virus to the infected host cell receptors, typically using different sialic acids to bind on the cell membranes [40]. After receptor binding, HA will then facilitate the entry of the viral genome into the target cells, leading to the fusion of host endosomal membrane with the viral membrane [41,42]. In addition to virus binding, HA may help to clump red blood cells together *in vitro*. The NA of IAVs is an enzyme that cleaves the sialic acid groups from glycoproteins, releasing the virus from the host cell after influenza virus replication [43]. NA also prevents the aggregation of the viruses based on its ability to cleave the sialic acid residue from viral proteins.

In addition to HA and NA proteins, other IAV proteins play indispensable roles in different processes of viral replication, assembly and budding. For example, M1 forms an intermediate layer interacting with other components of viral proteins. It binds RNA, directs virus budding, and regulates the transport of ribonucleoprotein (RNP) into and out of the nucleus, with its middle domain being responsible for binding NP protein and self-association [44]. M2 protein, a 97 amino acid protein in the form of hemotetramer, serves as an important ion channel for virus assembly and budding [45]. Particularly, its cytoplasmic tail plays a crucial role in the production of infectious influenza virus particles by interacting with M1 protein and influencing virus assembly at the site of virus budding [46].

The classification of IAVs has been well defined. Based on the surface HA and NA proteins, all IAVs are further classified into 16 different HA subtypes (H1 to H16) and 9 different NA subtypes (N1 to N9). Among the 16 different HA subtypes, the first three HAs, including H1, H2 and H3, are found in human influenza viruses, while H16 is the newest H type isolated from black-headed gulls caught in Sweden and the Netherlands in 1999 [47].

IAVS HAVE CAUSED GLOBALLY HISTORIC PANDEMICS AND EPIDEMICS, DEMONSTRATING A CONSIDERABLE THREAT TO PUBLIC HEALTH

Many influenza pandemics have happened throughout history, resulting in enormous morbidity and mortality, as well as economic losses. In fact, more than thirteen influenza pandemics have occurred since 1500 [41]. Three major influenza pandemics and epidemics occurred in the 20th century, including the 1918 Spanish influenza, the 1957 Asian flu and

the 1968 Hong Kong flu. A significant human influenza pandemic, known as the 2009 swine flu, occurred in the last decade.

The 1918 Spanish influenza, which killed approximately 50 million people, was caused by an A/H1N1 variant which is still pandemic in both human and pig populations [48–52]. A/H2N2 avian influenza is responsible for the 1957 Asian flu, with the pandemic outbreak originating from China and spreading worldwide, causing 1.5 – 2 million deaths [50–52]. The 1968 Hong Kong flu was caused by an A/H3N2 variant and killed approximately 1 million people worldwide (http://www.who.int/foodsafety/micro/avian/en/index1.html). Most recently, Tumpey and colleagues at the Center for Disease Control and Prevention (CDC) of the United States isolated a novel swine-origin influenza A H3N2 variant [A(H3N2)v] from humans with increased capacity to infect Calu-3 cells that were derived from human bronchial epithelium, raising a great concern over the pandemic potential of these viruses [53].

The outbreak of the 2009 influenza A/H1N1 originated in Mexico during March and early April of 2009 and spread quickly to other countries. According to the World Health Organization (WHO), this H1N1 pandemic has caused a global outbreak, involving over 212 countries with a total of at least 18,209 deaths (http://www.who.int/csr/don/2010_06_25/en/index.html). WHO has thus declared the 2009 flu pandemic to be the first global influenza pandemic of the 21st century.

As far as the highly pathogenic avian influenza (HPAI) H5N1 is concerned, a 3-year-old child died from respiratory failure in Hong Kong in May 1997 [43], bringing the first known human case of influenza A/H5N1, with the outbreak resulting in 6 deaths in total among 18 confirmed infections. Since 2003, H5N1 has infected humans frequently with fatal consequences. As of June 07, 2012, the virus has caused 357 human deaths among a total of 606 infected cases, with the death rate reaching 60% (http://www.who.int/influenza/human_animal_interface/EN_GIP_20120607CumulativeNumberH5N1cases.pdf). In addition to humans, this virus is the leading cause of avian deaths, as well as infections and deaths for several other mammals, such as cats [44].

The recent worldwide outbreak of swine origin 2009 H1N1 and the increasing numbers of H5N1 human infections illustrate that influenza remains a significant public health problem. Because of the high morbidity and mortality of HPAI H5N1, its wide mutation or reassortant within or across strains, and its broad host reservoirs and intimate interaction with human populations, the H5N1 virus has been listed as the biggest risk for future influenza pandemic. Although human-to-human transmission of H5N1 has been very rare, recent research has confirmed the efficient transmission of the adapted H5N1 virus via aerosols or respiratory droplets among mammals, such as ferrets, the animal species that most closely resembles humans in flu studies [54–57], indicating the potential transmission of the H5N1 virus among humans. Thus, the current concern of flu research has focused on the possibility that H5N1 could transmit through humans and causes a future flu pandemic.

IMPORTANCE AND NECESSITY FOR ESTABLISHING BIOSAFETY-RELATED PROTOCOLS FOR IAVS

The prevention and control of potential future flu pandemics caused by IAVs, particularly by potential transmission of H5N1 virus via aerosols among humans, require more extensive investigation of IAVs from the perspectives of epidemiology, virology, pathology, laboratory diagnosis, clinical features, transmission analysis, and vaccine and antiviral therapeutic development. As a result of increased research activities related to influenza virus, many safety-related problems are bound to occur. Current reports have shown a

number of occupationally-acquired, nosocomial infections of influenza [58–60]. Although no laboratory animal-associated human influenza viral infections have been reported, the possibility of human infections from infected animals, such as ferrets, mice, and cynomolgus macaques, and then transmission among humans, cannot be ruled out. The potential for human-to-human transmission results from the fact that IAVs may present in respiratory tissues or secretions of infected animals or birds, as well as in a variety of organs in some infected animal species. As a consequence, major laboratory biosafety problems arise from handling or manipulating virus-containing specimens or by aspirating virus via aerosols from infected animals. In addition, direct inoculation of mucus membranes through gloves contaminated with virus-contained tissues or secretions becomes another important source of laboratory infections (http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5_sect_VIII_e.pdf). Therefore, establishment of biosafety-related protocols for IAV studies should be considered as a practical necessity.

CURRENTLY ESTABLISHED BIOSAFETY PROTOCOLS FOR IAVS AND SUITABILITY

Currently, IAV-related biosafety protocols are well established in the majority of the laboratories handing live IAVs. CDC, National Institutes of Health (NIH) and WHO have set up strict biological safety practices for research involving potentially highly pathogenic influenza virus strains, including noncontemporary human influenza strains, such as H3N2, H2N2 and 1918 influenza virus, as well as circulating HPAI viruses, such as H5N1 and H7N7 (http://www1.umbi.umd.edu/research-development/compliance/images/biosafetypracthighpathflu.pdf). Some of these biosafety protocols and precautions are summarized below (http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5_sect_VIII_e.pdf).

Levels of biosafety labs used for influenza study depend on the virus strains

Biosafety labs were classified into four different categories according to the degree of risk posed by the research and the involvement of graded levels of protection for personnel, the environment and the community. These biosafety labs contain an array of laboratory practices and techniques, safety equipment and laboratory facilities suited to the operations performed (http://www.cdc.gov/flu/about/qa/1918flupandemic.htm). Influenza A viruses with different degrees of pathogenicity should be handled in the corresponding levels of biosafety labs. In general, biosafety level 1 (BSL-1) is the least stringent, while BSL-4 provides the most stringent conditions. BSL-2 and animal BSL-2 (ABSL-2) facilities are recommended for diagnostic and production research activities of low pathogenicity avian influenza (LPAI) H1–4, H6, H8–16 strains, as well as equine and swine influenza viruses and virus-related work in animal models. H5 and H7 subtypes with low pathogenicity and reassortant avian IAVs not categorized as select agents can also be conducted in BSL-2 and ABSL-2 laboratories (http://www.selectagents.gov/resources/Guidelines%20for%20Avian%20Influenza%20Viruses_2011-11-4.pdf). Aliquot and dilution of specimens or nucleic acid extractions involving untreated specimens containing IAVs could be performed in BSL-2 laboratories (http://www.who.int/influenza/resources/documents/guidelines_handling_specimens/en/index.html). Non-contemporary and wild-type human H2N2 influenza strains should be handled using BSL-3 and ABSL-3 practices, while experiments using cold-adapted, live attenuated H2N2 vaccine strains, as well as those involving the seasonal human influenza viruses such as H3N2, may be conducted under BSL-2 and ABSL-2 practices and facilities. Since the pandemic potential caused by the 1918 influenza strain is considered to be very significant, the practices and procedures related to the full reconstruction of this virus type and related animal research should strictly follow the practices, procedures and facilities recommended for BSL-3 and ABSL-3. HPAI

virus H5 and H7 strains are highly pathogenic with an equally high risk of potential transmissibility of these strains among mammals and humans. Therefore, the manipulation of these IAVs and chimeras containing genetic elements from other highly pathogenic influenza virus strains requires at least BSL-3 and ABSL-3 facilities. It should be mentioned that any procedure potentially generating aerosols or droplets, such as sonication and vortexing, should be carried out in BSL-3 cabinets and that large laboratory animals, such as nonhuman primates, should be housed in primary barrier systems in ABSL-3 facilities (http://www.who.int/influenza/resources/documents/guidelines_handling_specimens/en/index.html; http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5_sect_VIII_e.pdf). BSL-4 is used for work on novel or exotic pathogens without suitable treatment or vaccines (http://www.cdc.gov/flu/about/qa/1918flupandemic.htm). As far as laboratory-generated transmissible mutant H5N1 viruses are concerned, some experts recommended for the viruses be handled in at least BSL-3 enhanced containment facilities, the second-highest level of biosafety, and strict review be performed before any work restarts, while others suggested that these viruses be conducted only in labs with the highest biosafety rating of BSL-4 [20,61].

Respiratory protection procedures for people working in BSL labs

In addition to handling and manipulation of specific virus strains in specified BSL labs, all personnel working in BSL-3 labs should rigorously adhere to respiratory protection, which includes the use of negative pressure, high efficiency particulate air (HEPA)-filtered respirators or positive air-purifying respirators (PAPRs), as well as the use of HEPA filtration for treatment of exhaust air. Research related to reverse genetics of the 1918 influenza virus strain and HPAI viruses requires extreme caution (http://www1.umbi.umd.edu/research-development/compliance/images/biosafetypracthighpathflu.pdf; http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5_sect_VIII_e.pdf).

Personal protection regulation for handling IAVs

Based on the risk of exposure from aerosols or droplets when performing specific manipulations, it is strongly recommended that all BSL-3 laboratory workers wear personal protective equipment, such as disposable gloves, solid-front or wrap-around gowns, scrub suits, or coveralls with sleeves fully covering the forearms, head coverings and shoe covers or dedicated shoes, eye protection and surgical masks, or full-face shield (http://www.who.int/influenza/resources/documents/guidelines_handling_specimens/en/index.html). Amendment of personnel practices also includes clothing change protocols and personal showering protocols prior to exiting the laboratory to reduce the risk of fomite transmission of highly contagious agents (http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5_sect_VIII_e.pdf; http://www.selectagents.gov/resources/Guidelines%20for%20Avian%20Influenza%20Viruses_2011-11-4.pdf). In addition, all BSL-3 precautions described above should be followed for work in BSL-2 with IAV/H5 virus specimens (http://www.who.int/influenza/resources/documents/guidelines_handling_specimens/en/index.html).

Precautions for handling equipment and specimens containing IAVs

Work related to handling or manipulating IAV-containing specimens may include diagnostic tests involving viral agent propagation *in vitro* or *in vivo,* any work involving influenza A/H5 virus replication in cell culture and/or storage of cell culture isolates, recovery of viral agents from cultures of influenza A/H5 specimens, as well as manipulations involving growth or concentration of IAV/H5 virus. Thus, specific protocols should be followed when handling or manipulating the above specimens or equipment used for IAV studies. According to WHO, all centrifugation of IAV-containing specimens should be carried out in

sealed centrifuge rotors or sample cups. These rotors or cups should be unloaded in a biological safety cabinet. In addition, it is strongly recommended that all work surfaces and equipment be completely decontaminated after processing specimens. Freshly prepared bleach solutions may be used as standard decontamination agents. (http://www.who.int/influenza/resources/documents/guidelines_handling_specimens/en/index.html).

Protocols for IAVs as select agents

A few strains of IAVs are classified as select agents. Since they are highly pathogenic to humans, all HPAI viruses and reverse genetic constructs containing all eight gene segments of the 1918 Spanish influenza virus (reconstructed 1918 influenza virus) were regulated as select agents by the U.S. Department of Agriculture and U.S. Department of Health and Human Services (DHHS), respectively (http://www.selectagents.gov/select%20agents%20and%20Toxins%20list.html) [46]. It is recommended that any experiments related to these IAV select agents be performed using BSL-3 or ABSL-3 practices, procedures and facilities, plus enhancements with special procedures, strictly following the protocols and regulations of these facilities. These BSL-3 facilities with specific enhancements include primary (safety cabinets, isolation cabinets, gloves, gowns) and secondary (facility construction) barriers to protect researchers from accidental exposure. These procedures, such as clothing change and shower-out requirements, as well as the use of a powered air purifying respirator, provide appropriate containment for conducting influenza-related research (http://www.cdc.gov/flu/about/qa/1918flupandemic.htm).

Special occupational health considerations

In addition to the above established protocols and precautions, a specific medical surveillance and response plan should be implemented for work involving HPAI viruses, recombinant and reassortant non-contemporary human influenza strains, and reverse genetic constructs of the 1918 pandemic virus strain. These plans include storage of baseline serum samples from individuals working with the IAV strains, annual vaccination of such individuals with the currently licensed influenza vaccine, employee counseling services concerning disease symptoms (such as fever, conjunctivitis) and respiratory symptoms, establishment of a protocol for monitoring personnel with the symptoms, and establishment of a clear medical protocol for responding to suspected laboratory-acquired infections. Antiviral drugs (e.g., oseltamivir, amantadine, rimantadine, zanamivir) should be available for treatment and prophylaxis, if necessary. In addition, the sensitivities of the virus being studied to the antivirals should be ascertained. It is also required that all personnel working with such IAVs be enrolled in an appropriately constituted respiratory protection program (http://www.cdc.gov/flu/h2n2bsl3.htm; http://www.cdc.gov/biosafety/publications/bmbl5/BMBL5_sect_VIII_e.pdf).

FUTURE PERSPECTIVES AND CONCLUSIONS

The establishment and performance of the above comprehensive protocols for handling and manipulating infectious IAVs have minimized the risk of accidental transmission of the influenza virus and maximized protection of the individuals working on such viruses from potential viral infections. However, more work needs to be done to improve current IAV biosafety protocols. The biosafety-related facilities and procedures in all laboratories where researchers are working on HPAI H5N1 viruses should be continually monitored and improved. For example, researchers are not recommended to work on HPAI H5N1 strains having low or no transmissibility in humans with influenza viruses containing high capacity of human-to-human transmission, such as the pandemic H1N1/2009 strains or the seasonal IAV strains, simultaneously in the same laboratory, due to the possibility to accidentally generate a reassortant HPAI virus with high human-to-human transmissibility. The recent

laboratory-created H5N1 mutants with airborne transmissibility in ferrets have already raised serious concerns about the potential transmission of the mutants or reassortant of H5N1 virus in human populations [62–64].

As research scientists, we should strictly follow all biosafety procedures in handling the biohazardous materials and use active methods to avert biological terrorism or other disease outbreaks [65]. At the same time, we strongly appeal to all governments of countries around the world and the administrators of research institutions to evaluate the projects related to the dual-use research before the approval of funding or communication of the dual-use research of concern, as the NSABB of the United States has done recently on the publication of the studies on laboratory generation of the influenza A/H5N1 mutants with increased airborne transmissibility [4]. Every effort should be taken to maximize the benefits and minimize the risks in the dual-use research on highly pathogenic influenza viruses and any other emerging and re-emerging infectious agents.

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Abbreviations used

(A)BSL-3	(animal) biosafety level 3
HA	hemagglutinin
HPAI	highly pathogenic avian influenza
IAVs	influenza A viruses
nAbs	neutralizing antibodies
NSABB	national science advisory board for biosecurity
PB2	polymerase basic protein 2

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