Use of Highly Pathogenic Avian Influenza A(H5N1) Gain-Of-Function Studies for Molecular-Based Surveillance and Pandemic Preparedness

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Zoonotic influenza viruses circulating in poultry and swine pose an ever present threat to human health. In particular, the rapid geographical expansion of highly pathogenic avian influenza (HPAI) A(H5N1) throughout Asia and then into Europe, the Middle East, and Africa during the 2000s galvanized the global community in an attempt to control this rapidly growing threat. Despite successful control efforts in some countries, the virus remains endemic in poultry in at least six countries and continues to cause human illness and deaths as well as countless outbreaks in birds. During the past decade, 668 cases and 393 deaths were detected and reported to the World Health Organization (WHO) (1). During the 17 years since human infections with HPAI A(H5N1) were first identified in Hong Kong, Special Administrative Region, People's Republic of China, in 1997, these viruses have evolved substantially through mutation and reassortment, resulting in multiple divergent genotypes and clades (2).

Ongoing H5N1 circulation has appropriately resulted in a focus on sequencing viral genomes to understand the evolution of these viruses and the significance of observed genetic changes. Expanded laboratory capacity for high-throughput Sanger sequencing and recent technological advances, such as next-generation sequencing and parallel computing, have revolutionized the quantity, quality, and availability of gene sequences and our ability to quickly and accurately analyze these data (3). Consequently, the number of animal and human influenza virus sequences available in publically accessible databases has dramatically increased over the years, as have the bioinformatics tools required for efficient investigation (4, 5). These advances in laboratory and analytical methods provide strong incentives to utilize molecular data for pandemic risk assessment of zoonotic influenza viruses at the animal-human interface (6).

However, examination of influenza sequence data alone does not allow us to assess the pandemic potential of a virus. Pandemic risk assessment that utilizes sequence data can take place only after critical genetic signatures are identified through laboratory research into the consequences for relevant biological properties (or phenotypes). These critical genetic features include those that based on previous experimental validation are predicted to confer virulence and/or have the ability to transmit efficiently in mammals. In this context, genomes are sequenced, mutations are detected relative to earlier viruses and prototype strains, significance is appraised based on prior knowledge of genetic markers, and phenotypes are tested using a variety of *in vitro* and *in vivo* experiments. Viruses possessing phenotypes of interest or concern often become candidates for reverse-genetics studies, which are essential to elucidate the precise molecular correlate(s) of a given phenotype (Fig. 1). From a molecular epidemiological perspective, this process is at the heart of how the public health community makes informed decisions about the threat posed by zoonotic influenza viruses and which interventions might be most effective (7).

Laboratories worldwide have employed reverse genetics to study the mechanisms by which HPAI H5N1 and other zoonotic influenza viruses evolve and how these mechanisms influence host receptor specificity, antigenic variation, replication, pathogenesis, drug susceptibility, and transmission (8–11). Besides being used to create vaccine viruses for the development of live, attenuated (12) and inactivated prepandemic H5N1 influenza vaccines (13), reverse-genetics methodologies also have been used for many years to study the phenotypic consequences of particular mutations, including genetic changes that confer a gain of function (GOF). Influenza virus GOF studies have focused on several research areas: *in vitro* and/or *in vivo* replication in mammalian cell culture or animal hosts, adaptive mutations conferring changes in host susceptibility, alteration of receptor binding profiles and/or tropism for mammalian airway tissues, enhanced polymerase activity, changes in host antiviral response (e.g., cell signaling pathways), susceptibility to antiviral drugs, and pathogenesis and/or transmissibility in mammalian animal models. Such GOF experiments have elucidated key biological principles and provided the scientific basis for genomic sequence-based risk assessment of zoonotic viruses with pandemic potential. For example, the molecular basis for avian versus mammalian influenza virus receptor binding (α2,3 versus α2,6 sialylated glycans) has been elucidated largely through GOF experiments, and some recent studies that identified specific HA mutations conferring a switch from avian to mammalian host receptor specificity also demonstrated the impact of these mutations on the ability of H5N1 virus to more efficiently infect the human upper respiratory tract (14–19). Mutations conferring enhanced virulence in mammalian models or inhibition of the host antiviral response with the potential to cause more serious human illness have been described in other studies (20–22). Still other GOF work characterized mutations that confer resistance to neuraminidase (NA) inhibitors (23–26). These data are critical to make effective drug treatment decisions and to inform stockpiling of antiviral medications. Finally, many publications have described mutations that confer adaptation of H5N1 viruses to mammalian hosts and transmissibility in guinea pigs or

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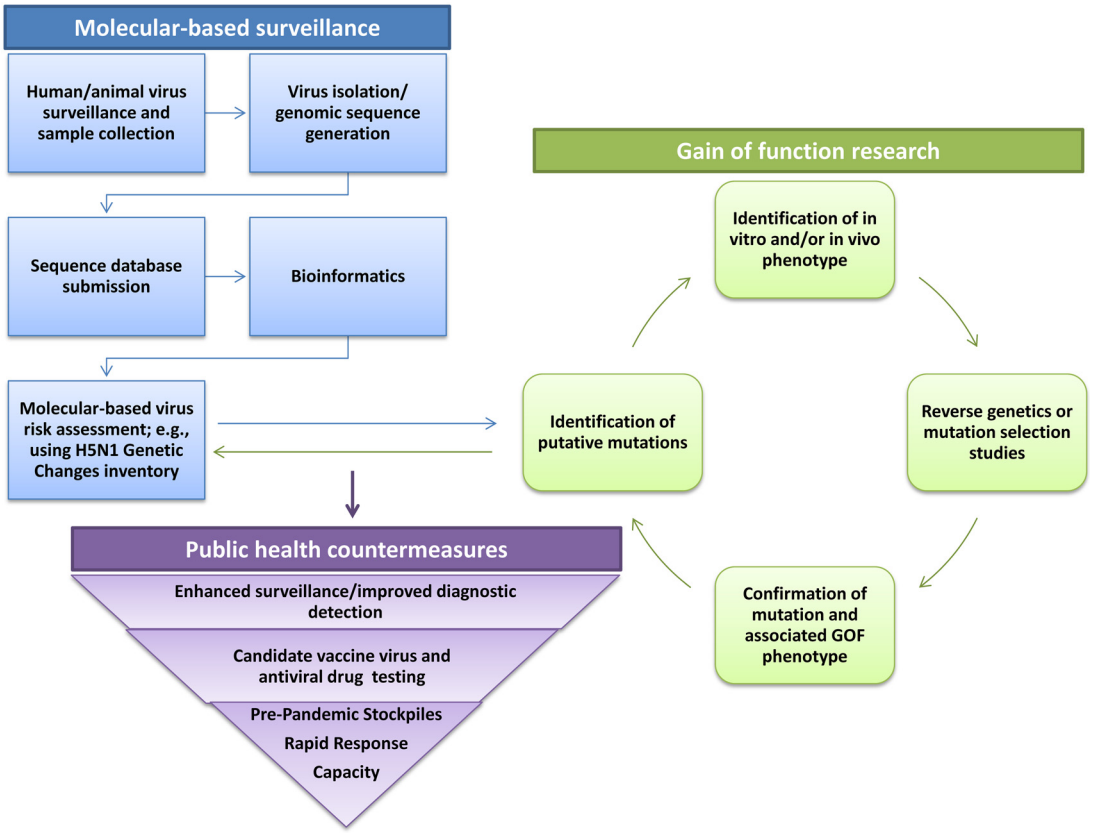

FIG 1 H5N1 or other influenza A virus molecular-based surveillance, gain-of-function research, and pandemic preparedness decision making.

ferrets (19, 24, 27). It should be noted that in many cases, this research can demonstrate loss of function, which is also valuable for risk assessment. These types of studies provide vital data with which to monitor circulating viruses for features that may suggest increased capability for human-to-human transmission or more long-term adaptation of H5N1 viruses in humans or other mammalian hosts, such as pigs.

Reverse-genetics experiments using the well-established ferret model to measure H5N1 respiratory droplet-mediated transmission were, until recently, unable to identify specific mutations involved in this process because the laboratory-derived viruses, like their wild-type counterparts, demonstrated a lack of or limited capacity for aerosol transmission (18,19, 28). However, studies from 2012 by Herfst et al. (29) and Imai et al. (21) were able to demonstrate that genetically modified H5N1 viruses could be transmitted relatively efficiently via respiratory droplets in a mammalian host following acquisition of specific mutations in the hemagglutinin (HA) and/or in combination with the presence of lysine at position 627 in the PB2 protein sequence. The controversy sparked by these studies continues to reverberate (21, 29). Some have argued that benefits associated with H5N1 GOF transmission studies outweigh the risks (30–32), while others have stated that public health derived little to no benefit to justify the research (33–35).

Some GOF research resumed following the end of a voluntary global moratorium (36). However, a new moratorium on funding certain types of GOF research on influenza, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS) viruses is now in place in the United States (37). This pause provided an opportune time to describe how the molecular markers identified by the controversial ferret transmission studies and many other GOF studies have provided important information for public health risk assessment of naturally occurring viruses detected in animals and humans. Here we outline how we utilize a molecularly based surveillance approach focused on knowledge and insights gained from GOF research to inform influenza pandemic risk assessment, as well as risk management and pandemic preparedness. We present the dramatic increase in the number of human cases caused by HPAI H5N1 in Cambodia and an outbreak of influenza A(H7N9) in China during 2013 as examples of how the results of GOF studies supported a more rapid and accurate risk assessment and response to these situations. In both instances, molecularly based surveillance identified naturally occurring mutations in avian influenza viruses isolated from humans that had been demonstrated by GOF studies to increase transmissibility in the ferret model, prompting the public health actions described below.

To keep up with the large body of data from H5N1 GOF publications, the Centers for Disease Control and Prevention (CDC) in collaboration with domestic and international researchers compiled published amino acid mutations and protein motifs that were tested experimentally and shown to alter relevant functional aspects of H5N1 virus phenotypes in the H5N1 Genetic Changes Inventory (38). While this type of data had been used at CDC for many years to conduct molecularly based surveillance of naturally occurring influenza viruses, the end result of the H5N1 Genetic

Changes Inventory was to provide a single point of reference to facilitate the efficient identification of specific mutations or motifs in viral proteins that might signal adaptation to mammalian species, changes in susceptibility to antiviral medications, or changes in viral pathogenesis and transmissibility. By focusing on mutations specifically identified as conferring GOF phenotypes, veterinary and public health experts have used the Genetic Changes Inventory to help assess the relevance of molecular markers identified in naturally occurring viruses. During pandemic risk assessment, viral sequence data are weighed with respect to other virologic and epidemiological traits of a newly identified virus.

Results from more than 15 years of H5N1 GOF studies were compiled to assist researchers at institutions worldwide in their risk assessment of naturally occurring influenza viruses to facilitate an early response in the face of emerging zoonotic influenza virus threats. Individuals or institutions tasked with risk assessment and pandemic planning must weigh the significance of the mutations detected via molecular surveillance. For example, mutations associated with some human H5N1 fatalities (e.g., PB2 627K) (39, 40) may carry more weight than those found to enhance virulence only in a mouse model (e.g., the presence of the NS1 PDZ binding domain motif) (41). Similarly, mutations previously found to alter the receptor-binding preferences of H5N1 viruses from an α2,3 (avian) to α2,6 (human) preference would be of particular concern. Of greater concern would be the identification of mutations associated with enhanced mammalian transmissibility, including the specific amino acid residue combinations identified by Herfst et al. and Imai et al. to confer H5N1 respiratory droplet transmission in ferrets (21, 29).

A recent instance of enhanced surveillance and response by the CDC and regional partners occurred following the abrupt rise in human cases of H5N1 that occurred in Cambodia in 2013. At the same time that increased case numbers were detected, public sequence database mining by researchers identified viruses from several 2013 human infections that possessed the same mutations shown by GOF studies to alter receptor-binding specificity toward an α2,6 preference (K189R and Q222L) and enhanced respiratory droplet transmission of a clade 1 virus in ferrets (N220K with Q222L) (42). Additional amino acid sequence comparisons of these viruses to those of previously circulating Cambodian clade 1 viruses revealed three other HA substitutions conserved in all 2013 viruses. These three mutations were also shown by GOF experiments to increase binding of H5 viruses to mammalian host cell sialic acid receptors in α2,6 linkage either alone (S133A and S155N) or in combination with other mutations (S123P) (43–45). These sequence findings directly led the CDC to dispatch a team of three subject matter experts to Cambodia to conduct an epidemiological investigation of sources of exposure to poultry for these human cases, case contact trace-back, including serologic analysis for H5 antibody, and retrospective investigations of poultry deaths and outbreaks in locations where cases were discovered. In addition, an intensive effort was undertaken to consolidate and analyze human and animal epidemiological and sequence data through collaboration across public health and veterinary sectors, as well as local, regional, and global agencies (42). Although comparisons of the viral genomes of poultry and environmental samples to human samples demonstrated that the K189R, N220K, and Q222L mutations (i) were absent in poultry viruses, (ii) were likely to have arisen during human infection, and (iii) did not transmit from person to person, the enhanced surveillance, improved laboratory capacity, and financial resources that resulted from this investigation highlight the utility of GOF data for pandemic preparedness (42). Because of the finding that these GOF mutations likely occurred during replication in humans and the possibility that they might arise again, a candidate vaccine virus (CVV) was developed against A/Cambodia/X0810301/2013, the virus that possessed two of the markers described by Imai et al. as enhancing ferret aerosol transmission and three mutations shown to alter avian receptor-binding specificity (21, 46). While a vaccine stockpile was not manufactured using this particular CVV, having a vaccine virus available that has been developed for human use and excluded from Select Agent Regulations reduces the time required for vaccine development and testing by at least 1 month, thus enhancing global pandemic preparedness. This reduction in the time required for vaccine development is the basis for the creation of a library of CVVs for emerging influenza pandemic threats, an approach taken by WHO's Global Influenza Surveillance and Response System for many years (46).

Due to the continuous evolution and antigenic drift detected in many subtypes of avian and swine influenza viruses, prioritization of CVV development is required. To meet this need and prioritize other research decisions, the Influenza Risk Assessment Tool (IRAT) was developed by the Influenza Division at the CDC together with a global consortium of animal and public health experts to offer a standardized set of considerations to be applied when evaluating viruses with pandemic potential (47). The tool uses an additive model, based on multiattribute decision analysis, to integrate weighted elements from both laboratory and field observations. Collectively, assessment of the molecular characteristics of circulating virus, together with factors such as incidence of human infections, population immunity, geographic or host distribution, antigenic variation, and/or extent of overall genetic diversification, provides an objective approach to measuring potential risk of a given strain, subtype, or group of viruses (47).

The recent emergence of low-pathogenicity avian influenza (LPAI) H7N9 virus causing human infections in China, like the emergence of pandemic influenza A(H1N1) virus in 2009, was instructive from a molecular surveillance perspective because the HA and neuraminidase (NA) genes were not closely related to those of previously recognized influenza A viruses (48). Despite the lack of high sequence identity of this H7N9 virus to other known viruses, many structural and functional motifs between this LPAI H7N9 and HPAI H5N1 viruses remain conserved, particularly the amino acid domains making up the three major structural elements of the receptor binding site (RBS) (49). In addition, NA enzymatic active sites, which are targets of NA inhibitors, share homology with NA proteins from H5N1 and other influenza A subtype viruses (48). Finally, the internal genes described for H7N9 viruses share common ancestry with H9N2 lineage viruses, ancestry that is retained in the internal genes of many H5N1 genotypes. Thus, many of the GOF mutations described for both surface and internal protein sequences of H5N1 viruses were assessed in the context of H7N9 virus sequences as they were deposited in databases during the early wave of human infections in China.

Within hours of the posting of the H7N9 sequence data, CDC and other investigators using the H5N1 Genetic Changes Inventory identified several of the mutations described previously in H5 viruses shown to possess GOF phenotypes, such as increased respiratory droplet transmission in ferrets (using H5 numbering:

HA T156A and HA Q222L), mammalian host adaptation (PB2 E627K and PB2 D701N), and enhanced virulence in mice (M1 N30D and M1 T215A) (48, 50). Early detection of these molecular markers in H7N9 viruses isolated from humans gave public health authorities evidence that these viruses posed an immediate pandemic threat. Based on H7N9 sequence data alone, development of a candidate vaccine virus began within a day using synthetic biology (51). H7N9 viruses obtained from human cases in China were subsequently shared with international partners within 2 weeks so that additional virologic characterization could be performed. Over a period of approximately 8 to 10 weeks from the initial reports of human infection, numerous laboratories identified H7N9 viruses (and the responsible mutations) with binding affinity to α2,6 host cell receptors (48, 52), replication without prior adaptation in mouse and ferret models (52), limited respiratory droplet transmission in ferrets (52), and reduced susceptibility to antiviral drugs (53), all substantiating inferences obtained from molecularly based surveillance using knowledge gained from GOF studies. The IRAT was used, as data accumulated, to assess and reassess the risk posed by H7N9 viruses compared with other zoonotic influenza viruses, and it was determined that the risk was greater than for H5N1 subtype viruses. More recently, GOF studies have focused specifically on H7N9 viruses and have identified other mutations (i.e., PB2 K526R) associated with enhanced mammalian replication (54). Researchers also assessed the antigenic relationships of the novel viruses with existing H7 prepandemic vaccine candidates. These collective virologic findings, along with the molecular markers identified, led WHO Collaborating Centers for Influenza and vaccine manufacturers to rapidly develop candidate vaccine viruses (55) and then led the Department of Health and Human Services to perform human clinical trials (56) and to stockpile H7N9 vaccine in the United States (57) for pandemic preparedness, shaving months off the time required to deploy this vaccine, should it be needed.

As coordination of international surveillance activities and global sharing of viruses improve (especially in the wake of the 2009 pandemic and emergence of H7N9 viruses in China), molecularly based surveillance has great potential for rapid risk assessment of samples collected as part of active and passive surveillance systems. Real-time feedback to investigators in the field or authorities making policy decisions related to poultry outbreak containment, clinical intervention, and diagnostic methodology, to cite a few examples, will remain critical in the future. In addition, as building laboratory capacity is prioritized in countries impacted the most by endemic H5N1 virus circulation and the higher incidence of human infection, the ability to screen viruses for amino acid markers or lineage-associated molecular determinants by sequencing, pyrosequencing, mass spectrometry, and real-time reverse transcription (RT)-PCR assays will become the reality for more and more laboratories.

In recent years, both the range and speed of molecular surveillance for H5N1 and other avian influenza viruses have continued to improve. Notwithstanding, GOF studies are needed to inform our interpretation of genetic data obtained from naturally occurring viruses. Despite recent gains in our understanding of the molecular basis for phenotypic properties of HPAI H5N1 and LPAI H7N9 viruses, more data are required to fully elucidate the mechanisms by which influenza viruses with pandemic potential cause severe disease and how they evolve during replication in mammalian hosts. This is especially true for studies that offer insight into the virologic and molecular changes associated with increased capacity for mammalian transmission, a hallmark of pandemic influenza viruses. As outlined above, GOF studies have provided critical information for molecularly based surveillance, as well as for research groups sequencing, characterizing, or experimentally testing these viruses. Besides answering fundamental questions about the molecular basis for key phenotypic characteristics of H5N1 and other avian influenza viruses, GOF data have been used to launch outbreak investigations and allocate resources (e.g., H5N1 in Cambodia), to develop criteria for the Influenza Risk Assessment Tool, and to make difficult and sometimes costly pandemic planning policy decisions, such as preparing CVVs and purchasing prepandemic vaccine stockpiles (e.g., H7N9 in China). The detection of GOF mutations in HPAI H5N1 and LPAI H7N9 viruses prompted immediate public health responses that differed from the actions that would have occurred with a rise in case numbers alone because concurrent detection of GOF mutations with an increase in human cases could be a signal that human-to-human transmission had begun, a situation where rapid response is paramount. By coupling results obtained from GOF studies with enhanced surveillance and preparedness, we as a community of scientists, veterinary and public health experts, regulators, and policy advisers have an opportunity to use the most advanced methodologies available to address the continuing threat posed by influenza viruses with pandemic potential.

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