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Review

Trends in influenza A virus genetics: Can we predict the natural evolution of a H5N1 Z?

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ABSTRACT

We reflect here on the issues pertinent to the evolution and host interactions of influenza A viruses (FLUAV) in general but with a focus on the current highly pathogenic avian influenza genotype H5N1 Z and its risk of becoming a determinate human pathogen. Influenza viruses evolve primarily by adaptation and reassortment that involves nucleotide or genome segment substitutions respectively. Although the introduction of a new subtype into a new host has been presumed to involve reassortment of genome segments, we propose a model of evolution to a new host that is mainly dependant on adaptive processes. Chronicling observations of previous human adaptive mutations in H5N1 Z, as well as mammalian and pandemic FLUAV strains of the past, we indicate that evolution can be tracked and the emergence of a pandemic strain may be at least in some sense predicted. Thus we propose that the greatest likelihood of H5N1 Z-like strains emerging in humans is by adaptation in humans although this may involve subsequent reassortment. We further extrapolate from studies of experimental evolution that 5 to 10 mutations through 10 to 20 passages in humans are required to allow a pandemic strain to evolve. We suggest that the high and increasing prevalence of H5N1 Z in domestic and wild aquatic bird reservoirs in concert with human and animal transmission will lead to the spread of virulence promoting genes to viruses infecting these species associated with increased severity of influenza infections.

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ABBREVIATIONS

influenza A virus, FLUAV; highly pathogenic avian influenza, HPAI; RNA polymerase subunits PB2, PB1, and PA; hemagglutinin, HA; nucleocapsid, NP; neuraminidase, NA; matrix proteins 1, and 2, M1 and M2; nonstructural protein 1, NS1; nuclear export protein, NEP/NS2; nucleotide, ntd; maximum diversity threshold, MDT

KEYWORDS: influenza A virus, genetics, evolution, H5N1 Z, host-range, adaptation, pandemic

INTRODUCTION

Of the approximately 200 different viral respiratory pathogens of humans, influenza A viruses (FLUAV) are among the most frequent causes of severe morbidity and mortality as a result of regular and well-defined yearly cycles of worldwide infection and consequent evolution [1-5]. Typically causing tracheobronchitis; influenza virus infection is usually limited to a portion of the airway epithelium and resident macrophages but may spread to the lower respiratory tract where the severity of disease is relative to the extent of tissue damage resulting from both direct infection and the indirect effects of the immune response [6]. Individuals who are

at high risk of influenza virus morbidity and mortality normally are at the extremes of the age spectrum or possess co-morbidities such as chronic conditions of the lungs and heart, diabetes, pregnancy or immunosuppression [6].

Human FLUAV infections follow highly constant annual temporal and geographic patterns of infection in which attack rates are high in non-immune populations yet mortality rates are typically below 0.1% in the normal healthy population with increases in those with underlying health conditions [7]. On a more sporadic basis, FLUAVes evolve into so-called pandemic strains. Over the last 100 years, three such strains have been documented [8, 9]. In 1957 and 1968, the pandemic FLUAV strains showed a remarkably higher incidence in the immunologically naïve population, yet the mortality rate remained near normal levels. In stark contrast, the pandemic of 1918-19 demonstrated an average mortality rate of about 2.5%, but varied up to 40% in some populations [7, 10]. An estimated 50 million deaths globally were recorded.

A new 'pandemic' strain has yet to come to fruition; however all eyes are focused on one particular lineage of Highly Pathogenic Avian Influenza (HPAI), H5N1 Z. The concern is that this virus will become a human pathogen through either direct contact between humans and birds or, through a more indirect pathway, involving a combination of mammals, birds and the environment. Although the mechanisms for the development of a pandemic strain are unknown, extrapolation from both historical and experimental data may provide us with a blueprint of how a pandemic strain may evolve and more importantly, whether this transformation is possible.

FLUAV

FLUAV has a genome composed of 8 segments of single stranded, negative sense RNA encapsidated in a lipid envelope (see reviews [11-14]). The virus encodes 11 proteins: 3 polymerase subunits, PB2, PB1, and PA encoded by segments 1, 2, and 3 respectively (as well as the recently discovered PB1-F2 protein from a second start codon in segment 2 [15]); hemagglutinin (HA, segment 4); nucleocapsid (NP, segment 5); neuraminidase (NA, segment 6); matrix proteins 1, and 2 (M1 and M2, both encoded by segment 7); nonstructural proteins 1 and nuclear export protein (NS1 and NEP/NS2, both encoded by segment 8). The 2 small bicistronic segments express 2 mRNAs by splicing in the cell nucleus, the site of replication. The segmented genome allows the formation of reassortant progeny during infection.

FLUAV evolution

As FLUAV are segmented RNA viruses the 2 main means of evolution are through: 1) adaptive evolution through point mutations; or 2) reassortment of genome segments between parental FLUAV viruses that coinfect the same host cell. Both are highly relevant to the development of an H5N1 human strain although as will be illustrated, the likelihood of one route may be favored over the other.

Adaptive evolution

Point mutation is a constant feature of virus replication where the RNA polymerase error rate of around 10^{-4} misincorporations/nucleotide results in about 1 nucleotide substitution per genome [16]. Thus RNA viruses exist as mutant swarms or quasispecies that are centered on consensus sequences [17, 18]. As a consequence of this genetic variability, virus populations are well adapted to their particular host environment but have the ability to rapidly evolve to adapt to changes in this environment. Given that the probability of a mutation in each genome is approximately 1 and there are 3 possible nucleotide substitution at each position, there are 3 x genome length, or about 40,764 possible single nucleotide polymorphisms for prototype A/PR/8/34 (3 x 13,588 ntd) that will all be present in a population of the same size (approximately 41,000 mutants) for any given influenza virus. Using the Poisson distribution the probability that all mutants are present in a population exceeds 99% for 5 x 41,000 infectious particles (2×10^5) and can be considered to constitute the maximum diversity threshold (MDT) needed to supply all possible single nucleotide substitution variants for selection.

Adaptive evolution proceeds via single nucleotide changes as multiple changes are not observed because of their low prevalence where the probability of multiple mutation is the product of the probability of each mutation ($P = 1/41{,}000^n$,

n = number of mutations) (reviewed [19, 20]). For evolution to proceed there must be infection with large populations of virus (that meet or exceed the MDT) or alternatively replication following low-dose infection must generate this level of infectious virus. The initial infections must therefore be at least minimally productive and not abortive. Secondly, to begin the process of selection of mutants that possess enhanced replication abilities in the new host, single nucleotide substitutions must confer a selective advantage in the new host. Furthermore each adapted mutant must replicate to levels such that it is shed and spread to a new host to continue the adaptive process. In the process of adaptive evolution, the sequential selection and accumulation of point mutations incrementally increases replicative fitness to an optimal point of effective replication to allow spread and thus survival within the new host species. Adaptation to a new host is both virus and host dependent where closely related viruses differ in their adaptability to a new host. Such is the case with the first H3N2 isolated in humans, A/Hong Kong/1/68, which readily adapts to the mouse using a starting does of 10^5 pfu. After twelve serial passages the adapted virus population is growing to >100 fold higher yields in mouse-lung and is >10,000 fold more virulent [21] (Fig. 1). In contrast the H3N2 strain isolated after 14 years of natural passage in humans, the A/Philippines/2/82 H3N2 strain, is resistant to mouse adaptation and is lost due to insufficient replication and/or host clearance under identical passage conditions (Fig. 1) or when 10 fold more virus is used to initiate the mouse infections (data not shown). FLUAV's therefore differ in their adaptability to new hosts.

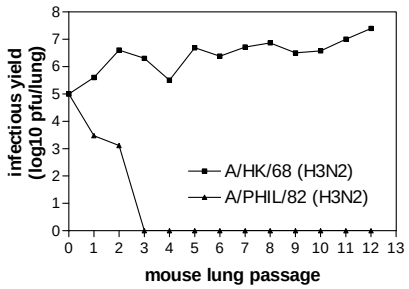

Fig. 1. Differences in adaptability of the A/Hong Kong/1/68 (A/HK/68) and A/Philippines/2/82 (A/PHIL/82) prototype H3N2 strains in the mouse lung. Groups of 3 mice were infected intranasally with 10^5 pfu of each virus and incubated for 3 days. Serial passages employed 1/10 dilutions of each pooled lung homogenate. The A/Hong Kong/1/68 FLUAV could be adapted to increase growth as well as virulence in the mouse whereas the A/Philippines/2/82 virus did not adapt and was lost after 2 passages (1 of 2 duplicate experiments is shown).

H5N1 Z viruses have demonstrated an ability to infect and evolve in human respiratory tracts indicating that continued evolution in humans is possible but is dependent on environmental conditions that provide the opportunities for evolution to become a human pathogen.

Genetic reassortment

Genetic reassortment occurs when genome segments and thus their encoded genes are exchanged among viruses, and is termed antigenic shift when it involves replacement of the surface antigens [12, 13]. Co-infection with different subtypes of FLUAV can result in segment exchange through reassortment to produce a novel human virus that may evade prior immunity or acquire new biological abilities. Antigenic shift results from reassortment between distinct influenza subtypes, however, FLUAV reassortment between sister viruses within a population is a common occurrence and significantly contributes to an enhanced rate of evolutionary change. This presumably is one of the main advantages of segmented versus non-segmented genomes where instead of requiring that each mutant genome reach the maximum diversity threshold required to generate the next beneficial mutation (as is the case for non-segmented genomes), segmented viruses can co-evolve all genome segments within the same population and then bring these together through reassortment into a single genome [21]. The cassette nature of the influenza virus genomes thus also allows exchange of genome segments among viruses to assemble multiple adaptive changes.

In studies of reassortment between human strains and their corresponding virulent mouse adapted derivatives, single gene segment replacements (that differ due to single mutations), each result in incremental enhancements in virulence through

increased replication in mouse lung tissues [11, 21-23]. Although similar findings come from studies done using reassortants derived from human and HPAI strains there is the additional observation of gene constellation effects where groups or "constellations" of genes from parental strains are required to confer virulence to reassortants [24-27]. For example in reassortment studies of systemic and neurological infection in the mouse, crosses of mouse-adapted H1N1 and H2N2 human viruses with HPAI A/chicken/FPV/Rostock/1934 (H7N1), virulence segregated with various combinations of polymerase subunits from the mouse-adapted strains plus segments 4 and 7 (encoding HA and M1, 2 genes) from the pathogenic avian strain [28]. Gene constellation effects have also been described for other avian viruses, where virulence in reassortant progeny requires the maintenance of the parental combinations of polymerase subunits [27]. This indicates that there are coordinated processes comprised of interconnected functions that are controlled by groups of genes which have co-evolved and must be maintained to produce the virulence phenotype in reassortant progeny. Thus virulence is not only multigenically controlled but some genes must function in context with other interacting genes, indicating constraints on transferring virulence by reassortment. In aggregate the genomic and reassortants studies of adaptation to increased virulence identify groups of genes, which implicate all genome segments, in the control of adaptation to increased virulence in a new host [21, 23, 26, 29]. Transfer of virulence from a highly pathogenic avian strain, such as H5N1 Z, by reassortment would require the transfer of the major determinants of virulence to a human strain while maintaining the ability of the resultant reassortant virus to replicate and transmit in the human host.

This stricture may impede the genesis of novel reassortants between H5N1 Z x human influenza virus that maintain the bird-selected virulence phenotype while maintaining the property of human-transmission (presumably from the human virus genome), as some of the required functions for both human transmission and bird derived virulence may be controlled by the corresponding genome segments obviating the acquisition of both properties in a single reassortant progeny. In particular the hemagglutinin protein controls both host range and virulence and thus exchange of the H5N1 surface proteins into human reassortants requires additional adaptive evolutionary changes within humans to bind effectively to human receptors [30-32]. The virulence determining properties of the H5 HA may thus be subsequently changed on becoming a human receptor binding molecule. In such instances, the H5N1 x human FLUAV reassortant can either be highly virulent for avian hosts and not replicate in humans or alternatively replicate in humans but at a loss of high virulence. Neither of these scenarios gives rise to a highly virulent pandemic strain. It is expected that an H5N1 Z reassortant that has derived human genome segments during coinfection (that has not yet been documented) would need to subsequently adapt to human tissues to acquire the host specificity determining mutations in some of the avian genes, such as HA. As this scenario encompasses the restrictions of reassortment and also requires adaptive steps in humans, it would be more likely that the first events in human evolution of H5N1 Z will be adaptation by accumulation of point mutations that enhance human replication, shedding and spread. Once H5N1 Z has adapted to humans this human virus could reassort with human strains to enhance replicative fitness for humans. Furthermore we propose that the evidence, outlined below, indicates that reassortment normally occurs between strains that have been adapted to the same host species.

Insertion, Deletion and Recombination

Influenza viruses may also undergo variations of recombination to produce deleted, inserted and hybrid structures of individual genome segments especially under conditions of serial, high multiplicity of infection, passage as first described by von Magnus [33]. Influenza viruses have also been seen to possess additional subgenomic fragments that are mosaics of genome segments [34]. Influenza recombination does not involve breaking and reunion but rather employs a "copy-choice" mechanism involving RNA template switching of polymerase in complex with a nascent RNA strand and, although very infrequent, can be highly significant as seen for a highly

pathogenic influenza A virus that had acquired a portion of ribosomal RNA in the hemagglutinin cleavage site [35] or viral translocations into the HA cleavage site as occurred for H7N3 in Chile [36] and again in Canada [37].

Host Switching in FLUAVes

As viral proteins interact with host factors, adaptive mutations will, in some instances, be required to enhance these interactions in order to optimize infection of a new host species that differ with respect to their corresponding proteins and factors. In terms of host specificity the mutations that are necessary for host switching are only now being identified. Most investigations have mainly addressed the roles of the hemmagglutinin protein and the polymerase subunits, which are responsible for entry into the cell and replication respectively [38].

Hemagglutinin (HA)

As the first step in infection is cell binding, HA binds to receptors possessing terminal N-acetyl neuraminic acid, more commonly known as sialic acid (SA), linked to galactose through either α2-3 or α2-6 linkage (SA α2,3 Gal; SA α2,6 Gal) [39]. HA preferentially binds to the linkages that are abundant in their natural host. Avian strains preferentially bind SA α2,3 Gal (abundant in duck epithelium) and human strains preferentially bind SA α2,6 Gal (predominant in pig and human lung epithelium) [40]. The differences in binding are partial as the avian and human viruses can bind sialic acid in both linkages but with about 10 fold difference in affinity.

Current analyses of the nature of sialic acid receptors in human respiratory epithelium has yielded differing results with one study demonstrating that SA α2,6 Gal is primarily associated with non-ciliated epithelium and SA α2,3 Gal is primarily found on ciliated epithelium [41]. Another study showed α2,6 sialic acid was also found on ciliated and goblet cells [42]. Avian FLUAV preferentially infect and are restricted to the ciliated epithelium, but human strains have a predilection for the non-ciliated epithelium with subsequent spread to the ciliated epithelium [41]. Although influenzaviruses from different hosts can differ with respect to binding specificity, this is not a strict determinant of ability to infect.

Two binding sites are identified in the crystal structure of H3 HA where the SA α2,6 Gal receptor shows stronger HA binding (K_d of 1 mM) to the primary site at the tip of the HA, whereas the SA α2,3 Gal receptor binds to this site (K_d of 2 mM) as well as a secondary site below the primary site at the subunit interface but with lower affinity (K_d 8 mM) [43]. The secondary site is of unknown significance, but has been speculated to account for the increased binding affinity seen for branched sialosides [43]. The primary binding site contacts sialic acid via 3 secondary structural components, the 190 helix (aa residues 190-198), the 130 loop (aa residues 135-138) and the 220 loop (aa residues 221-228). The amino acid contacts in the primary sialic acid binding site of HA are completely conserved among all avian subtypes [44]. In contrast to the binding pocket, sites that contact other sugars in the oligosaccharide chain can vary among influenza viruses to increase receptor specificity for SA α2,3 Gal (reviewed in [44]).

Polymerase

Influenza host-range is multigenically controlled, where early reassortant studies have identified the polymerase genes PB2, PB1 and PA as well as the NP and NS1 genes in differences in host range [45]. Genomic analyses of experimental FLUAV evolution from human to the mouse showed that the mouse-adapted variant of A/FM/1/47 (FM-MA) grows better in mammalian tissues (mouse lung and dog cells) but was attenuated in avian tissues (chicken allantoic cavity) [23] (Fig. 2). The avian attenuation was due to a pair of mutations in the PB1 and PB2 polymerase subunits (D538G and K482R, respectively) that were host specific and functioned to increase replication and virulence in the mouse lung [23]. This demonstrates that these mutations affect avian versus mammalian host specificity, and could contribute to the conversion of an avian strain to a mammalian strain. Other findings from experimental mouse-adaptation of a virulent H7N7 avian strain, showed that mutations in the PB2 polymerase subunit, D701N and S714R, and the NP protein, N319K, increased polymerase activity, replication and virulence in mice in addition to replication in mammalian tissues without affecting replication in avian tissues [46]. However, a previous genetic study of a virulent mouse-adapted variant of the A/HK/1/68 H3N2 human clinical isolate that

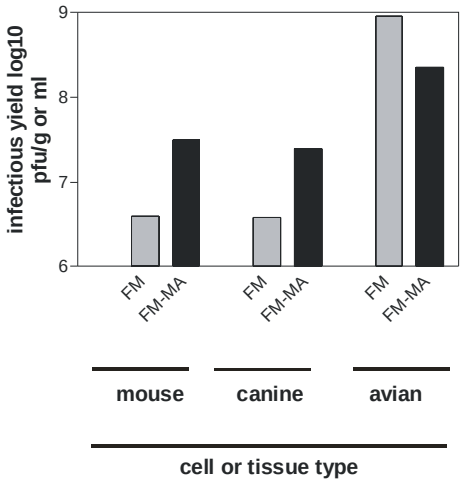

Fig. 2. The yield of mouse adapted versus human parental strains in mouse-lung, MDCK cells and chicken allantoic activity. The growth properties of A/FM/1/47 (FM) and A/FM/1/47-MA (FM-MA) are shown for infections where FM-MA has enhanced ability to replicate in mammalian tissues but reduced ability to replicate in avian tissue. Each value is the average of 2-3 titrations, data derived from Brown and Bailly [23].

demonstrated the same growth properties as FM-MA also possessed the PB2 D701N mutation along with other PA and NP mutations [21]. The mammalian adaptive D701N, K702R[47] and S714R PB2 mutations have been functionally characterized and reside in a nuclear localization signal where these mutations enhance binding to the human importin 5α, that binds and imports the polymerase into the nucleus [48].

H5N1 Z evolution

To date, HPAI viruses are exclusively members of the H5 and H7 subtypes. The first HPAI H5N1 FLUAV was isolated in Scottish chickens in 1959 [49]. The independent emergence of HPAI H5 viruses has occurred repeatedly since 1959, however the fact that H5N1 Z is the first HPAI to arrive in Asia and that it continues to spread worldwide despite all efforts to control it is rather remarkable. All other HPAI outbreaks (of either H5 or H7 strains) have been local, episodic and generally controllable through aggressive eradication and infection control measures that drive the virus into extinction [50]. In the case of H5N1 Z, these measures have been largely inadequate, such that H5N1 Z persists in wild and domestic birds and is expanding its geographic distribution. Part of the reason for this widespread prevalence involves the inclusion of various avian species, including migratory birds, as hosts. The first dramatic demonstration of wild bird involvement was the outbreak of A/Bar-headed Goose/5/05-like H5N1 Z in 2005 in Qinghai Lake that killed thousands of Bar headed geese, ducks and gulls [51].

History of H5N1 Z

A/Hong Kong/156/97-like H5N1 viruses first appeared in humans in 1997 to infect 18 people in Hong Kong with 6 fatalities [52]. Since then, other related H5N1 strains of the H5N1 Z genotype, have evolved and are endemic to numerous areas throughout Eurasia including Indonesia. In the process, >300 humans have been infected and there have been >200 fatalities. These viruses possess a demonstrated potential for systemic infection and enteric shedding [6, 53, 54] that parallels the disease in chickens, indicating common features of pathogenesis among avian and mammalian hosts.

In all cases of H5N1 Z infection, transmission has been mediated through close contact with either infected avian species or genetically related individuals. There has been no evidence of general human to human transmission [55].

Genetic analysis of human HPAI H5N1 viruses

The first human H5N1 infections were caused by A/Hong Kong/156/97-like viruses (HK156) that had derived surface protein genes from A/Goose/Guangdong/1/96-like (Gs/Gd/96) viruses (the first HPAI H5N1 subtype isolated in Asia) [56]. Gs/Gd/96 was the product of both extensive adaptation and reassortment among domestic aquatic and terrestrial poultry where it had derived surface proteins from A/Swan/Hokaido/5/96-like (H5N3) and A/Duck/Hokaido/55/96-like (H1N1) viruses with its internal genes being most closely related to those of A/Dk/Nanchang/1681/92 (H3N8) and A/Dk/Nanchang/1904/92 (H7N1) [57]. The internal protein genes of HK156 were derived from early A/Chicken/Beijing/1/94 and later A/Quail/HK/G1/97 H9N2 viruses that were the first disease causing avian influenza viruses reported in China in 1992 [58]. Although these

H9N2 viruses were low pathogenic avian influenza strains (LPAI) they did cause 30% mortality in infected flocks [59].

The first H5N1 Z genotype strains arose in birds in 2002 and in humans in 2003 (A/HK/213/03-like viruses) and were traceable back to the H5N1 viruses that caused the 1997 human outbreak because they shared the same lineage of surface proteins, however, the internal genes were derived from multiple subtypes of avian influenza viruses derived from an extensive interbreeding population of viruses that involved bidirectional transmission of viruses resident in aquatic and terrestrial poultry [60]. Cycles of co-infection and reassortment among many strains of avian influenza may have provided the extreme host range and virulence properties of HPAI H5N1 Z by sampling the most fit mutant genes from among independently evolved avian influenza viruses of elevated pathogenicity that were resident in terrestrial and aquatic poultry. The reassortment of genome segments amongst many avian FLUAV serotypes appears to have been favored by the prevalence of small-scale poultry production in south-east Asia in combination with emerging large-scale intensive poultry rearing operations that were sharing influenza viruses through bi-directional shipping of immature and adult animals to fattening farms and live markets.

Over the last 5 years H5N1 Z viruses have predominated among H5N1 viruses [61] indicating that this lineage has maintained its genome segment composition but has continued to evolve by adaptation and widen its host specificity. For example, four isolates from a human outbreak in Southeast Asia in 2004 (A/Vietnam/1203/04-like viruses), also had an elevated virulence for ferrets, a mammalian model for influenzavirus infection [62, 63]. As the number of hosts continues to increase, the possibility of reassortment in other alternate hosts leading to tropism to human tissue may increase. Epitomizing this latter point is the recent discovery that domesticated cats are susceptible to systemic and enteric H5N1 Z infection [64]. The domestic nature of the cat predicates a new risk for exposure of humans to the virus [65]. The potential for adaptation may rise over time as may the subsequent potential for co-infection and reassortment between H5N1 Z and any of the common human FLUAV strains.

Despite these changes, in general the genomic composition of H5N1 Z has remained relatively constant since its discovery. For example, analysis of all avian H5N1 Z strains since 1997 have demonstrated a preservation of avian receptor specificity [66, 67]. Sporadic human infections continue to occur, presumably due to the fact that human alveolar tissues possess avian-like α2,3 SA receptors making the lower lung more susceptible to infection with H5N1 Z [42, 41]. The genome of H5N1 Z thus has become the dominant H5N1 lineage, and consequently has become more restricted in its ability to produce successful reassortant combinations even among avian strains. The H5N1 Z genotype also may be more restrictive against reassortment with human influenza viruses. Thus the evolution of H5N1 Z since 2002 has been one of continued adaptation where point mutations continue to accrue within a common genetic lineage that has diverged into several antigenically differentiated clades [61].

Laboratory studies of influenza evolution to high virulence in a mammalian host

The laboratory mouse has been used for isolating and propagating human influenza viruses since the first isolation in 1933 [68]. Intranasal inoculation of mice with human clinical strains has demonstrated an ability to establish low-level infections with replication that is not sufficient to cause disease [21]. However, after a process of rapid directed evolution consisting of 12 to 20 serial cycles of high dose infection (using viral populations that contain all possible single nucleotide substitutions), the virus adapts to the mouse host and causes fatal pneumonia at low dosages [21, 22, 29]. Such viruses have acquired multiple mutations and can be said to be hypervirulent causing fatal primary viral pneumonia at low doses. As this property is shared with HPAI H5N1 Z, the mouse provides an excellent model for study of H5N1 Z evolution.

Mouse-adapted variants of the prototypical H3N2, (A/HK/1/68), became > 10^4 fold more virulent for mice on acquiring 5 mutations after 12 serial passages (LD_{50} = $10^{3.7}$) and became hypervirulent after 20 mouse-passages to kill at dosages (LD_{50} = $10^{2.5}$) near the minimal infections dose

(a necessary characteristic of any natural highly virulent virus) due to the selection of an additional 6 mutations [21]. Mouse adaptive mutations were shown to primarily occur not only at sites of RNA but also viral and host protein interactions, such as is repeatedly seen in nuclear localization signals [21, 46]. Such viruses have evolved due to mutations that achieve both host switching and high virulence [21]. Similarly, the hypervirulent mouse-adapted variant of an H1N1 virus (A/FM/1/47), that had a prior history of mouse passage, showed that it had acquired single mutations in 5 genes, each of which made the virus incrementally more virulent [23].

Genetic analysis of the A/FM/1/47-mouse-adapted variant showed that all 5 mutations increased replicative abilities and virulence in the mouse lung [23]. Furthermore, studies of growth properties in tissues from other hosts showed that a pair of mutations in subunits of the RNA polymerase was host specific (as described earlier) and only enhanced replication in mouse lung whereas the remaining 3 mutations enhanced replication in avian and other mammalian tissues [23]. Thus adaptation selects for variants possessing mutation that enhance replication in the new host, however, many of these mutations work in a host independent manner.

The implication of these mouse-adaptation studies to the evolution of a highly pathogenic influenza is highly significant; many mutations that enhance virulence in one host will also enhance virulence in other hosts. Presumably only host specific adaptive mutations, which have been shown to primarily involve polymerase genes in mouse studies, as well as HA mutations, would be needed for an avian virus such as H5N1 Z to acquire the ability to replicate and spread among humans. The mouse-adaptation studies also demonstrated that over a relatively small number of serial infections, viruses acquire mutations that control host range switching as well as increased virulence.

Another study of adaptation to the mouse of a virulent avian H7N7 strain showed the selection of 8 mutations were responsible for mammalian host switching [46]. Generalizing from these experiments would indicate that both host switching and high virulence requires as few as 5 mutations for each property, to change a human strain into a highly virulent mouse-adapted variant. Extrapolating from experimental mouse-adaptation of human and avian strains, to human adaptation of the highly virulent H5N1-Z lineage, it could be expected that a minimum of 5 to 10 mutations are required, including mutations in each of the polymerase and HA subunits to switch to humans.

Natural evolution by adaptation

In considering movement of FLUAV into novel host species we see that wild FLUAV strains from aquatic birds have been repeatedly introduced into farmed poultry [50]. Whereas natural avian influenza strains replicate poorly in farmed poultry, HPAI have repeatedly evolved in the last century. Where molecular data is available, the repeated patterns are ones of adaptation where viruses that originated from the natural reservoir have been transmitted within domestic populations of poultry to lead to the selection of highly virulent viruses by adaptation. This was first shown for highly pathogenic H5N2 in Pennsylvania in 1983 and for several subsequent independently evolved outbreaks in Mexico, Italy, Chile, Pakistan, and Canada (reviewed by Greger [69]). Thus HPAI are generated through adaptation of newly introduced strains under high density host conditions that favor continued high-dose transmission, that lead to the genesis of FLUAV species that have become adapted to high virulence in poultry species.

There have also been at least two independent occurrences of adaptation of H3N8 avian influenza to equines. An avian H3N8 adapted directly to horses in China in 1989 and 1990 causing high mortalities. The etiological strain, A/Equine/Jilin//89 possessed 8 genome segments of avian origin that were closely related to avian H3N8 [70, 71]. This follows the previous and permanent establishment of an avian H3N8 in horses in 1977 [72] suggesting that some particular avian serotypes may be more suited for switching to mammalian hosts. In a similar vein, the classical H3N8 equine FLUAV has adapted to canines to generate A/Canine/Florida//2004.

This strain continues to spread and cause canine influenza in the United States [73]. The index cases originated in racing greyhounds that were housed at racetracks with equines that provided the proximity and population density that both favor infection and establishment of equine viruses into canines. This is the first known canine influenza strain in recorded history and it possesses 8 genome segments of equine origin. This evidence epitomizes influenzavirus evolution due to adaptive changes rather than reassortment [73].

It is difficult to remove reassortment from the evolution equation as a recent large-scale genomic sequencing project has revealed that viruses within the American population constitute multiple lineages that are born from reassortment [74]. In this regard, the human North American seasonal outbreak of 2003-4 was primarily caused by an A/Fujian/411/2002-like (H3N2) strain that was a reassortant between a co-circulating minor clade of H3N2 and the existing dominant H3N2 strain [74]. This observation indicates that dominant strains are the product of both adaptive evolution and reassortment among co-circulating lineages but that would not be recognized as reassortants without genomic sequence and phylogenic analysis.

In general, viral evolution appears to be the product of both adaptive mutations as well as reassortment with genetically compatible cocirculating strains from the same species. For reassortment to occur among influenza viruses they need to be closely compatible so that their constituent gene products can productively interact without negative influences and so reassortment must not result in a drop in replicative fitness or the resulting reassortants will not have a competitive advantage relative to the existing strains in the human or any other relevant species.

Evolution of pandemic viruses

Of the three pandemic strains that have arisen in the last 100 years, the 1918 H1N1 pandemic appears to have been caused by human adaptation of an avian strain [47, 75]. The 1957 H2N2 and 1968 H3N2 viruses were the products of adaptation and reassortment of avian strains. The reassortment events in both H2N2 and H3N2 were similar, as the PB1 and one or both of HA and NA were derived from an avian source with the remainder of the genome derived from the pre-existing human H1N1 and H2N2 influenza strains respectively. Although these general statements can be made, the detailed steps that culminated in the generation of these viruses are largely obscured because they occurred before the advent of molecular biology and the large scale surveillance required to isolate the various progenitor and intermediate reference viruses needed to determine genealogical events. Several adaptive mutations have been mapped with respect to receptor specificity changes of the HA proteins on becoming human pathogens. Receptor specificity switching of H2 and H3 subtypes was primarily due to two mutations, Q226L and G228S [76-80].

Genetic studies of the 1918 pandemic strain, including genealogical and codon usage, have shown that the genome is composed entirely of avian type genes with a group of 10 point mutations in the 3 polymerase subunits, PB1, PB2, and PA, that confer adaptation to human tissues [47]. In addition, two amino acid substitutions in the HA gene, D190E and G225D, have been shown to reverse receptor specificity to avian-like SA α2,3 Gal [81]. One particularly significant PB2 mutation, E627K, was identified. Interestingly, most avian viruses possess the 627E amino acid while mammalian viruses normally possess the 627K (reviewed [38]). Further research into the E627K mutation shows a preference for mammalian cell replication and has been shown to be important for optimal replication at the lower temperatures of the mammalian respiratory tract [82]. Although most point mutations are not this dramatic, these examples show the potential for large changes in biology caused by the accumulation of single amino acid changes.

Evolution of H5N1 Z in humans

Adaptive HA mutations have been observed that affect receptor specificity of H5N1 Z isolates from infected patients. The most prevalent mutation has been the S223N substitution (also called S227N in H3 numbering) [31]. This mutation was observed in a fatal infection in a man and morbid infection in his son in Hong Kong in 2003, a fatal case in Vietnam during 2005 and in Turkey [83]. H5N1 Z patients also

have been shown to possess L129V plus A134V [30] and N182K plus Q192R [32] mutations that in both instances increase binding to human type receptors.

The E627K mutation has been identified in approximately 35% of human H5N1cases and appears to have stabilized in both human and avian populations as evidenced by the 2005 Qinghai Lake outbreak, thus breaking the rule of this mutation being mammalian specific but furthering the human-adaptive potential of this lineage [51]. As suggested earlier, this E to K mutation may confer tropism to the lower or upper respiratory tract, respectively. This was shown in one human patient from the 2004 Southeast Asia outbreak (Vietnam), where a divergence in FLUAV populations between the upper and lower respiratory tract was caused by the E627K mutation [84]. The mutation also was present in the one person, who died during an outbreak of H7N7 in the Netherlands in 2003 [85], suggesting a possible link with virulence. However, based on studies of the highly virulent Vietnam isolates of H5N1 obtained from humans and birds in 2004 [63, 62], the E627K mutation is not strictly necessary for the property of virulence in mammals.

Several other human specific polymerase subunit mutations [47] have also been identified in human H5N1 infections when compared to human strains using BLAST analysis of human H5N1 genes in the Genbank database. An A199S mutation was selected in 5 of 18 HK156-like viruses but none of H5N1 Z isolates have demonstrated this mutation suggesting a difference in the evolution of these 2 groups. H5N1 Z possesses another specific adaptive polymerase mutation in the nuclear localization signal, K702R, which has been found in 4 patients. Of these, one patient possessed both the K702R and the E627K mutations in viruses isolated from his nasal and pleural cavities, (Genbank gi:113496325 and 113496355 respectively) but possessed only the 627 K mutation in his throat isolate (gi:113496380).

Eight of 67 isolates from patients have selected the human like N375S PB1 mutation [47]. Directional mutations (mutations at the same site but to a different amino acid) at sites of human adaptation have also been seen for 18 of 80 sequenced human H5N1 strains, where the V100I mutation in the PA amino acid sequence is directional with the human adaptive V100A mutation [47]. An Indonesian isolate possessed a PB2 L475F mutation that is directional with respect to the mammalian L475M mutation. Many other mutations have been identified in the polymerase genes that are near to previously identified adaptive sites [47]; however, the relevance to human adaptation is not yet elucidated.

All of these H5N1 mutations have been selected in humans and therefore appear to be human adaptive. In addition, as with the mutations in the 1918 strain, a portion of H5N1 Z mutations may provide the threshold for human infection and spread. More importantly, these may only be the first steps in H5N1 Z human adaptation. The fact that these mutations have been selected repeatedly and independently indicates that one passage in humans has a significant impact on the genotype of this virus. The repeated instances of multiple adaptive mutations also indicated a more extensive level of human adaptation is occurring in some infected patients but is not yet sufficient to allow sustained human-to-human spread.

H5N1 Z today

The H5N1 Z lineage has become the dominant H5N1 lineage in poultry and has stabilized with respect to its genetic complement of genome segments [61]. It is no longer reassorting with other subtypes, indicating that it is becoming more genetically distinct and its genome may now be considered to constitute a unique gene constellation that is a highly evolved group of interdependent interacting genes.

More direct data on the nature of H5N1 Z reassortants that possess human genome segments comes from reverse genetic studies of the behavior of engineered human reassortants between A/Udorn/72 (H3N2) and H5N1 Z [86] where it was possible to generate several H5N1 Z x human reassortants that could infect ferrets; however replication, and clinical signs were reduced for most reassortants and the viruses did not transmit to uninfected ferrets in contrast to prototype human strains. One reassortant with the human virus HA and NA and 6 internal genes of H5N1 grew as well or better than the human virus in cell culture and ferrets indicating that it is possible to derive viable human reassortants [86].

The consequences of a more virulent virus with human surface proteins may be highly significant but more controllable using conventional vaccines. Interestingly attempts were made to replicate the reassortant events that occurred to generate the H2N2 and H3N2 viruses by generating human viruses containing the H5N1 HA and/or NA plus PB1 genes; however rescue was not possible indicating that such reassortants were too severely attenuated to be viable [86]. This is similar to the gene constellation effects seen in reassortant studies of virulence for avian strains. This indicates that there may be severe constraints on reassortment such that only a subset of possible genome segment combination are viable and may severely restrict the development of avian x human reassortants.

The future of H5N1 Z – what to expect

The last 2 pandemics in 1957 and 1968 were the result of adaptation and avian-human reassortment whereas the singularly virulent 1918 virus was appears to have been the product of adaptation of an avian virus to the human host. Looking at H5N1 Z, either route may end up in an unwelcome result, although one may be far more severe than the other.

Should H5N1 reassort, the virus may be composed predominantly of human influenza genome segments with novel surface H5N1 genes. In both the 1957 and 1968 pandemics, the H2N2 and H3N2 viruses (respectively) acquired genes for both surface and polymerase proteins from an avian strain [8]. Should an H5N1 human reassortant virus arise in a similar manner, a more typical virulence pattern would be expected in humans. The fact that many humans have antibodies to a distantly related N1 antigen found in current human H1N1 strains may also offer some further protection against serious disease in much of the human population.

That being said, given the known natural history of influenza [8], the risk from H5N1 seems to be one of adaptation to humans. Adaptation will result in a human variant of H5N1 Z with the possibility of maintenance of the extreme virulence phenotype of the avian H5N1 Z variant.

For viruses spread by direct or close contact as is the case for respiratory infection, hypervirulence is generally at cross-purposes to transmissibility as severe disease limits movement and contact with new susceptible hosts, H5N1 would be expected to attenuate to maintain the pattern of shedding that favors good transmission and subsequent survival. Yet one cannot surmise whether this expected attenuation would be rapid or relatively protracted. In the case of the 1918 virus, both successful transmission and high virulence were kept throughout the time of the pandemic.

Predicting H5N1 Z pandemicity

Based on the theorized mutational thresholds as well as current clinical evidence, some 10 to 20 cycles of infection in humans (through human-to-human transmission) may be sufficient to fully adapt the virus to the human host. This may not seem likely as close contact with H5N1 Z infected poultry has been the rule and thus appears to be one prerequisite for infection [87]. Yet facilitated transmission may provide an increased opportunity for mutation. As previously evidenced by the SARS outbreak of 2003, the virus may optimally spread in a healthcare setting through nosocomial or iatrogenic routes. Indeed, the introduction of the virus into an immunocompromised human population may be sufficient to cause primary and secondary infection, drive adaptation and lower the minimal infectious dose.

CONCLUSION

The simple observation that HPAI H5N1 viruses have been prevalent in Asia without transmitting stably to humans suggests that the risk of a pandemic is lower than perhaps anticipated from the first flurry of human infections in 1997 in Hong Kong. However, this observation may be more apparent than real as this virus continues to defy measures to control its spread and is now been found throughout Eurasia and its subcontinents. This lineage also continues to evolve in changing environments in birds and other animals such as pigs and various farmed poultry thus affecting its ability to evolve to become endemic in other species including humans.

Re-entry of HPAI back into the environment is unprecedented and constitutes a genetic corruption of the feral waterfowl reservoir of FLUAV such

that high pathogenic viruses are now resident in both wild birds and domestic poultry. On the one hand, this virus has broken many of the conventional views of host range and virulence for influenza A viruses. Its prevalence is increasing on a global scale in birds with occasional infection of humans and other mammals; the species barrier seems not to apply rigorously to this virus. Because influenzaviruses constitute one large interbreeding population of virus variants, a concern is that the increased presence of HPAI in general and H5N1 Z in particular, represents a threat that is growing as more species become infected with FLUAVes of enhanced virulence. Just as "a rising tide raises all boats" we propose that this trend may lead to more serious FLUAV disease in more species primarily as a function of this increased prevalence of HPAI H5N1 Z in the natural avian reservoir. Virulence promoting genes may thus flow through adaptation and subsequent reassortment to viruses in humans and other mammals.

Reports from Europe are suggesting that H5N1 is becoming endemic in domestic geese and ducks and is spread to poultry to cause HPAI [88]. This increasing endemicity is likely to fundamentally change the ecology of avian influenza. As influenza is an interbreeding genetic pool the concern is that the FLUAVes genus is becoming more fit in general which will take a toll among diverse FLUAV host species. FLUAVes thus will become more aggressive to cause increased morbidity and mortality rates with an increased likelihood of the genesis of novel human influenza to which humans lack pre-existing immunity. Some signs of movement of H5N1 Z genes into viruses of other species already has been observed. For example, swine H9N2 isolates have been shown to possess the internal H5N1 Z genes associated with systemic and fatal infections [89]. Duck isolates of H3N8 viruses that have increased avian and mammalian virulence and that also possess internal proteins encoded by H5N1 Z genes have been isolated in Beijing in 2004-5 (submitted J. Pu, E.G. Brown, and J. Liu). Although the next pandemic may be H5N1, this potential continues to be questioned. Yet, as demonstrated by the H1N1 1918 pandemic strain, as little as one specific mutation may be sufficient to change host and tissue specificity beyond the threshold limit needed to jump the species barrier. In combination with another 5 to 10 human adaptive mutations, which could be selected through a series of human transmissions, one can predict that the virus will indeed become transmissible and possibly virulent.

Vigilance and preparedness on a global scale are the cornerstones for a successful response to an unknown threat. In 1918, the world was unprepared; today, all relevant scientific faculties are working to stop the pandemic before it starts. Unfortunately, what is lacking is an in-depth knowledge of the evolution of the virus in the environment as well as the biological implications of this evolution. Although laboratory studies provide model data for extrapolation, and an understanding of the past may provide some direction, we cannot truly understand the future of H5N1 Z. Further research specifically directed at the influence of the environment on evolution will lead to a greater understanding of the virus and possibly develop models to interpret and predict future movement and evolution of the virus.

Human influenza continues to be the number one concern for annual epidemics and less frequent but dramatic pandemics with high attack rates and sometimes high mortality. But unlike previous times, we may be able to prevent the pandemic from happening. Multi-disciplinary research is the future of pandemic preparedness and should be a goal for not only this pandemic generation, but for all to come.

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