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Importance of 1918 virus reconstruction to current assessments of pandemic risk

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Abstract

Reconstruction of the 1918 influenza virus has facilitated considerable advancements in our understanding of this extraordinary pandemic virus. However, the benefits of virus reconstruction are not limited to this one strain. Here, we provide an overview of laboratory studies which have evaluated the reconstructed 1918 virus, and highlight key discoveries about determinants of virulence and transmissibility associated with this virus in mammals. We further discuss recent and current pandemic threats from avian and swine reservoirs, and provide specific examples of how reconstruction of the 1918 pandemic virus has improved our ability to contextualize research employing novel and emerging strains. As influenza viruses continue to evolve and pose a threat to human health, studying past pandemic viruses is key to future preparedness efforts.

Keywords

1918 influenza virus; Pandemic virus; Influenza viruses; Pathogenesis

1. Introduction

Unlike other viral diseases including smallpox, polio, and measles, which circulate exclusively in humans, influenza A viruses are not a target for eradication (1993). The segmented genome of influenza A viruses, coupled with an error-prone polymerase and high tolerance for mutations, means that antigenic and genetically distinct viruses are constantly generated in both humans and zoonotic reservoirs (Brooke, 2017; Petrova and Russell, 2018), posing a public health risk when humans come into contact (direct or indirectly) with virus shed from these animals. Most zoonotic transmission events are limited, resulting from close contact with infected poultry or swine, and due to both virus and host barriers, do not result in subsequent or sustained human-to-human transmission. Emergence of a pandemic influenza virus is dependent on three criteria: little to no pre-existing immunity to the virus in the human population, the ability for the virus to cause infection in humans, and the capacity for sustained human-to-human transmission throughout the population (Belser et al., 2010). The third criterion is the only one lacking among zoonotic influenza viruses.

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Reports of epidemics in humans believed to be caused by influenza viruses date to ancient times (Potter, 1998). However, our current understanding of pandemic influenza viruses has been largely obtained by studying the last four pandemics in human history, which occurred during the 20th and 21st centuries; analyses of pandemic influenza from the 19th century are limited (Valleron et al., 2010). The H1N1 pandemic from 1918-19 is well-known as the most devastating infectious disease event in history, lowering the life expectancy in the United States by more than 10 years and causing an estimated 50 million deaths worldwide (Glezen, 1996; Johnson and Mueller, 2002). Subsequent pandemics in 1957 and 1968, caused by H2N2 and H3N2 strains, respectively, resulted in slightly lower attack rates but dramatically lower case fatality proportions compared with those recorded in 1918–19 (Biggerstaff et al., 2014; Mills et al., 2004). Advances in modern medicine, laboratory science, and public health practices have contributed to our understanding that not all pandemic viruses will pose a uniformly severe threat to human health. Most recently, the 2009 H1N1 pandemic illustrated both the strides made in preparedness efforts during the last century, and the unpredictability of identifying a causative pandemic virus prior to widespread detection in humans.

Given the frequency and diversity of circulating influenza viruses in zoonotic populations (Webby and Webster, 2003), a virus possessing the properties necessary to cause a pandemic could emerge at any time. Understandably, it is difficult to prepare for a target you cannot identify in advance. There are many zoonotic reservoirs from which a pandemic virus could emerge following interspecies transmission, including but not limited to those in avian and swine populations (Yoon et al., 2014). From these reservoirs, select virus subtypes have already demonstrated an enhanced ability to cause human infection. Among these are H5N1 subtype highly pathogenic avian influenza (HPAI) viruses, responsible for > 850 cases with a > 50% fatality rate in the last 15 years (WHO, 2018). H7N9 viruses, first associated with human infection in 2013 as low pathogenic avian influenza (LPAI) viruses but now circulating in both HPAI and LPAI forms, have caused an even greater burden of human disease, with > 1600 human cases detected with a > 30% fatality rate (FAO, 2018). H2 subtype viruses, associated with the 1957 pandemic, have persisted in avian reservoirs (Schafer et al., 1993). Variant H1 and H3 viruses from the swine reservoir have additionally caused human infections over the last decade, and are antigenically distinct from currently circulating strains (Shu et al., 2012). It is clear that there is a multitude of potential candidates for the next influenza pandemic.

The more researchers and public health professionals learn about viruses from past pandemics, the better we will understand which molecular features are associated with a pandemic virus, the scope of mammalian disease elicited by pandemic influenza viruses, and the best ways to mitigate illness and infection via use of vaccination and antiviral drugs. Reconstruction of the 1918 virus has offered an invaluable tool in advancing our knowledge in these areas (Fig. 1). This virus represents in many ways the “worst case scenario” for a pandemic virus: a highly transmissible virus, causing severe disease in many infected individuals (notably in previously healthy, younger individuals) with a high mortality rate. Studying the molecular determinants of virulence and transmission for this virus, in comparison with other pandemic viruses and other contemporary viruses with pandemic potential, has afforded us a window into understanding why the 1918 pandemic

was so devastating, and has aided efforts to ensure an event of this magnitude is not repeated. In this review, we describe critical findings obtained from research utilizing the reconstructed 1918 virus, and discuss selected examples of improved understanding of novel and emerging strains due to prior and parallel research with this past pandemic virus, with an emphasis on mammalian pathogenicity and transmissibility.

2. Part 1: Unlocking the mysteries of the 1918 pandemic virus

2.1. 1918 virus reconstruction and mammalian pathogenicity

The causative strain of the 1918 pandemic was initially thought to be lost to time: identification and isolation of influenza virus was not reported until the 1930s (Shope, 1931), at which point no samples containing live virus from the pandemic remained. Accordingly, efforts in 1951 to culture infectious virus from lung biopsies collected from 1918 victims preserved in the permafrost were unsuccessful (Taubenberger et al., 2007). It was only via the use of archaevirology that enabled reconstruction of the 1918 virus (Tumpey and Belser, 2009). Coding sequence of 1918 viral RNA segments was obtained by RT-PCR from either formalin-fixed, paraffin-embedded autopsy tissues taken from victims at the time or isolated from a frozen lung sample of a 1918 victim buried in the permafrost from Brevig Mission, Alaska (Taubenberger, 2006; Taubenberger et al., 1997). Via the use of reverse genetics, infectious virus was rescued from plasmid-cloned 1918 virus influenza gene segments (Basler et al., 2001; Fodor et al., 1999). Initial assessments of coding sequences of 1918 viral RNA gene segments indicated that the virus shared high amino acid similarity with avian consensus sequences (Taubenberger et al., 2005), but did not possess many known markers of mammalian virulence (Taubenberger et al., 1997), necessitating full reconstruction of the live virus to identify genetic determinants associated with pathogenicity and transmissibility.

Infectious virus containing coding sequences of the eight gene segments from the 1918 pandemic virus was completed in 2005, permitting for the first time in vivo and in vitro examination of the fully reconstructed strain (Tumpey et al., 2005a); the virus has since been evaluated in mice, ferrets, guinea pigs, swine, and macaques (Kobasa et al., 2007; Tumpey et al., 2007; Van Hoeven et al., 2009a; Weingartl et al., 2009). It was readily apparent that the 1918 H1N1 virus possessed several unusual properties not typically associated with this subtype. Human influenza viruses generally do not replicate efficiently in mice without prior adaptation, however, the reconstructed 1918 virus was highly virulent in the BALB/c mouse model, with substantial morbidity (as measured by weight loss), rapid mortality, and viral titers in the lungs significantly higher than a seasonal H1N1 virus (Kobasa et al., 2007; Pappas et al., 2008; Tumpey et al., 2005a). Furthermore, compared with a seasonal H1N1 virus, mice infected with the reconstructed 1918 virus displayed enhanced proinflammatory and cell death responses, contributing to severe pulmonary pathology present in infected lung tissue (Kash et al., 2006). This work confirmed that the constellation of all eight 1918 genes yielded a virus of exceptional mammalian virulence.

Ferrets have been employed for influenza virus research for as long as the virus has been identified. Reports from the 1930s detail their use for pathogenesis studies (Shope, 1934; Smith et al., 1933) and for studies of virus immunity (Andrewes, 1939). Only 20 years

removed from the 1918 pandemic, these ferret studies sought to understand what made the 1918 causative virus so virulent in comparison with human and swine influenza viruses of that time. Ferrets are now recognized as an invaluable small mammalian model for the coincident study of influenza virus pathogenicity and transmissibility, attributed to several key features. The epithelia lining the ferret respiratory tract bear a distribution of sialic acid (SA) receptors that is generally similar to that of humans (Jayaraman et al., 2012; Shinya et al., 2006), with similar attachment patterns of human and avian influenza viruses to respiratory tissues from both species (van Riel et al., 2006, 2007). These features confer the susceptibility of ferrets to most human and zoonotic influenza viruses without the need for prior host adaptation. Furthermore, ferrets and humans share numerous clinical, laboratory, and virological features following influenza virus infection (Belser et al., 2011c).

Most human seasonal influenza viruses, LPAI viruses, and other zoonotic strains typically cause mild and transient illness in ferrets, with virus replication restricted to the respiratory tract (Belser et al., 2009). To better identify features unique to the causative virus of the 1918 pandemic, ferrets were inoculated with the reconstructed 1918 H1N1 virus and compared with human seasonal and avian H1N1 viruses (Tumpey et al., 2007; Watanabe et al., 2009). The 1918 H1N1 virus-inoculated ferrets had higher infectious viral titers in nasal specimens, with ferrets shedding infectious virus for a longer period of time, compared with seasonal or avian H1N1 viruses. Unlike many seasonal H1N1 viruses isolated prior to 2009, the reconstructed 1918 virus replicated efficiently throughout the ferret respiratory tract, including to high titer in the lung (Pearce et al., 2012a; Tumpey et al.,2007). Furthermore, ferrets infected with the 1918 H1N1 virus exhibited more pronounced clinical signs and symptoms of infection, including rhinorrhea, sneezing, fever, lethargy, anorexia, and severe morbidity, compared with other H1N1 viruses tested. A lethal phenotype in 1918 virus-infected ferrets is possible, though not all ferrets uniformly succumb to the infection (Pearce et al., 2012a; Pillet et al., 2011; Tumpey et al., 2007; Watanabe et al., 2009). Systemic spread of virus and induction of host responses in extrapulmonary tissues has been reported following 1918 virus inoculation in ferrets, likely contributing to the severe disease present in this species (de Wit et al., 2018).

While mice and ferrets represent the most frequently employed small mammalian models to study influenza virus pathogenicity, other species have been experimentally inoculated with the reconstructed 1918 virus to ascertain the full scope of disease progression and involvement of immune responses. Cynomolgus macaques developed a severe respiratory infection, leading to acute respiratory distress syndrome, following inoculation with the reconstructed 1918 virus (Kobasa et al., 2007). Infectious virus was detected at high titer throughout upper and lower respiratory tract tissues, with histopathological examination identifying severe alveolar damage in lung tissue; systemic spread of virus to the heart and spleen was detected among some inoculated animals. Microarray analysis of global gene expression profiles in the bronchi identified generally similar sustained host responses following 1918 virus infection as in mice (Kash et al., 2006), further supporting the role aberrant immune responses contribute to the severity of disease. In contrast, while guinea pigs could be infected by the reconstructed 1918 virus and displayed infectious virus in nasal washes and lung tissue, pronounced morbidity or mortality were not observed (Van Hoeven et al., 2009a). A similar phenotype of low virulence following HPAI H5N1 virus

infection in this species indicates that guinea pigs are limited in their utility as a model for influenza virus pathogenesis.

2.2. Molecular determinants of 1918 virus pathogenicity

Intensive efforts have been made to identify the role(s) each gene segment of the 1918 virus contribute to the overall virulence of the virus in mammals. Despite the sequence of the 1918 hemagglutinin (HA) and neuraminidase (NA) not displaying immediately conspicuous features that would suggest heightened virulence in mammals, it was quickly apparent that these genes, notably the HA, were instrumental in conferring a high-virulence phenotype. The first evidence that the 1918 HA glycoprotein was largely responsible for its high virulence came from a 2002 study which showed that recombinant H1N1 viruses possessing the 1918 HA were virulent in mice (Tumpey et al., 2002). Additional recombinant viruses bearing the 1918 HA gene were shown to enhance the virulence of a number of H1N1 and H3N2 viruses in mice, leading to increased pathogenicity, viral loads, and inflammatory responses (Kobasa et al., 2004; Tumpey et al., 2004). Enhanced replication efficiency and augmented elicitation of host inflammatory responses were also observed in vitro with reassortant viruses bearing the 1918 HA (Billharz et al., 2009; Geiss et al., 2002; Pappas et al., 2008; Tumpey et al., 2005a, 2005b). The influenza HA is a homotrimeric integral membrane glycoprotein that has multiple functions, including initiating virus binding to sialic acid-containing host cell receptors and mediating fusion of the viral and cell membranes. Because of the multifunctional nature of this protein, it has been difficult to elucidate the precise mechanism by which the HA gene of the 1918 virus enhances virulence and replication in mammals. Moreover, the 1918 virus does not possess the typical polybasic cleavage motif found to be associated with highly pathogenic avian influenza viruses. However, one major, yet underappreciated virulence factor is the structure and composition of glycans on the HA surface. It has been demonstrated that the low number of N-linked 1918 H1N1 glycosylation sites on top of the HA head directly contributes to its virulence in mice and may be one virulence-associated function of this protein (Sun et al., 2013). Thus, in the years following the 1918–19 pandemic, H1N1 virus evolution in humans has led to the steady acquisition of HA glycosylation sites that may have attenuated the pandemic virus over time (Cherry et al., 2009).

Among all the single-gene 1918 viruses tested, only recombinant viruses bearing the 1918 HA gene were shown to confer a lethal phenotype in mice (Pappas et al., 2008). However, other 1918 virus genes were found to contribute to 1918 virus replication and morbidity. In particular, the 1918 polymerase complex (PA, PB1, and PB2) has been identified as a contributor to mammalian pathogenicity. A reassortant virus bearing the polymerase complex and NP from the 1918 virus on the background of a seasonal H1N1 virus was sufficient to confer enhanced virulence in ferrets, enabling more efficient virus replication in both the trachea and lung than the seasonal wild-type strain (Watanabe et al., 2009). The addition of the 1918 PB1 virus gene to a seasonal H1N1 virus was able to increase plaque-size morphology compared to the wild-type seasonal virus (Pappas et al., 2008; Watanabe et al., 2009). Furthermore, the PB1 gene was found to be a determinant for virus replication in both the murine lung and human bronchial epithelial cells (Pappas et al., 2008).

The 1918 PB1 may function by increasing the polymerase activity of the 1918 virus or by way of PB1-F2. First identified in 2001, the PB1-F2 protein is an accessory protein encoded by the + 1 reading frame of the influenza PB1 gene, and is now recognized as possessing several molecular determinants of virulence (Chen et al., 2001). The PB1-F2 gene from the 1918 virus has been associated with increased replication efficiency in mice, resulting in higher rates of viral production and cell death in the lungs compared to infection with a laboratory-adapted H1N1 virus (Smith et al., 2011). In ferrets, expression of the 1918 PB1-F2 resulted in the moderate modulation of local cytokine responses but ultimately did not substantially augment the virulence of a seasonal H1N1 virus (Meunier and von Messling, 2012). However, expression of the 1918 PB1-F2 was found to enhance the pathogenesis of secondary bacterial pneumonia in mice, providing experimental evidence to support the high incidence of severe pneumonia in humans associated with this pandemic virus (McAuley et al., 2007).

The NS1 gene serves as a strong IFN-antagonist with broad roles in evading host antiviral responses; the 1918 NS1 gene closely resembles that of other avian viruses (Heikkinen et al., 2008). Reassortant viruses bearing the 1918 NS gene on the backbone of seasonal or laboratory-adapted H1N1 viruses did exhibit increased efficiency in blocking IFN-regulated genes in vitro, with concurrent upregulation of numerous proinflammatory genes (Billharz et al., 2009; Geiss et al., 2002). However, these reassortant viruses caused only little to modest increases in virulence or replication efficiency compared to the wild-type strain in mammalian in vivo or in vitro models (Basler et al., 2001; Brown et al., 2010; Meunier and von Messling, 2011; Pappas et al., 2008), indicating that the NS1 protein likely does not represent a critical virulence determinant for this virus. Similarly, the matrix gene (which encodes two proteins, M1 and M2) did not contribute to 1918 virus replication and virulence (Tumpey et al., 2004; Pappas et al., 2008). Moreover, sequence analysis of the 1918 matrix coding sequence did not reveal known amino acid changes associated with virulence (Reid et al., 2002).

While studies employing reassortant viruses have greatly improved our understanding of the contributions of individual 1918 genes in overall mammalian virulence, and have highlighted the importance of the unique constellation of genes contributing to this property, it is important to note that the virulence of the 1918 virus in humans was likely influenced by differences in preexisting/differential immunity in different age cohorts (Worobey et al., 2014). The role of host immunological responses and variation in cellular immunity between different age groups at the time of the pandemic must be taken into account (McAuley et al., 2015); however, this parameter is difficult to fully emulate in serologically naïve small mammalian models.

2.3. Molecular basis of 1918 virus transmissibility

Efficient virus transmissibility between humans by the airborne route represents a critical step for a zoonotic influenza virus to cause a pandemic. Most human infections with zoonotic influenza virus are self-limiting, with human-to-human transmission rare, typically occurring in family clusters (Koopmans et al., 2004; Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with

Avian Influenza et al., 2008; Wu et al., 2016). Ferrets emulate transmission patterns of many influenza viruses, with efficient transmission from infected to naïve ferrets typically occurring with seasonal, but not most zoonotic, influenza viruses (Andrewes and Glover, 1941; Herlocher et al., 2001; Maines et al., 2006). Furthermore, statistical analyses found that estimates of human secondary attack rates correlate with transmission results obtained in the ferret respiratory droplet model (Buhnerkempe et al., 2015), highlighting the importance of this readout in assessments of pandemic risk (Belser et al., 2018).

As expected, the reconstructed 1918 virus transmitted efficiently between ferrets via respiratory droplets (Tumpey et al., 2007). Virus was detected in recipient ferrets within 24 h of being placed in an adjacent cage with perforated side-walls (to permit air exchange in the absence of direct and indirect contact with inoculated ferrets), with comparable high titers of infectious virus in nasal wash specimens among either directly inoculated or contact animals. This pattern of virus transmissibility was more robust than a transmissible seasonal H1N1 virus, which required a longer duration of time for all recipient ferrets to become infected, and elicited only mild morbidity in these contact animals. The robust transmissibility of the reconstructed 1918 virus in the ferret model has provided necessary data for probability analyses assessing the likelihood of contemporary viruses with pandemic potential to spread efficiently among humans (Buhnerkempe et al., 2015).

Several studies have investigated the molecular determinants associated with the efficient transmissibility of the reconstructed 1918 virus (Tumpey and Belser, 2009). Among these, receptor specificity has been identified as a critical component modulating interspecies transmission of influenza viruses (Table 1) (Gambaryan and Matrosovich, 2015). Human-adapted H1 viruses, including the 1918 HA, contain Asp190 and Asp225 which confer strong binding to α2-6 linked SA (Stevens et al., 2006). Switching the receptor binding preference of the 1918 virus to resemble avian influenza viruses (Glul90 and Gly225) resulted in a change from strong binding to α2-6 linked SA to α2-3 linked SA, with a virus bearing these mutations replicating to high titers of infectious virus in nasal specimens from inoculated ferrets but the abolishment of virus transmissibility by the airborne route to naïve animals (Tumpey et al., 2007). Reduced transmissibility was also detected in a mutant virus bearing dual α2-3/α2-6 SA binding. This work supports the role of receptor binding preference in virus transmissibility by the airborne route as indicated in other studies (Maines et al., 2006).

While receptor binding preference of the HA represents a critical component of virus transmissibility, mutation of the two amino acids identified above (switching binding preference from α2-3 to α2-6) was not sufficient to confer an airborne transmissible phenotype to an otherwise non-transmissible avian H1N1 virus (Table 1) (Van Hoeven et al., 2009b). Similarly, a reassortant virus bearing the HA and NA of the 1918 virus on the backbone of a non-transmissible avian H1N1 virus failed to transmit via respiratory droplets in the ferret model (Table 2). However, inclusion of the 1918 PB2 gene (but not PB1 or PA) with 1918 surface glycoproteins yielded a virus that transmitted with comparable efficacy as the wild-type 1918 virus, identifying a role for both the 1918 HA and PB2 in the robust transmissibility observed with this virus. The presence of a lysine at position 627 in the PB2 (a previously identified marker of mammalian virulence (Subbarao et al., 1993)) was

identified as a critical feature of this gene for 1918 virus transmissibility, as mutation at this position to the avian consensus residue (K627E) greatly reduced virus transmissibility of the reconstructed 1918 virus (Van Hoeven et al., 2009b).

Delineating the coding sequence of the 1918 virus has made possible valuable studies to model and study the likelihood and impact of “1918-like” viruses emerging from the contemporary avian reservoir. Generation of a virus derived from recently isolated avian virus genes with high homology to the reconstructed 1918 virus possessed enhanced virulence in mammals compared with avian H1N1 viruses, though reduced compared with the fully reconstructed 1918 virus (Forero et al., 2015; Watanabe et al., 2014). While mutation of residues in the HA to confer α2-6 binding and mutation of E627K in PB2 increased pathology in respiratory tract tissues compared with the wholly avian “1918-like” virus, these changes were not sufficient to confer a transmissible phenotype by the airborne route. However, mutation of < 10 additional amino acids in the HA and polymerase genes resulted in enhanced tissue tropism, virus replication, and a transmissible phenotype in ferrets, highlighting the current circulation of avian influenza virus genes capable of acquiring a virulent and transmissible phenotype similar to the 1918 virus (Watanabe et al., 2014).

Collectively, manipulation of the reconstructed 1918 virus in the laboratory has greatly aided our ability to understand what contributed to the efficient worldwide spread of this virus, and has provided critical mammalian pathogenicity and transmissibility baseline data from which we can measure other influenza viruses that pose a threat to human health. In the second half of this review, we provide examples of just how valuable 1918 virus reconstruction has been to achieve this goal.

3. Part 2: Using the 1918 virus to understand contemporary pandemics and pandemic threats

3.1. What’s old is new again: H1N1 in the 21st century

Four years after the reconstruction of the 1918 pandemic virus, a new H1N1 pandemic virus swept the globe, this time emerging from North American swine populations (Garten et al., 2009). The causative virus contained a unique constellation of gene segments, derived from Eurasian swine lineage (NA, M), North American classical swine (NA, NP, NS), and swine triple reassortant lineages (PB2, PA, PB1). The virus spread rapidly worldwide, necessitating the WHO to declare a pandemic in June 2009, within two months of detection of the first human cases in North America. The case-fatality ratio of this virus in the first year of the pandemic was ultimately found to be low (Nishiura, 2010), but elevated compared with seasonal influenza mortality prior to the pandemic in children and adults < 65 years of age (Cox et al., 2011; Shrestha et al., 2011). Of course, the world was a very different place in 2009 compared with 1918, both to the benefit (modern medicine, availability of antiviral drugs, and antibiotics to treat secondary bacterial pneumonia (Viasus et al., 2012)) and detriment (increased global travel due to international airline transportation (Khan et al., 2009)) of the spread and management of influenza, making it difficult to compare degrees in virus virulence between both pandemic viruses.

Initial laboratory assessments of the 2009 H1N1 pandemic virus revealed that the virus was of generally mild-to-moderate virulence in small mammalian models, with the capacity for severe and fatal disease in some instances (Itoh et al., 2009; Maines et al., 2009; Munster et al., 2009). Unlike prior circulating H1 and H3 subtype viruses in humans, 2009 H1N1 viruses replicated efficiently in the mouse lung following inoculation, and were similarly detected throughout the ferret respiratory tract. Most virus isolates from this pandemic were found to transmit efficiently via respiratory droplets in the ferret model, with some variation depending on the strain or experimental conditions employed (Itoh et al., 2009; Maines et al., 2009; Munster et al., 2009). Separated by 91 years, reconstruction of the 1918 virus greatly aided efforts to improve our understanding of this most recent pandemic virus. Side-by-side analyses of viruses from both 1918 and 2009 has provided valuable insights into discovering features that are both shared and distinct between these two pandemic viruses, inclusive of virological, histopathological, and broader systems-level analyses (Peng et al., 2014; Tisoncik-Go et al., 2016).

The 2009 H1N1 pandemic virus was unusual in that it did not possess many known markers of mammalian virulence or transmissibility, including the presence of 627K and 701D in PB2, or a full-length PB1-F2 or NS1 protein (Garten et al., 2009). Furthermore, many studies that restored these virulence markers revealed that inclusion of molecular features associated with past pandemic viruses did not substantially augment virus pathogenicity or transmissibility of wild-type strains in mammalian models (Hai et al., 2010; Hale et al., 2010; Herfst et al., 2010; Pena et al., 2012). However, an Asp to Gly substitution at position 225 of the HA, detected in over 1% of 2009 H1N1 cases during the first year of the pandemic and also present in a variant reconstructed 1918 virus, was suggested as a virulence marker due to its association with severe pneumonia, ICU admittance, and fatal outcomes (Belser et al., 2011b; Chutinimitkul et al., 2010; Tumpey et al., 2007). Both 1918 and other H1N1 viruses bearing a Gly at this position exhibited dual binding to both α2-6 and α2-3 linked SA; the presence of this mutation on a 2009 H1N1 virus further enabled increased binding to human type II pneumocytes and alveolar macrophages in the human lung, and exhibited modest enhancements in virus replication in human respiratory tract epithelial cells (Belser et al., 2011b; Chutinimitkul et al., 2010), highlighting the potential advantage this mutation confers in lower respiratory tract replication fitness.

As expected by a pandemic virus, the 2009 H1N1 virus exhibited strong binding to α2-6 linked SA, and did not bind efficiently to α2-3 linked SA, though the binding to human receptors was less robust than the 1918 HA (Maines et al., 2009; Yang et al., 2010). Virus transmissibility between naïve ferrets via respiratory droplets was detected at levels slightly below or comparable to seasonal H1N1 viruses tested prior to the pandemic. Mutation of position 219 in the HA of a 2009 H1N1 virus (from Ile to Lys), to more closely match the receptor binding site of the 1918 strain, resulted in a virus with enhanced α2-6 SA binding and transmissibility by the airborne route in ferrets (Jayaraman et al., 2011). However, mutation of position 225 in the HA as described above did not modulate virus transmissibility in ferrets or guinea pigs (Belser et al., 2011b; Chutinimitkul et al., 2010).

Beyond modulation of amino acids at the receptor binding pocket, studies have shown that the presence of glycans in close proximity to receptor binding domains can modulate

binding affinity and specificity (Skehel and Wiley, 2000). Unlike both the reconstructed 1918 virus and 2009 H1N1 viruses, which possess only one glycosylation site on the HA globular head, seasonal H1N1 viruses circulating prior to the 2009 pandemic possessed additional glycosylation sites. The addition of two glycosylation sites to the 1918 HA resulted in reduced lethality of the virus in mice and reduced binding to α2-6 linked SA, indicating that modulation of glycans could influence pathogenicity and binding specificity in mice; furthermore, removal of two glycosylation sites from a 2006 seasonal H1N1 virus augmented morbidity and mortality of this virus and increased binding to α2-3 linked SA (Sun et al., 2013). Collectively, these are a few of many examples demonstrating the utility of having the reconstructed 1918 virus as a reference to better understand currently circulating influenza viruses.

3.2. Emerging pandemic threats: viruses from the swine reservoir

Swine have long been identified as a “mixing vessel” for influenza viruses (Scholtissek, 1995). The presence of both α2-3 and α2-6 linked sialic acids in the respiratory tract of swine, and close proximity and contact with both humans and avian species, make them an attractive intermediate for avian viruses to circulate and acquire mutations that might facilitate interspecies transmission events and human infection (Ito et al., 1998; Ma et al., 2008). Reports of high evolutionary rates of change among viruses isolated from swine (Ludwig et al., 1995) suggest that the generation of influenza variant viruses in swine could potentially enable introduction of a virus to humans with increased fitness compared with viruses that did not infect an intermediate host. The genes of all influenza viruses currently circulating in swine populations are of avian origin, introduced either directly from avian species or following circulation in humans (Janke, 2014). Notably, it appears that the classical swine H1N1 lineage is derived from the 1918 pandemic virus (Worobey et al., 2014).

Influenza virus infection in North American pigs was caused primarily by classical swine H1N1 viruses for most of the 20th century (Olsen, 2002). The emergence of seropositivity to and isolation of H3N2 viruses from the swine population ~1998 has resulted in an escalation of antigenic and genetic diversity of influenza viruses in this population (Zhou et al., 1999), with reassortment occurring between classical swine H1N1 and H3N2 viruses (Karasin et al., 2000). Triple-reassortant swine (TRS) influenza viruses (of human, avian, and swine origin) have led to infection of humans in close contact with infected swine or following exposure to locations where pigs were present; these are termed variant influenza viruses following isolation from humans (Shinde et al., 2009; Terebuh et al., 2010). Select variant viruses are capable of causing severe disease in humans, including lower respiratory tract infection and gastrointestinal complications (Pulit-Penaloza et al., 2018). As these viruses continue to exhibit substantial genetic and antigenic diversity compared with H1 and H3 viruses circulating in humans (Shu et al., 2012), their ability to jump the species barrier and cause human infection identifies them as a source of pandemic concern.

Swine influenza viruses have been known to infect, cause disease, and transmit between ferrets since their first detection in the 1930s (Shope, 1934). An early classical swine isolate from 1931 was found to possess comparable virulence in ferrets and mice as the

reconstructed 1918 virus, in contrast with a contemporary H1N1 virus (Memoli et al., 2009). Interestingly, North American H1 and H3 TRS viruses, in addition to recently isolated variant viruses that possess even greater genetic diversity, generally replicate efficiently throughout the ferret respiratory tract and typically exhibit mild to moderate virulence, similar to most pandemic 2009 H1N1 viruses (Belser et al., 2011a; Pascua et al., 2012; Pearce et al., 2012b; Pulit-Penaloza et al., 2018). These variant viruses are capable of efficient transmission between ferrets in direct contact, but virus transmissibility via respiratory droplets varies. Studies have identified that the lineage of surface glycoproteins can affect this property, with swine viruses bearing human-lineage HA and NA genes transmitted between ferrets more readily than those with swine-lineage surface glycoproteins (Barman et al., 2012). Furthermore, while the ability to bind human receptors appears to be a critical component, the HA-NA balance can also contribute to the resulting mammalian transmissibility of these H1 subtype viruses (Yen et al., 2011).

The diversity of viruses isolated from humans following exposure to swine represents a challenge for risk assessment, both with regard to antigenic variability within the surface glycoproteins and heterogeneity in internal gene constellations of these viruses (Rajao et al., 2018). This demonstrates the difficulty of studying the risk posed by these viruses and underscores the need for in vivo assessments to understand multifactorial traits like virus transmission. As establishment of the classical H1N1 swine lineage following transmission events between humans and swine dates to the 1918 pandemic, 1918 virus reconstruction offers (concurrent with swine virus isolates from the 1930s) a critical reference with which to compare the virulence and transmissibility of influenza viruses that continue to emerge from this population that pose a threat to human health.

3.3. Emerging pandemic threats: viruses from the avian reservoir

It was initially postulated that the 1918 pandemic virus transmitted from birds to humans in its entirety (Taubenberger et al., 1997; Webster et al., 1995), though this is difficult to confirm conclusively (Reid and Taubenberger, 2003; Vana and Westover, 2008; Worobey et al., 2014). Both subsequent 1957 H2N2 and 1968 H3N2 pandemic strains were products of virus reassortment with previously circulating influenza viruses, where the PB1 and HA (1957 and 1968) and NA (1957) were replaced with gene segments of avian origin (Webster et al., 1995). The avian reservoir of influenza viruses and perpetual threat of zoonotic transmission necessitates close examination of avian influenza A virus subtypes that appear to most readily jump the species barrier to cause human infection. While a diversity of H5, H7, H9, and H10 viruses have been associated with human infection to date (Uyeki et al., 2017), the two viruses most frequently associated with this property are H5N1 and H7N9, collectively responsible for > 2400 confirmed human cases in the last 15 years (FAO, 2018; WHO, 2018). Despite both of these viruses fulfilling the first two criteria of a pandemic virus (capacity for human disease in an immunologically naïve population), H5N1 and H7N9 viruses have not yet acquired the ability of sustained transmissibility, with only limited and sporadic detection of human-to-human transmission to date (Liu et al., 2017; Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza et al., 2008). However, a heightened ability for H7N9 viruses to transmit by the airborne route in ferrets compared with H5N1

viruses (Belser et al., 2013; Maines et al., 2006; Richard et al., 2013; Watanabe et al., 2013), underscores the importance of understanding if acquisition of specific molecular determinants would result in a pandemic strain.

Avian influenza viruses can exhibit both high and low virulence in mammalian models, depending on the inoculum strain. Most LPAI viruses cause generally low virulence in mammalian models, characterized by transient weight loss and fever, mild to moderate lymphopenia, pulmonary inflammation, and elicitation of proinflammatory cytokines and chemokines (Belser and Tumpey, 2013). In contrast, select avian influenza viruses (most frequently but not exclusively associated with HPAI strains) are capable of causing severe respiratory disease in many species. Beyond the clinical signs and symptoms of infection apparent with low virulence viruses, infection with these highly virulent avian strains often result in spread of virus to extrapulmonary organs, including the brain, with possible development of neurological complications necessitating humane euthanasia (Belser and Tumpey, 2013; Schrauwen et al., 2012). While the spread of 1918 virus to brain tissue of experimentally inoculated animals was not detected in our hands (Tumpey et al., 2005a, 2007), detection of infectious virus in the olfactory bulb and cerebrum of 1918 virus-infected ferrets in selected studies highlights the potential for 1918 virus to cause systemic disease (de Wit et al., 2018; Kristensson, 2006), and as such represents a valuable comparison virus for studies identifying molecular determinants of avian influenza virus virulence.

Numerous studies have furthered our understanding of avian influenza virus pathogenicity in mammals by studying the 1918 virus concurrent with contemporary avian influenza viruses, as the high conservation of many internal genes between these viruses facilitates identification of molecular determinants of virulence (Basler and Aguilar, 2008; Taubenberger et al., 2005). Sequence alignment of the PB1-F2 gene of H5N1 and H1N1 viruses possessing varied virulence in mammals identified one amino acid change (Asn to Ser at position 66) that correlated with virus pathogenicity of both the 1918 virus and recently isolated HPAI H5N1 viruses (Conenello et al., 2007). Similarly, associations between elicitation of inflammatory mediators in mice, non-human primates, and human cells infected with both 1918 virus and HPAI viruses identifies host factors that may be predictive of severe influenza virus infection with contemporary pandemic threats (Davis et al., 2015; Morrison et al., 2014; Perrone et al., 2013, 2008). These and other studies illustrate the utility in including the 1918 virus in assessments of virus virulence to better identify strain-specific and subtype-specific features that contribute to this property, while further underscoring the need for continued assessments with additional zoonotic viruses of concern that have crossed the species barrier to cause human infection (Yang et al., 2015).

While many virulence factors are shared between the 1918 virus and currently circulating avian influenza viruses, the lack of robust transmissibility among most avian viruses in mammals represents a critical difference between these strains. H5N1 influenza viruses are not readily transmissible via respiratory droplets in the ferret model, but can acquire a transmissible phenotype following virus reassortment and/or with a relatively limited acquisition of mutations (Herfst et al., 2012; Imai et al., 2012). Recent use of next generation sequencing has facilitated studies of bottlenecks which govern human and avian influenza

virus transmission dynamics, leading to a greater understanding of the selective pressures contributing towards within-host adaptation of avian influenza viruses in the context of virus transmissibility (Varble et al., 2014; Wilker et al., 2013; Zaraket et al., 2015). This is supported by research employing “1918-like” viruses, which have further identified that transmission bottlenecks can vary during the course of host adaptation, demonstrating that a transmissible phenotype can arise from multiple evolutionary pathways (Moncla et al., 2016).

4. Conclusions

Analysis of the reconstructed 1918 virus has contributed a wealth of information towards influenza viruses in general and current assessments of influenza virus pandemic risk in particular. Recent identification of a novel influenza accessory protein (PA-X) was facilitated in part by sequence delineation of the 1918 virus (Jagger et al., 2012), further illustrating the role 1918 virus reconstruction has played in enhancing our understanding of influenza pathobiology and identifying novel targets for antiviral therapies. Concurrently, these studies have illuminated areas where our knowledge of influenza virus pathogenicity and transmissibility are incomplete, and have identified new avenues and approaches to meet these needs. As evidenced throughout this review, most of our understanding of influenza virus pathology in mammals has been gained due to the intersecting and overlapping study of virus-infected humans and experimentally inoculated animals (Kuiken et al., 2010). Continued research efforts in this area, especially those that take into account factors including diverse immunological backgrounds and immunocompromised hosts, prior exposure histories, and the effect of coinfections, will further our ability to treat, prevent, and control both pandemic and interpandemic influenza viruses.

Antiviral drugs and vaccines represent critical tools to reduce the burden of influenza virus infection worldwide, and their use features prominently in pandemic preparedness and response frameworks (Holloway et al., 2014). 2009 marked the first influenza pandemic in history for which neuraminidase-inhibitor antiviral drugs were available (Hurt et al., 2012); recombinant viruses bearing the surface glycoproteins of the 1918 virus are similarly sensitive to these agents (Tumpey et al., 2002). The use of the reconstructed 1918 virus has contributed to both our understanding of correlates of protection following vaccination and in vaccine approaches to study cross-protective immunity (Bragstad et al., 2011; Pillet et al., 2011). This work has further provided supportive evidence of prior exposure to 1918 virus eliciting cross-reactive immunity against the 2009 H1N1 pandemic virus (Giles et al., 2012; Hancock et al., 2009). As the production process for pandemic influenza viruses can take several months, efforts to improve vaccine formulations are ongoing, including those that aim to elicit broadly protective antibodies (Krammer and Palese, 2015). In this vein, examination of the 1918 HA structure has contributed to the development of monoclonal antibodies and broadly reactive vaccines against influenza virus (Fleishman et al., 2011; Xu et al., 2010).

The genetic and antigenic diversity of viruses in zoonotic reservoirs continues to expand. As influenza viruses continue to emerge from zoonotic reservoirs to cause novel human infections, risk assessment rubrics have been created to assess the potential pandemic risk

of zoonotic influenza viruses, which are guided by data from both current pandemic threats and past pandemic strains (Cox et al., 2014; WHO, 2016). It is clear that reconstruction of the 1918 virus has provided invaluable information towards pandemic preparedness efforts. Continued examination of and reference to this notorious strain will further enable public health efforts to mitigate the burden of influenza virus infection worldwide.

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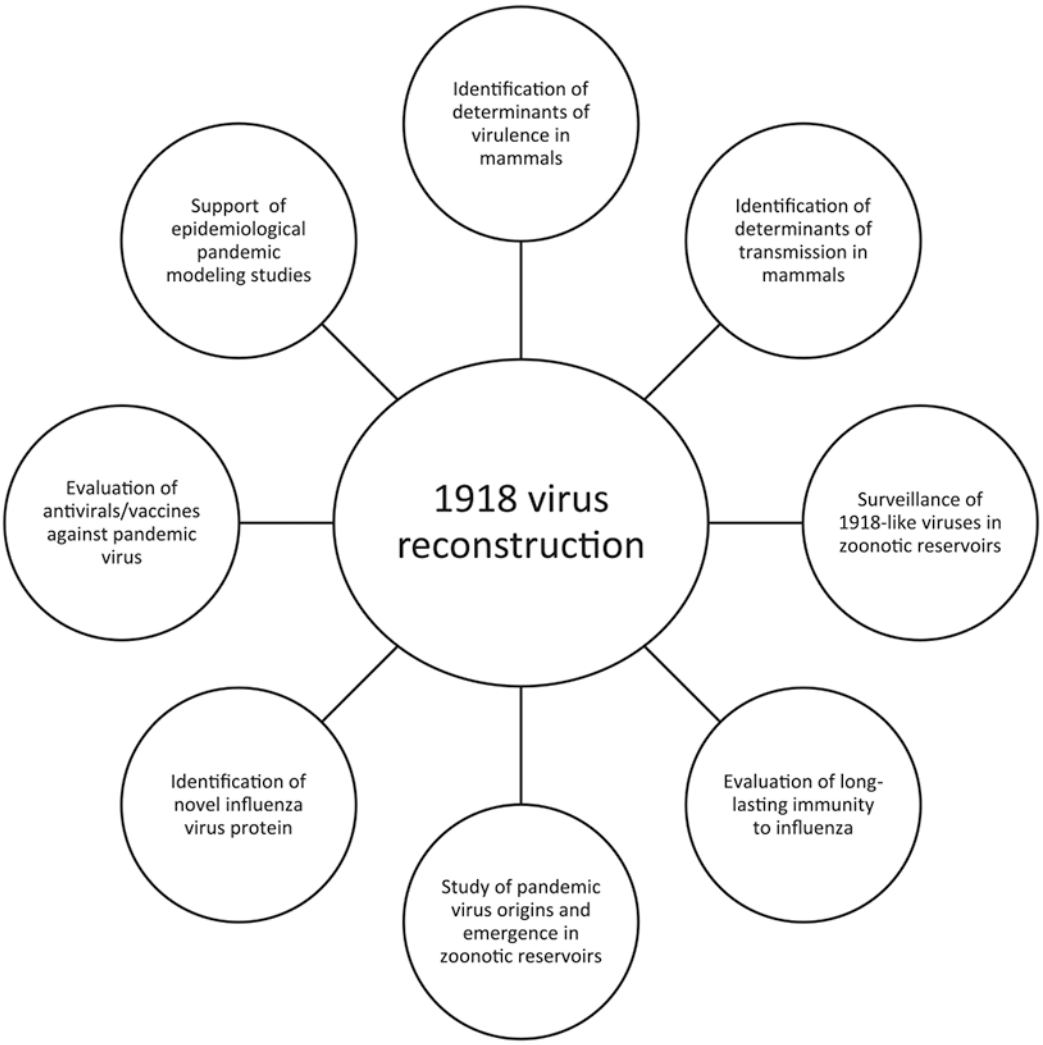

Fig. 1.

Selected benefits of 1918 virus reconstruction on contemporary public health pandemic preparedness. Individual studies supporting these and other roles of 1918 virus reconstruction are discussed throughout the text and may be found in (Buhnerkempe et al., 2015; Giles et al., 2012; Jagger et al., 2012; Pearce et al., 2012a; Tumpey and Belser, 2009; Watanabe et al., 2014; Worobey et al., 2014).

Table 1

Contribution of receptor binding to 1918 virus transmission in ferrets.

Virus[a]	Description	190[b]	225[b]	α2-6[c]	α2-3[c]	Transmission[d]
Tx/91	Seasonal H1N1 virus	D	D	Yes	No	3/3
SC/18	Reconstructed 1918 virus	D	D	Yes	No	3/3
NY/18	Natural variant 1918 virus	D	G	Yes	Yes	2/3[e]
AV/18	"avianized" 1918 virus[f]	E	G	No	Yes	0/3
Dk/NY	Avian H1N1	E	G	No	Yes	0/3
Dk/NY-DD	Avian H1N1 with HA mut	D	D	Yes	No	0/3

[a] Tx/91, A/Texas/36/1991; SC/18, A/South Carolina/1/1918; NY/18, A/New York/1/1918; AV/18, NY/18 virus with D190E mutation in HA; Dk/NY, A/duck/New York/15024-21/1996; Dk/NY-DD, Dk/NY virus with E190D and G225D mutation in HA.

[b] Amino acid position (H3 numbering). D, aspartic acid; G, glycine; E, glutamic acid.

[c] Presence or absence of hemagglutination using resialylated red blood cells. Data previously published in (Glaser et al., 2005; Tumpey et al., 2007; Van Hoeven et al., 2009b).

[d] Respiratory droplet transmissibility of reassortant viruses in the ferret model (number of contact ferrets with detectable virus in nasal wash specimens and seroconversion to homologous virus/total number of contact ferrets unless specified otherwise).

[e] One ferret represented in numerator seroconverted to homologous virus in the absence of virus detection.

[f] "avianized", variant SC/18 virus with two amino acid changes in the HA to change receptor binding specificity from *a*2-6 to *a*2-3.

Table 2

Molecular determinants of 1918 virus transmission in ferrets.

Gene segments from 1918 virus[a]	Transmission[b]	Conclusion
All	3/3	Efficient transmission of fully reconstructed 1918 virus
None	0/3	No transmission of avian H1N1 virus
HA	0/3	1918 surface glycoproteins not sufficient for transmission
HA, NA	0/3	
HA, NA, pol complex (PA, PB1, PB2)	3/3	Role for internals
HA, NA, PA	0/3	Role for PB2 and HA to confer efficient transmission
HA, NA, PB1	0/3	
HA, NA, PB2	3/3	
HA, PB2	3/3	
All but PB2	0/3	PB2 necessary but not sufficient for transmission
PB2	1/3	
All, PB2 K627E mutation	1/5	Role for 627K mutation

[a] Recombinant viruses bearing gene segments from the reconstructed 1918 virus on background of avian H1N1 virus (A/duck/New York/15024–21/1996).

[b] Respiratory droplet transmissibility of recombinant viruses in the ferret model (number of contact ferrets with detectable virus in nasal wash specimens and seroconversion to homologous virus/total number of contact ferrets). Data previously published in (Belser et al., 2010; Tumpey et al., 2007; Van Hoeven et al., 2009b).