Understanding Influenza

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Abstract

Influenza, a serious illness of humans and domesticated animals, has been studied intensively for many years. It therefore provides an example of how much we can learn from detailed studies of an infectious disease, and of how even the most intensive scientific research leaves further questions to answer. This introduction is written for researchers who have become interested in one of these unanswered questions, but who may not have previously worked on influenza. To investigate these questions researchers must not only have a firm grasp of relevant methods and protocols, they must also be familiar with the basic details of our current understanding of influenza. This article therefore briefly covers the burden of disease that has driven influenza research, summarises how our thinking about influenza has evolved over time, and sets out key features of influenza viruses by discussing how we classify them and what we understand of their replication. It does not aim to be comprehensive, as any researcher will read deeply into the specific areas that have grasped their interest. Instead, it aims to provide a general summary of how we came to think about influenza in the way we do now, in the hope that the reader's research will help us to understand it better.

Keywords

Influenza, Introduction, History, Taxonomy, Replication Cycle

1 The Need for Methods and Protocols

Methods and protocols are tools for asking questions, not ends in their own right. A book like this should only exist – and can only be worth your attention – if you have a need to apply those tools. For influenza viruses, as with most pathogens, the need to keep on asking questions comes from three sources.

Firstly, there is your own interest. Influenza viruses, like anything else when observed carefully enough, are fascinating and, in their own way, beautiful. If you are reading this book at all, it is likely that you are grappling with at least one unknown aspect of influenza virus biology, a puzzle that drives you into work and stays with you when you leave. Currently no-one knows what is going on there, and you want to find out. Hopefully the contents of this book may help you with this.

Secondly, there is the wider relevance of solving any problem in biology. Science is only worthwhile because we find ways to take our isolated findings, made in a small number of systems on a small number of occasions, and use them to say something that is generally applicable. In biology this is particularly true when studying molecular parasites such as viruses [1], as their replication and transmission require the exploitation of features of the host that we might otherwise fail to notice. In the case of influenza, understanding the biology of the virus has helped us to understand aspects of the biology of the host including the identification of interferons [2,3], the concept of original antigenic sin [4,5], details of nuclear import [6] and glycan distribution in the respiratory tract [7]. In addition, our accumulated understanding of influenza means that the virus and its derivatives have provided a wealth of tools for studying other biological problems, from influenza-specific tetramers as a tool to analyse immune responses and immunological memory [8,9], to the widely-used HA epitope tag for protein detection and biochemical purifications [10]. Even if influenza itself does not capture your attention, it can be the key to the problem that does.

There is of course a third reason why we need methods and protocols for understanding influenza viruses, and why governments and charitable bodies are often prepared to fund our work. Influenza kills and sickens vast numbers of people and domesticated animals, and has been doing so for hundreds of years.

2 The Need to Understand Influenza

You will be familiar with this. Not only because of your interest in influenza viruses, but because it is very likely that influenza has already infected you at least once. The normal course of an influenza infection in a healthy adult is not pleasant, but nor is it particularly dramatic. The symptoms that you probably experienced – including sudden onset of fever, muscle pain, headaches and exhaustion – were for the most part collateral damage caused by your own immune response, and experienced only once the replication and shedding of the virus had been brought under control [11]. After an unpleasant week, you most likely made a full recovery.

Despite its uncomplicated normal course, two points in particular make human influenza more than an inconvenience. Firstly, influenza is an extremely common disease (Fig. 1A), and the cumulative economic effects of influenza-related absenteeism, to say nothing of the cumulative unhappiness it causes, are considerable. Secondly and more troublingly, influenza causes serious illness in small proportion of cases. This can be a direct effect of viral pneumonia but is often through indirect

effects, particularly secondary bacterial infections and the exacerbation of underlying cardiovascular and respiratory illness (Fig. 1B). Influenza can and does cause serious illness in previously healthy individuals, but it is most likely to cause problems in at-risk groups – infants, older adults, the immunocompromised and the pregnant (Fig. 1C). Among these groups, particularly older adults, influenza-related deaths are a major cause of excess winter mortality (Fig. 1D).

Influenza in this form is an ongoing public health issue, causing seasonal epidemics each winter in temperate countries and with a more complex pattern in the tropics [12]. A more unpredictable threat arises from influenza pandemics, which occur every few decades when a new variant of the virus adapts to transmit efficiently between humans (Fig. 1E). Such pandemics are unpredictable not just in their timing but in their severity. The pandemic of 2009 caused an extra influenza season, resulting in hundreds of thousands of excess deaths globally, but on a case-by-case basis it was no more severe than seasonal influenza [13]. In contrast, the 'Great Influenza' of 1918 (Fig. 1E) initially caused such severe disease that within 18 months it had killed around one in thirty of the global population [14]. The prospect of another pandemic of this magnitude is a cause of great concern, as noted in a recent assessment by the UK government which ranked pandemic influenza as the most likely civil emergency risk in its most severe impact category [15].

For all this, influenza is not primarily a disease of humans, and its effects on domesticated animals are also severe. Epizootic (animal) outbreaks of influenza were recorded for as long as human epidemics, though changes in the ways we use animals have changed the impacts of epizootic influenza [16]. Equine influenza, which caused major infrastructure problems when horses were widely used working animals [16], is now a notable problem for high-value horses in the racing industry [17]. Swine influenza has been an ongoing problem since at least 1918, though it is unclear whether it caused swine epizootics before this [17,18]. The historical incidence of influenza in poultry is unclear, but in recent decades frequent outbreaks have been reported, including in high-density farms and live bird markets which allow for the rapid spread of the virus [16]. Troublingly, a number of recent avian influenza strains, notably of the H5N1 and H7N9 subtypes, have been capable of causing severe disease in humans.

For all these reasons, there has long been a pressing need to understand influenza, and to turn every method at our disposal to that task.

3 Failing to Understand Influenza

The problem of influenza has been both urgent and readily identifiable for an extremely long time [16]. As such, our attempts to understand influenza mirror developments in how we think about disease. As with all attempts to understand natural phenomena, our thinking about influenza is a progression of flawed and incomplete ideas. Few of these are without any value but none of them, including any of our current ideas, are likely to be completely correct.

Initially, influenza was described in rather general terms as outbreaks of an acute febrile respiratory illness with high incidence in all groups of society but little mortality except among the infirm [19,16]. It was recognised that the disease appeared sporadically and it gained its name from the correct assumption that this was due to the action of some external factor – an 'influence' or, in Italian, an '*influenza*.' (The French term '*la grippe*,' from *gripper*, 'to seize,' captures instead the experience of the symptoms.) The alignment of the stars was sometimes blamed – and although we do not now think that this is causative, influenza is due to an external cause and there is truth in the

idea that influenza is strongly seasonal. A noxious change in the environment was also suspected – and while influenza is not caused directly by a change in the quality of the air, its transmission is indeed influenced by temperature and humidity [20,21]. Despite difficulties in clearly distinguishing influenza from other diseases, it was recognised that animals suffered from a similar illness and there were suggestions that human and animal outbreaks might follow each other closely in time [16,22].

During the nineteenth century, the germ theory of disease provided a radical new explanation for how the physical world could influence disease – through the action of invisibly small living beings. Epidemiological studies provided strong evidence that influenza was caused by the transmission of an infectious agent rather than by the emergence of harmful miasmas [23]. During this time, technical improvements in medical diagnosis allowed influenza to be discussed in terms that are more familiar to modern readers, though there was a stronger emphasis on the psychological and neurological effects of the disease, with great popular and medical interest in reports of psychosis, suicide and depression following attacks of influenza [24].

Following the enormous successes of nineteenth century microbiologists, it was quite reasonably assumed that influenza was caused by a bacterium. In 1892 a causative bacterium was even identified by Richard Pfieffer – *Bacillus influenzae* (or 'Pfieffer's bacillus,' now *Haemophilus influenzae*) [22]. Despite subsequent work showing that this is not the primary cause of influenza, this finding is still important. A retrospective study of lung tissue sections suggests that severe secondary bacterial pneumonia was a consistent feature of fatal cases in the 1918 influenza pandemic [25], and even in today, during seasonal influenza outbreaks, secondary bacterial infections are a major cause of influenza-related illness. The interactions between influenza viruses and co-infecting bacteria are now an area of active research [26].

At the end of the nineteenth century a new idea about microbes began to gain favour – that certain infectious diseases were caused not by bacteria but by something which could pass through the finest of filters – perhaps a contagious living fluid or, as the idea developed, some exceptionally small particulate material [27,22]. This became the modern concept of a virus (from the Latin *virus*, or 'poison'). Among the earliest animal diseases to be associated with a virus was fowl plague, which was shown to be caused by a filterable agent as early as 1901, though it was only identified as a form of avian influenza virus some fifty years later [22]. In 1931 swine influenza was shown to be caused by a virus [28], a finding which prompted the identification of human influenza as a viral disease in 1933 [29,30].

Once identified, the influenza viruses proved to be particularly easy to propagate. At first this was done in experimental animals such as ferrets, chosen because of superficial similarities between the symptoms of influenza and canine distemper, which was known to pass to ferrets from dogs. Small rodents such as mice that could be easily reared for laboratory work were also popular experimental hosts, as were embryonated chicken eggs, which had long used by physiologists and embryologists as they could be opened to examine the living embryo [31]. Once tissue culture systems were developed, it was found that influenza viruses thrived in them. Particular use has been made of Madin Darby Canine Kidney (MDCK) cells, a cocker spaniel carcinoma cell line which, despite bearing no relation to the natural site of infection, proved particularly permissive to the replication of a wide range of influenza viruses [32-34].

The isolation and propagation of the primary causative agent of human influenza in 1933 laid the groundwork for eighty-five years of intensive study. It accelerated our efforts to understand

influenza, giving us a description of the disease which is richly detailed – though still, inevitably, rich in unanswered questions.

4 A Basic Understanding of Influenza

4.1 Classification of Influenza Viruses

4.1.1 Family

Improvements in understanding influenza have allowed us to classify influenza viruses. Doing so captures a great deal of our current understanding of their evolution, ecology and host interactions.

The disease influenza is caused by members of the orthomyxovirus family. As well as four genera of influenza viruses, named A – D (Influenza A virus in the genus *Alphainfluenzavirus*, Influenza B virus in the genus *Betainfluenzavirus*, etc.),, the *Orthomyxoviridae* also includes the *Thogotovirus, Isavirus* and *Quaranjavirus* genera [35]. Originally, influenza viruses were assigned to the now-defunct myxovirus family of negative-sense RNA viruses, named from *myxa*, Greek for 'mucus,' due to the ability of the virions to bind to and degrade mucins [36,37], the same property that allowed their detection through a haemagglutination assay [38]. However, this family was divided in two when it was discovered that influenza viruses had marked structural, biochemical and genetic differences from the other known myxoviruses [39]. Many of these differences can now be attributed to the fact that orthomyxoviruses such as influenza viruses have a segmented genome (eight segments for the influenza A and B viruses, seven for the influenza C and D viruses) which replicates in the nucleus. In contrast, the genomes of the remaining 'paramyxoviruses' (such as mumps virus, measles virus and the human parainfluenzaviruses) are formed from a single molecule of RNA and replicate in the cytoplasm.

4.1.2 Types

The four known genera (or 'types') of influenza viruses are genetically isolated from each other (Fig. 2), and have accumulated substantial differences which are reflected in the antigenic properties of their nucleoproteins (NP) and matrix proteins (M1) [40,35]. The influenza A viruses are the ancestral genus and propagate for the most part in waterfowl, particularly those in the orders Anseriformes (including ducks and swans) and Charadriiformes (including waders and gulls) [16,41]. Influenza A viruses are extremely common in these birds, in which they often cause enteric infections with no apparent disease. Waterfowl can excrete considerable quantities of virus into mud and water, in which it can remain stable for some time [42,41]. Recently, a divergent group of influenza A viruses was discovered in New World leaf-nosed bats, apparently also causing enteric infections [43,44].

The avian influenza A viruses have an unusual facility for cross species transmission, and have been found circulating in a wide range of warm-blooded vertebrate hosts (Fig. 2). Cross-species transmission of the viruses appears to be strongly encouraged by the domestication of animals. Influenza A viruses have been found in domesticated birds, notably chickens, turkeys, ducks, and quail, and mammals, notably pigs and horses [16,41]. It is presumably through these routes that influenza A viruses first infected humans, and influenza pandemics continue to result from cross-

species transmission events involving farmed animals [41]. After emerging as a pandemic, strains of influenza A virus continue circulating in human populations for decades as seasonal epidemics (Fig. 1E).

Some of these epidemic influenza viruses circulated in human populations for long enough to evolve into distinct genera (Fig. 2). The influenza B viruses cause annual seasonal human epidemics as well as occasionally infecting seals. The influenza C viruses have diverged still further from the influenza A viruses (Fig. 2). They infect humans and pigs, and while quite common in humans they typically do not cause severe disease [45]. Recently a novel influenza C-like virus was detected circulating in cattle [46]. These viruses were sufficiently different from other influenza C viruses to assign them to a new genus, the influenza D viruses [35]. Influenza D viruses appear to cause only mild disease in cattle [47], and while many humans are seropositive for the viruses it is not known if this is due to infection or merely to exposure [48].

4.1.3 Subtypes

Each influenza genus (or type) contains one only species, but they are further differentiated into subtypes and lineages. While these distinctions were originally immunological observations, they are best understood in terms of the molecular anatomy of the influenza virion, which in the case of influenza A virions is assembled from ten viral proteins, eight segments of viral RNA (vRNA), and proteins and lipids derived from the host cell (Fig. 3A) [49].

Importantly for understanding subtypes, the virion is bounded by a dense fringe of viral glycoproteins. For influenza A and B viruses these are haemagglutinin (in UK English; in US English 'hemagglutinin;' HA), a trimer named for its ability to agglutinate red blood cells in an experimental setting, and neuraminidase (NA), a tetramer named for its enzymatic activity. Influenza C and D viruses have a single haemagglutinin-esterase-fusion protein (HEF) that fulfils the functions of both HA and NA. For all influenza viruses, these 'spike' glycoproteins are abundant on the surface of the virion, are required for viral entry and exit, and extend further out from the surface than other, less abundant viral transmembrane proteins (the ion channel M2 and its orthologues in all genera, and the NB protein of the influenza B viruses). For these reasons, spike glycoproteins are the major targets for neutralising antibodies and the major focus of interest when designing vaccines.

Selection to avoid adaptive immunity causes the spike glycoproteins to undergo rapid diversification. This 'antigenic drift' reduces the effectiveness of antibodies raised in response to a previous exposure or vaccination, necessitating frequent reformulation of influenza vaccines. Over time, the HA and NA genes of the influenza A viruses have diversified enough to form multiple antigenically distinct groups or 'subtypes,' each containing a wide range of variants (HA subtypes are shown in Fig. 2). Antibodies against the glycoproteins in one subtype offer little if any protection against viruses of another subtype. Due to their importance in understanding antiviral immunity, the HA and NA subtypes are recorded in an abbreviated form when naming an influenza A virus, for example H1N1, H3N2 or H5N1. Currently circulating seasonal influenza A viruses are of the H1N1 or H3N2 subtype, and both subtypes are included in vaccines.

4.1.4 Lineages

While no other influenza virus genes have diversified to the same degree as the HA and NA genes of influenza A virus, all genes of influenza viruses can rapidly acquire mutations and many have evolved into distinct lineages. Of particular note is the influenza B virus HA gene, which separated into two distinct co-circulating lineages in the early 1980s [50-52]. Due to the importance of HA as an antigen the divergence of these lineages, referred to as Victoria and Yamagata after the reference strains B/Victoria/2/87 and B/Yamagata/16/88, has had an impact on the design of seasonal influenza vaccines. At the time of writing, there is an increasing tendency to move from trivalent vaccines (H1N1, H3N2 and a single B) to tetravalent vaccines (H1N1, H3N2 and both lineages of B).

4.1.5 Reassortment

Due to the segmented nature of the influenza genome, coinfection of a cell with two or more influenza viruses can create reassortant progeny carrying a mixture of genome segments from more than one parental virus. It is becoming increasingly clear that reassortment between closely-related variants of the virus plays a major role in the normal replication of influenza viruses within a single host, allowing virions carrying only a partial set of functional genome segments to contribute to an infection through complementation [53].

Reassortment of gene segments between genera does not appear to be possible. However, if a cell is coinfected by two influenza A viruses, one adapted to humans and the other from a non-human host, progeny can arise which acquire most of their genes from the human-adapted virus but carry the glycoprotein genes of a subtype not currently circulating in humans. This 'antigenic shift' creates a novel influenza A virus which is well-adapted to human growth but to which humans have no prior immunity. Most influenza pandemics appear to arise in this way [41].

Reassortment is also used in vaccine development, where HA and NA genes (and sometimes the PB1 gene) from recent clinical isolates are combined with the remaining viral genes from a 'backbone' strain adapted for high growth in chicken eggs and low pathogenicity in humans [54,55].

4.1.6 Naming Conventions

The classification of an influenza virus strain is encoded in its full name, in the form:

[*GENUS*] / [*HOST OF ORIGIN*] / [*GEOGRAPHICAL ORIGIN*] / [*SEQUENTIAL NUMBER OF ISOLATION*] / [*YEAR OF ISOLATION, either as two or four digits*] ([*SUBTYPE, IF AN INFLUENZA A VIRUS*])

Some conventions apply to the naming of hosts [40]. If the host was a human, the host description is omitted. If the host was an animal, its common name is given, lowercase, rather than the Latin binomial name (e.g. 'duck'). Certain names have gained prevalence in the literature, for example 'equine' for isolates from horses, 'swine' for isolates from pigs and 'bovine' for isolates from cattle. Finally, if the virus was isolated from non-living material this is specified instead of the host (e.g. 'lake water').

For example:

- A/duck/Guangxi/53/2002 (H5N1) is the fifty-third influenza A virus strain isolated from ducks in Guangxi in 2002 and is of the H5N1 subtype.

- B/Florida/04/2006 is the fourth influenza B virus strain isolated from a human in Florida in 2006.
- C/Paris/1/67 is the first influenza C virus isolated from a human in Paris in 1967.
- D/bovine/Mississippi/C00046N/2014 is an influenza D virus isolated from a cow in Mississippi in 2014. In this case, a more complex sequential numbering system has been used by the reporting laboratory.

Abbreviations are sometimes used in strain names, particularly after the first use, for example A/BEL for A/Bellamy/1942(H1N1) or A/HK/156/97 for A/Hong Kong/156/97(H5N1). In addition, some widely studied strains have common abbreviations, notably:

- PR8: A/Puerto Rico/8/1934(H1N1); a number of variants of this extensively studied strain exist, with somewhat different properties [56].
- Udorn: A/Udorn/307/72(H3N2). A laboratory-adapted strain that, unusually, retains the filamentous virion morphology of clinical isolates (Fig. 3B).
- WSN: A/WSN/33. A virus isolated in 1933 by (W)ilson (S)mith and colleagues which subsequently underwent intracranial mouse passage to become (N)eurotropic [57]. Unlike most low-pathogenicity strains of influenza virus, WSN does not require the addition of trypsin to cleave and activate HA, as it has acquired mutations in NA that can bind plasminogen and in HA that allow its plasmin-mediated cleavage [58-61].
- X-31: PR8 with the HA and NA genes from A/Aichi/2/68(H3N2). The first example of developing an egg-adapted influenza vaccine candidate by reassortment, as well as a commonly used strain for laboratory study [62,55].

4.2 Replication of Influenza Viruses

4.2.1 Entry

Despite their differences, all of the influenza viruses have a similar replication cycle (Fig. 4; reviewed in [63]). In mammals, influenza virus is typically acquired via respiratory droplets or contact with fomites, after which it infects the respiratory epithelia. In waterfowl, the virus replicates in the intestinal epithelia and can be acquired by contact with faeces [64]. In all cases, to establish an infection an influenza virion must diffuse through the mucus layer which protects epithelial cells and then bind to a receptor. In most cases this is sialic acid, a negatively charged monosaccharide, chiefly *N*-acetylneuraminic acid, present at the terminal position of glycans on glycoproteins and glycolipids at the apical cell surface. An exception to this is the highly divergent bat influenza A viruses, which do not recognise sialic acid and whose receptor has yet to be identified [65].

Binding is the first step in entry, which encompasses virus binding up to import of the viral genome into the nucleus. There are multiple forms of sialic acids and different influenza viruses have different binding preferences. Influenza A and B virions bind to *N*-acetylneuraminic acid through their HA proteins, while influenza C and D virions bind to *N*-acetyl-9-*O*-acetylneuraminic acid through their HEF [45]. Sialic acids in the human respiratory tract are predominantly linked to glycans through an α2,6 linkage, whereas in the avian intestine sialic acids are predominantly α2,3 linked, and the binding preferences of human and avian influenza viruses reflect this [7,66,67]. Pigs present abundant sialic acids with both α2,3 and α2,6 linkages, making them potentially susceptible to infection with viruses from a wide range of hosts [67].

Binding triggers receptor-mediated endocytosis of the virion, allowing it to enter early endosomes [68]. As endosomes mature their pH falls and the concentration of potassium ions rises [69], triggering two changes in the virions.

Firstly, an influx of hydrogen and potassium ions through the viral ion channel M2 (Fig. 3A) weakens the interactions between the matrix protein M1 and the ribonucleoproteins (RNPs) which encapsidate the viral genome [70]. The importance of this process meant that M2 was the target for the first generation of influenza antiviral drugs, the adamantine-derived ion channel inhibitors (amantadine and rimantadine). Unfortunately, the evolution of resistance mutations means these drugs are now essentially obsolete [71,72].

Secondly, the decreasing pH of the endosome causes protonation of the stem of HA, destabilising its tertiary structure. This leads to an irreversible conformational change, releasing a hydrophobic fusion peptide that inserts into the endosomal membrane. The HA stem then folds back on itself, drawing the viral and endosomal membranes together and bringing about membrane fusion [73]. Membrane fusion is followed by matrix disassembly which, aided by host mechanisms, allows the release of the viral genome into the cytoplasm [74,75,69,76,77]. There, it binds to cellular nuclear import factors and gains entry to the nucleus [78].

4.2.2 Transcription and Replication

Influenza viruses have a negative sense RNA genome (vRNA), which is not a substrate for cellular transcription or translation. They must therefore use a viral polymerase for primary transcription of the incoming genome, as well as all subsequent transcription and replication of the genome [79]. The viral polymerase, a trimer of three subunits (Fig. 3A), is therefore an attractive target for antiviral drugs, though at the time of writing none have been licensed for routine use. Transcription of the viral genome relies on 'cap snatching,' in which a short capped sequence is cleaved from a host mRNA and used to prime transcription of a segment of the viral genome. Polyadenylation of viral mRNA occurs through stuttering on a short polyuridine tract near the 5' end of each segment of vRNA. As noted above orthomyxoviruses transcribe their genomes into the nucleus, an unusual strategy among the RNA viruses, and as a result influenza virus mRNA can undergo splicing.

Viral mRNAs are translated by host ribosomes, and polymerase and NP proteins are then imported back into the nucleus to encapsidate full-length uncapped transcripts of the viral genome into new RNPs (Fig. 3A). This allows replication of the viral genome – initially into positive sense complementary genomes (cRNA) and then, following a second round of replication, into new negative-sense (vRNA) genomes. Newly-synthesised RNPs interact with M1 and with the viral Nuclear Export Protein (NEP; formerly known as NS2), which allows them to be exported from the nucleus through interactions with the nuclear export factor Crm1 (XPO1) [78].

In addition to priming transcription, cap-snatching depletes mRNA transcripts of host genes and so contributes to host shut-off. This is one of the large number of mechanisms, many mediated by the multifunctional NS1 protein, which contribute to the virus' control of the host cell's immune response (reviewed in [80,81]). While well-adapted influenza viruses are normally highly effective at limiting the host immune response, some highly pathogenic strains of the virus can instead trigger excessive immune activation, causing a pro-inflammatory 'cytokine storm' which can be fatal [82,83].

4.2.3 Assembly and Egress

Once RNPs have entered the cytoplasm they interact with Rab11-positive membranes. Rab11 is a regulator of exocytic and recycling processes, and this interaction transports the RNPs to the apical plasma membrane of the cell [84-89]. The trans-Golgi network transports viral transmembrane proteins to the same site. At the cell surface, the transmembrane proteins, RNPs, M1, and NEP assemble into virions, also taking up host proteins and NS1, which is highly abundant in the cytoplasm [49].

Newly formed virions emerge with a dense fringe of HA or HEF, which tethers them to sialic acid on the cell surface. Like many viruses, influenza viruses therefore have receptor-cleaving activity, catalysed by the NA enzyme for influenza A and B viruses and the esterase function of HEF for the influenza C and D viruses. Sialic-acid analogues, notably oseltamivir (Tamiflu) and zanamivir (Relenza), act as neuraminidase inhibitors and are the main class of drug currently licensed to treat influenza infections. Without NA inhibition or antibody binding, newly formed virions are shed into the mucus, to spread within and between hosts.

5 Improving Our Understanding of Influenza

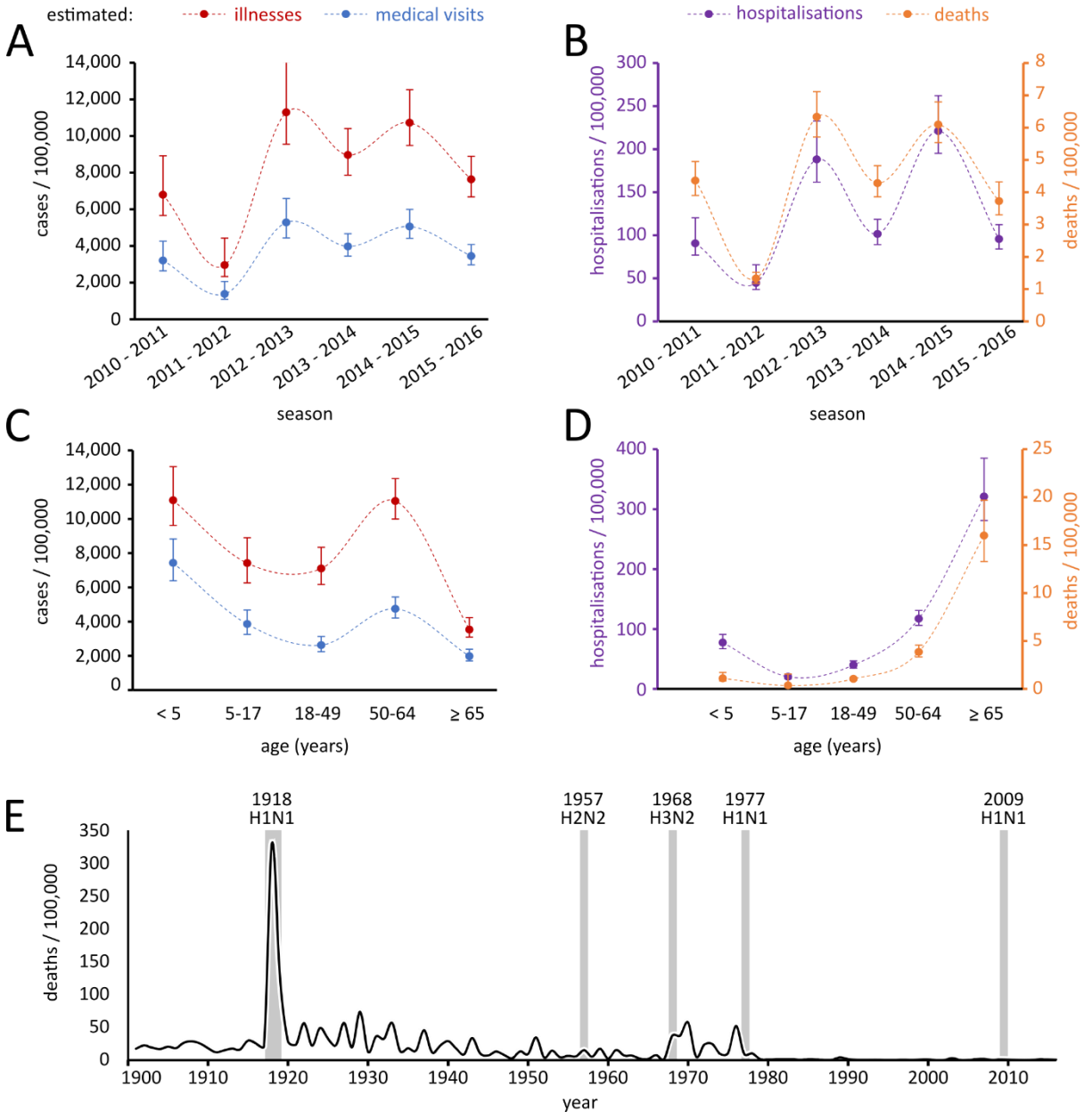
Despite all of these developments in understanding influenza, there remain many unanswered questions, and new discoveries reshape our understanding of the virus on a regular basis. To take just two examples, at the time of writing the structure of the viral polymerase [90-93] and the existence of the influenza D genus [46] are both very recent findings.

Other questions have remained unanswered for decades. These range from questions of basic biology – such as why clinical isolates of influenza form virions with a variety of morphologies, including highly elongated filaments (Fig. 3B; reviewed in [94]) – to fundamental questions about which factors determine viral transmission. More practical questions also remain unanswered. What affects the likelihood of a new pandemic strain emerging, and can effective pre-pandemic monitoring forestall this? Could new classes of antiviral drugs avoid the problems of drug resistance? Can vaccines be produced in a timely fashion and be well-matched to both seasonal and pandemic strains of the virus, and is long-lasting immunity through a universal vaccine possible?

This is by no means a comprehensive list, and it is not intended to be one. As a reader of this book, you will hopefully be particularly interested in improving our understanding of some particular aspect of influenza. To answer questions that have not been answered after eighty-five years of intensive research you will require tools – both well-established resources, and the latest cutting-edge developments that have not previously been applied to your problem. You will need to know what can be done and, in order to go out and do it yourself, you will need protocols that are clearly written and do not leave you to reinvent every minor detail for yourself. Hopefully, the methods and protocols described in this book will help you with this.

Figures

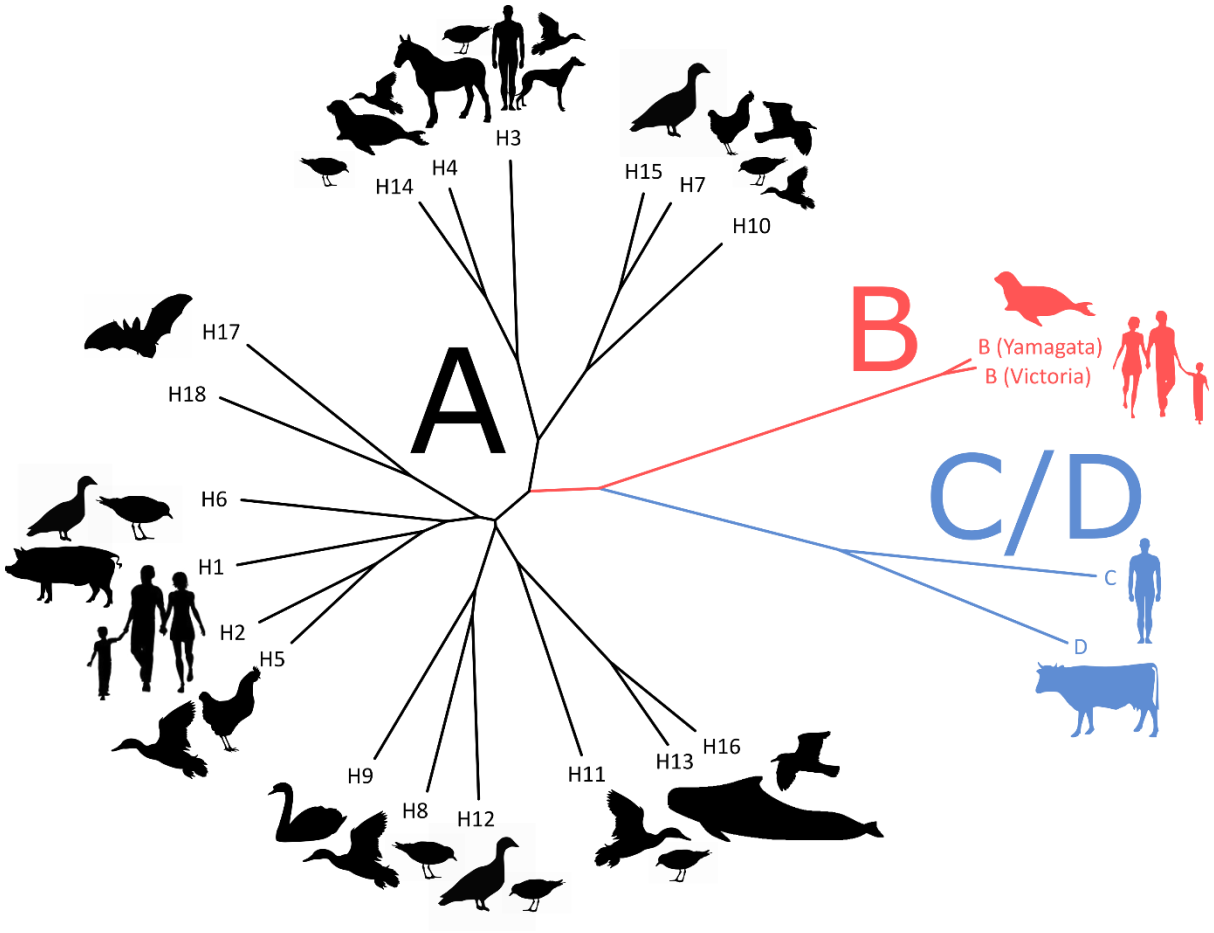
Figure 1: The Burden of Human Influenza



Influenza causes widespread illness, and severe disease in a small proportion of those cases. Data from the USA show (A,B) recent estimates of the burden of influenza and (C,D) the burden of influenza for different age groups in the 2015-2016 influenza season. These figures compare estimates of cases, made by the US Centers for Disease Control, to the total resident population size, using data from the US Census Bureau [95]. Figures for deaths refer only to deaths caused directly by pneumonia and influenza deaths, and do not include deaths from cardiovascular or respiratory complications, or cases where influenza was not tested for. The true number of influenza-related deaths is estimated to be 2-4 fold higher [95]. Estimates are shown with 95 % confidence intervals or, for the deaths in (D), 95 % credible intervals. (E) Historical deaths from influenza in the UK, calculated from the causes of death recorded in death certificates and adjusted for total population

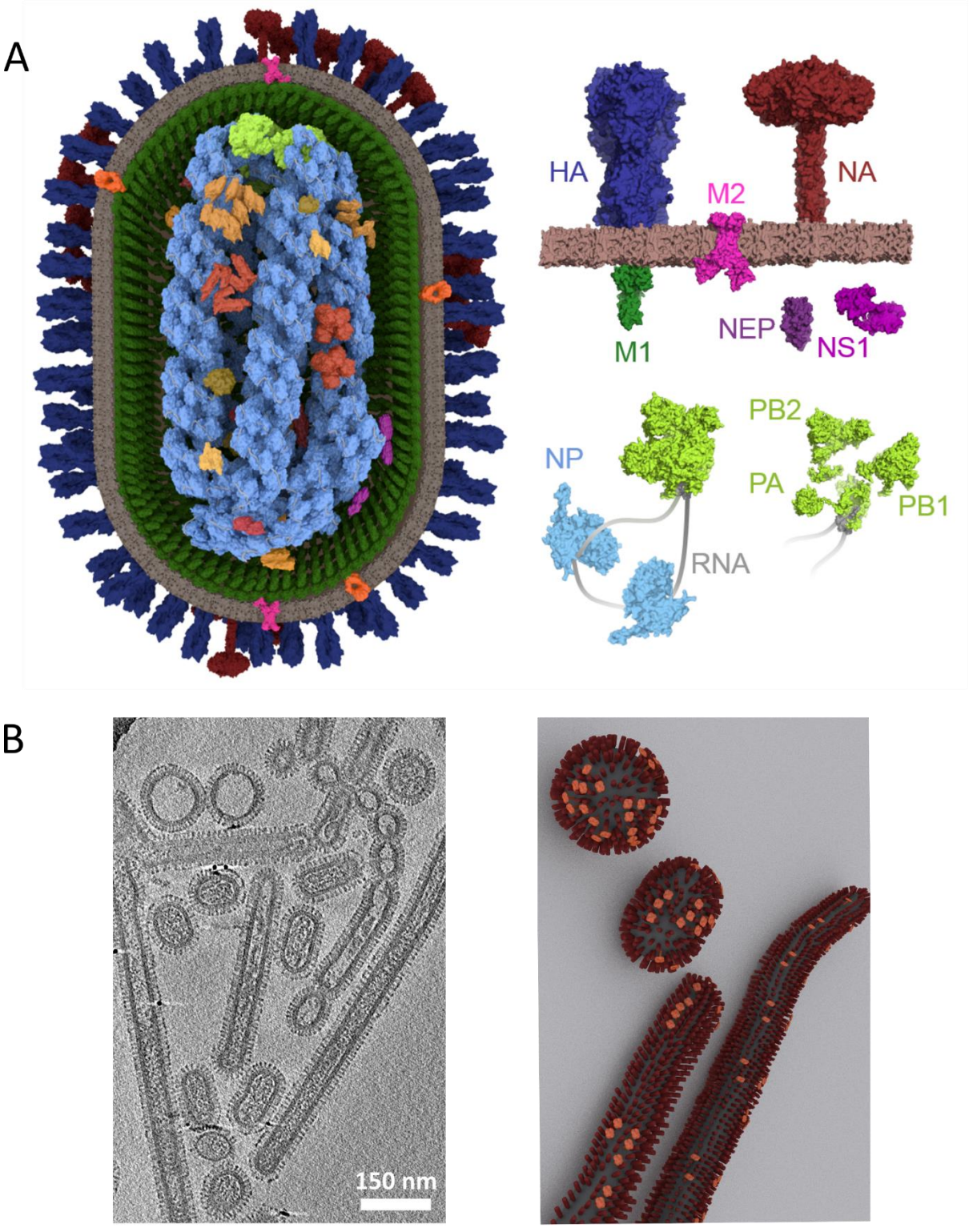
size (data from the UK Office for National Statistics [96-98]). This will underestimate the true number of influenza-related deaths for similar reasons to (B,D). Influenza A virus pandemics and the subtype they introduced into the population are indicated with shaded bars; note the peak for the ‘Great Influenza’ of 1918.

Figure 2: Taxonomy and Ecology of Influenza



A phylogeny of influenza viruses, constructed by neighbour-joining from a multiple sequence alignment of a full length HA sequence randomly selected for each influenza A virus subtype, for the Yamagata and Victoria lineages of influenza B virus and for a full-length HEF sequence of influenza C virus and influenza D virus. Silhouettes of representative hosts are indicated around the phylogeny.

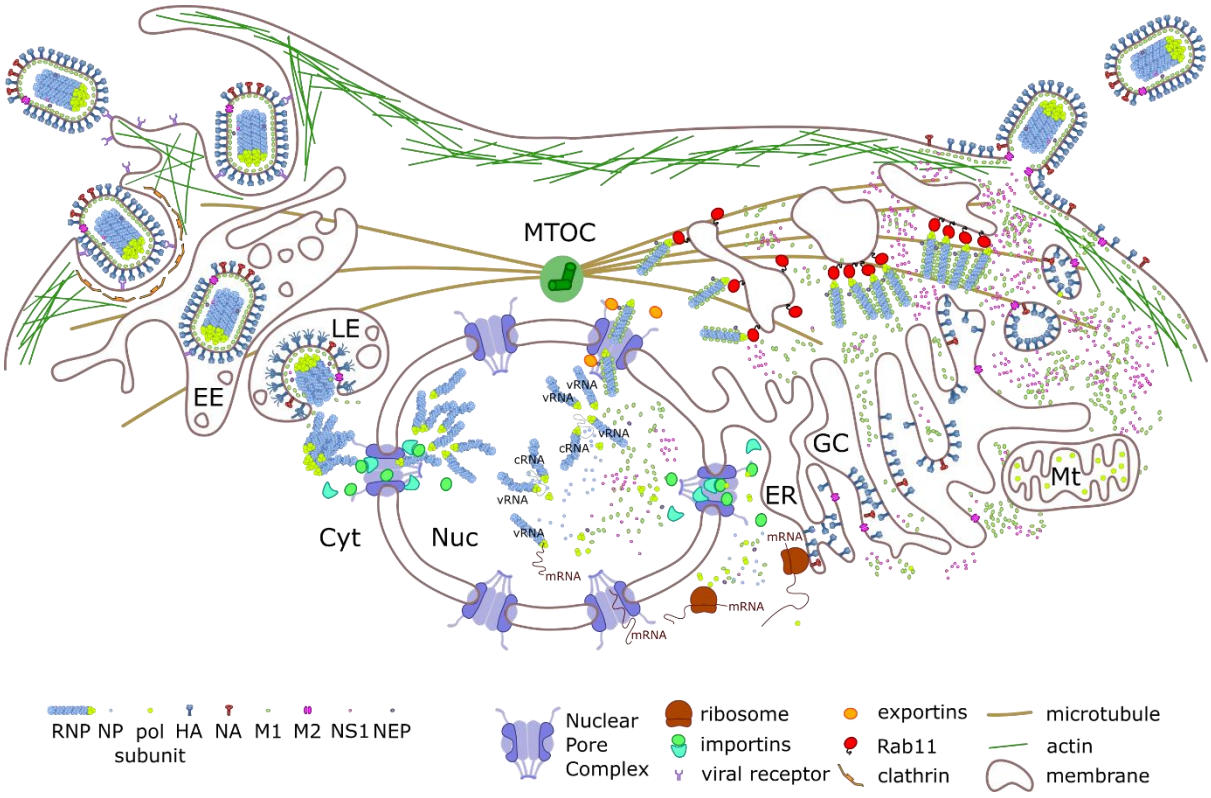
Figure 3: Molecular Anatomy of an Influenza Virion



(A) The structure of an influenza A virion, modelled using data from ref [49]. The viral proteins are shown individually on the right hand side of the image, along with membrane and RNA. In addition, the virion contains numerous host proteins, which are coloured in different shades of orange. (B) Cryoelectron tomogram showing unfixed virions of the pleomorphic Udorn strain [99], and an artistic representation of three classes of virion morphology: spherical, 'bacilliform' and filamentous. While

the full range of morphologies can be observed in clinical isolates, laboratory-adapted strains of influenza typically form only spherical and bacilliform virions. (Virion images provided by Naina Nair, under a Creative Commons Attribution 4.0 International License, 2017.)

Figure 4: The Influenza Virus Replication Cycle



Schematic overview of the influenza virus replication cycle, with viral proteins coloured as in Figure 3A. A virion enters the cell via the apical plasma membrane at top left and new virions emerge from the apical plasma membrane at top. The cell is given structure by actin filaments and microtubules and its surface carries sialic acid-bearing proteins and lipids that act as viral receptors. Endocytosis is mediated by clathrin or by macropinocytosis, after which the virus travels from early to late endosomes where it fuses, uncoats, and vRNPs penetrate into the cytosol. Transport of RNPs through nuclear pore complexes is mediated by importins and the exportin Crm1 (XPO1), and microtubule-dependent membrane traffic for RNP export is facilitated by Rab11. Proteins are translated from mRNA by ribosomes. While some are imported into the nucleus others are inserted into membranes at the endoplasmic reticulum, diffuse through the cytoplasm or, in the case of the polymerase subunit PB2, enter the mitochondrial matrix [100]. For further details see text. Abbreviations: cRNA: (positive-sense viral) complementary RNA; Cyt: Cytoplasm, EE: Early Endosome, ER: Endoplasmic Reticulum; GC: Golgi Complex; LE: Late Endosome, mRNA: (viral) messenger RNA; Mt: Mitochondrion; MTOC: Microtubule Organising Centre; Nuc: Nucleus; vRNA: viral (negative-sense) RNA.

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