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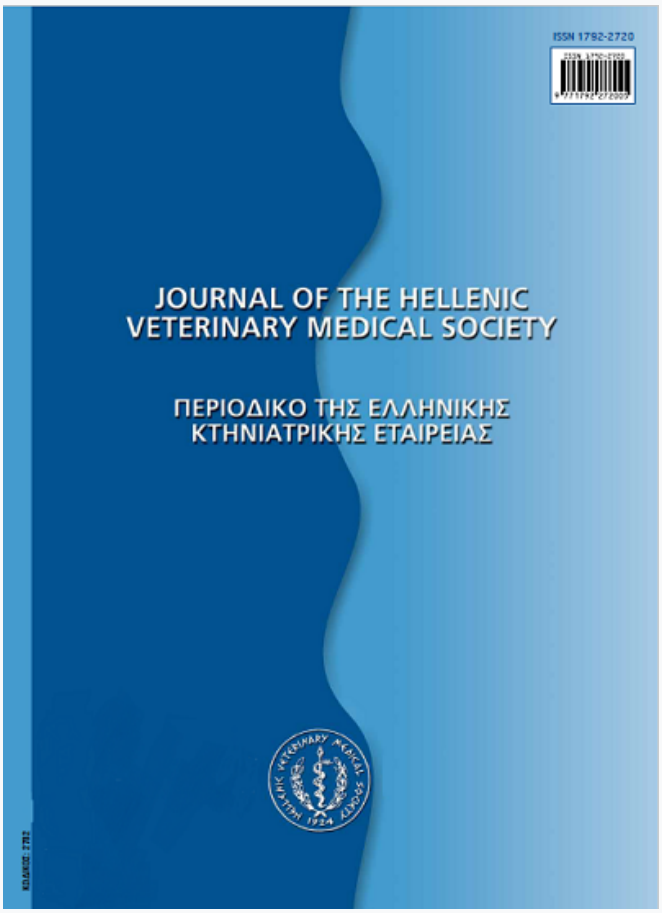

Influenza in birds, pigs and humans: how strong is the species barrier?

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Γρίπη πτηνών, χοίρων και ανθρώπων: πόσο ισχυρός είναι ο φραγμός των ειδών;

Van Reeth K., De Vleeschauwer A., Kyriakis C.S., Pensaert M.

ΠΕΡΙΛΗΨΗ. Οι πρόσφατες επιζωοτίες της γρίπης των πτηνών από τον υψηλής λοιμογόνου ικανότητας H5N1 και τα τυχαία κρούσματα ανθρώπων και άλλων θηλαστικών, συμπεριλαμβανομένων των χοίρων και των αιλουροειδών, έχουν θέσει σε συναγερμό τη διεθνή επιστημονική κοινότητα. Νέα ερωτήματα έχουν προκύψει σχετικά με τη μετάδοση των ιών της γρίπης μεταξύ των ειδών και ο ρόλος του χοίρου ως "αναμιγνείον δοχείο" των ιών των πτηνών και του ανθρώπου έχει υποστεί κριτική. Ο κύριος σκοπός αυτής της ανασκόπησης είναι να εκτιμήσει την πιθανότητα της γρίπης των πτηνών και του χοίρου στο να προκαλέσουν αντίστοιχο λοιμώδες αναπνευστικό νόσημα στον άνθρωπο. Η μετάδοση των ιών της γρίπης μεταξύ των ειδών είναι σπάνια εξέλιξη γεγονότων και πολύ λίγοι ιοί έχουν πετύχει να εγκατασταθούν σε νέο είδος ξενιστή. Μέχρι την εμφάνιση του ιού H5N1 το 1996 μόνο 3 περιπτώσεις ασθενών ανθρώπων από ιούς πτηνών είχαν αναφερθεί. Η μη μετάδοση από άνθρωπο σε άνθρωπο του H5N1 αποδεικνύει ότι απαιτούνται εκτεταμένες αλλαγές του γενώματος του ιού για να υπερπηδηθεί ο φραγμός των ειδών. Αν και οι ιοί γρίπης των πτηνών έχουν απομονωθεί από χοίρους, μόνο σε μία περίπτωση ο ιός γρίπης των πτηνών H1N1 που μεταδόθηκε από άγριες πάπιες σε χοίρους ήταν ικανός για περαιτέρω εξάπλωση σε χοίρειο πληθυσμό. Η ευαισθησία των χοίρων στους ιούς υψηλής και χαμηλής λοιμογόνου ικανότητας των πτηνών έχει επιβεβαιωθεί σε πειραματικές μελέτες, αλλά η μετάδοση από χοίρο σε χοίρο δεν έχει αποδειχθεί. Επίσης, περιγράφεται η πειραματική και η φυσική μετάδοση, των υψηλής λοιμογόνου ικανότητας ιών, στα αιλουροειδή, μυς, νυφίτσες και πιθήκους του γένους μακάκους. Ακόμη παρουσιάζονται οι κύριες διαφορές της λοιμογόνου ικανότητας του ιού ανάμεσα στα διάφορα είδη των θηλαστικών. Έτσι, η μελέτη της λοιμογόνου ικανότητας μπορεί να προσφέρει ιδέες και γνώσεις σχετικά με τα αίτια της περιορισμένης εξάπλωσης του ιού σε ένα νέο ξενιστή. Μπορούμε να συμπεράνουμε ότι, αντίθετα προς την κοι-

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ABSTRACT. The recent epizootics of the highly pathogenic H5N1 avian influenza in poultry and the occasional infections of humans and other mammals, including pigs and felines, have alerted the international scientific community. New questions over the interspecies transmission of influenza viruses have been raised and the role of the pig as a "mixing vessel" of avian and human viruses has been criticized. The major aim of this review is to evaluate the zoonotic potential of avian and swine influenza. Interspecies transmissions of influenza viruses are rare virus-evolution events and very few viruses have succeeded to become established in new host species. Until the appearance of the H5N1 virus in 1996 only 3 cases of humans infected with avian viruses were recorded. The lack of human-to-human transmission of H5N1 demonstrates that extensive changes in the virus genome are required in order to overcome the species barrier. Although avian influenza viruses have been isolated from pigs, only in one occasion an avian H1N1 virus transmitted from wild ducks to pigs was able to further spread in the swine population. The susceptibility of swine to highly and low pathogenic avian viruses has been confirmed in experimental studies, but pig-to-pig transmission has not been demonstrated. Experimental and natural transmission of highly pathogenic avian viruses to felines, mice, ferrets and maqacues are also discussed, showing the major differences in the virus pathogenesis among different mammalian species. The study of this pathogenesis may offer insights to the reasons of limited virus spread within a new host. We may conclude that, contrary to common believes, the species barrier remains a serious obstacle for the spread of novel influenza viruses in new host species, including humans. Our experience with H5N1 and H7N7 has tested old established theories, proving them insufficient. Further study of the factors which influence and limit the transmission of influenza viruses from one species to another is needed to better understand and

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νή άποψη, ο φραγμός των ειδών παραμένει ένα σοβαρό εμπόδιο για την εξάπλωση των νέων ιών γρίπης σε νέα είδη-ξενιστές, συμπεριλαμβανομένου και του ανθρώπου. Η εμπειρία μας με τους ιούς H5N1 and H7N7 θέτει σε αμφιβολία τις παλιές θεωρίες και αποδεικνύει ότι είναι ανεπαρκείς. Χρειάζεται περαιτέρω μελέτη των παραγόντων που επηρεάζουν και περιορίζουν τη μετάδοση των ιών της γρίπης από το ένα είδος στο άλλο για να γίνει περισσότερο κατανοητό και να αξιολογηθεί ο κίνδυνος της εμφάνισης νέας πανδημίας από ιούς γρίπης.

Λέξεις ευρετηρίασης: γρίπη πτηνών, πτηνά, χοίροι, άνθρωπος, φραγμός ειδών

evaluate the risk of the emergence of new pandemic influenza viruses.

Key words: avian influenza, birds, pigs, humans, species barrier

INTRODUCTION

Influenza A viruses are enveloped, single stranded RNA viruses in the family Orthomyxoviridae (reviewed in 1). Two other types of influenza-type B and C- also occur in humans causing mild or subclinical infections, but they are unimportant for animals and are beyond the scope of this review. Influenza A viruses are further classified into subtypes based on the antigenic properties of the "external" glycoproteins haemagglutinin (HA) and neuraminidase (NA). The HA and NA are very important for the entrance and exit of the virus in the host's cells and for the induction of an antibody response in the host, but they are also highly variable. In constrast, the "internal" proteins like the nucleoprotein (NP) and matrix (M) proteins are more conserved between different influenza A viruses. Sixteen different HAs (H1-H16) and 9 different NAs (N1-N9) have been recognised among all influenza A viruses. The HA and NA can occur in different combinations, this combination designates the subtype of the virus.

Influenza is the classic example of a genetically unstable virus and there are 2 genetic mechanisms that allow the virus to alter its antigenic constitution. Antigenic drift involves the gradual accumulation of small mutations in the genes encoding the HA and/or NA. This may result in subtle antigenic changes, which enable the virus to multiply significantly in individuals with immunity to preceding strains and thus to cause an influenza epidemic. Antigenic shift is a much more dramatic and less common antigenic change, which refers to the introduction of a completely novel influenza virus subtype in the human or animal population. One possible mechanism of antigenic shift is genetic "reassortment" between two different influenza virus subtypes that simultaneously infect the same cell. Influenza viruses have a segmented genome, which means that their genetic information is not on a single RNA strand, but on 8 separate segments of RNA, each of which contains the information for one or two viral proteins. During a mixed infection the individual gene segments of both viruses can mix and match to produce a progeny virus with part of its genes from one virus and part from the other virus. Reassortment between a circulating human virus and an avian virus can lead to a "hybrid virus", including the avian gene segment that encodes a novel HA. Because humans are immunologically naive to such a novel subtype, it may spread worldwide and cause a pandemic.

Influenza A viruses infect a large variety of bird species, humans, pigs, horses, seals, ferrets and mink, and occasionally other species, like cats and dogs. Birds, humans and pigs are among the most important influenza virus hosts, in which the infection can have serious economic impact. Influenza virus transmission between these species is rare, but it may have serious consequences if it occurs. The direct transmission of an avian influenza (AI) virus to humans, for example, or reassortment between human and avian virus strains may set the stage for an influenza pandemic in humans. Pigs are thought to serve as "mixing vessels" for the reassortment between human and avian viruses and as the source of pandemic influenza viruses for humans. A highly virulent AI virus, subtype H5N1, has recently become endemic in poultry in South-East Asia. The H5N1 virus has also crossed the species barrier to humans, for which it is extremely virulent, pigs, cats and tigers. It is feared that H5N1 may become the next human pandemic influenza virus if it would acquire the capacity to spread from human to human, which it lacks so far.

Knowledge of the factors that limit avian virus transmission to and between mammals, and of the pathogenesis of AI in mammals is essential in the prevention of influenza pandemics. Since the emergence of H5N1, there has been a renewed interest in both issues and several old dogmas have been

criticised. In this paper, we first compare the pathogenesis of avian, human and swine influenza viruses in their native hosts. The second part discusses the pathogenesis of AI viruses in mammals, with special attention for the recent findings with H5N1 in humans, pigs and other animals. The importance of swine influenza as a zoonosis and general facts on host species barriers to influenza virus infections are also discussed.

The nature of influenza viruses in birds, pigs and humans

Wild and domestic birds [2,3]: There are large pools of influenza viruses covering all known subtypes in wild birds, especially ducks and geese, which function as the reservoir for influenza viruses in domestic birds and mammals. While influenza viruses that have become established in mammals show a restricted combination of HA and NA subtypes, 82 different combinations have been isolated from wild birds. Influenza viruses also evolve at slower rates in wild ducks than in mammals and they are said to be in "evolutionary stasis". The influenza virus replicates in both the respiratory and intestinal epithelia of these ducks and high amounts of virus are shed in the faeces (up to $10^{8.7}$ 50% egg infectious doses (EID50)/g duck faeces) for 3 to 4 weeks. Influenza viruses have been isolated from untreated lake water where large numbers of waterfowl are found and they are efficiently transmitted between birds through the faecal-oral route via surface waters. Surveillance studies in wild birds in North America and Europe have revealed a high prevalence of viruses of low virulence for poultry. Isolation rates may reach up to 15% in ducks and up to 2.8% in other wild birds, but they also depend on the bird species and age, time and place. Several wild bird species have the potential to distribute influenza viruses between countries or even continents, because they are generally asymptomatic virus carriers.

Domestic poultry, such as chickens and turkeys, commercially reared ducks and geese, ratites and caged pet birds are also susceptible to influenza. Influenza viruses infecting domestic poultry can be divided into two distinct groups based on their clinical manifestations in chickens. The highly pathogenic avian influenza (HPAI) viruses have been restricted to subtypes H5 and H7, though not all viruses of these subtypes are HP, and cause severe disease with mortality up to 100%. All other viruses are low pathogenic (LP) and cause a much milder, primarily respiratory disease. The HA plays a central role in the pathogenicity of AI viruses, as further explained in the section on pathogenesis. The cases of both HPAI and LPAI in domestic poultry appear to result from the introduction of influenza virus from wild birds. Once introduced in the poultry population, influenza viruses can spread between poultry farms by a number of methods and particularly by mechanical transfer of infective faeces. Importantly, LP H5 and H7 AI viruses, that have been introduced in poultry flocks from wild birds, may mutate into HP viruses after a short or long time of circulation in the poultry population. Until recently, H5 or H7 subtypes isolated from free-living birds, were almost invariably of low pathogenicity for poultry.

The Asian HP H5N1 virus, on the other hand, is somewhat exceptional[4,5]. This virus has been circulating in Asia since 1996 and it led to the culling of millions of poultry in Hong Kong in 1997. Despite the eradication of H5N1 from Hong Kong, its precursors continued to circulate in ducks and geese in South China in which they caused largely subclinical infections. In the winter of 2003-2004 very severe outbreaks of H5N1 were almost simultaneously reported in 8 countries in South-East Asia – South Korea, Vietnam, Japan, Cambodia, Indonesia, Thailand, China and Laos – and the virus is now enzootic in several of them. The virus has subsequently spilled over from poultry into the wild bird population and, unexpectedly, has killed thousands of migratory birds at the Qinghai Lake nature reserve in China[3]. From mid 2005 until mid 2006 H5N1 infections in wild birds and/or poultry have also been reported in Russia, Turkey and several Eastern European countries, and some countries in Western Europe and Africa. Only a few cases of H5N1 in wild birds occurred in June 2007 in Germany, the Czech republic and France.The H5N1 cases in wild birds in European countries without outbreaks among poultry suggest that wild birds may play a role in the spread of H5N1 to unaffected regions. However, the recent outbreaks of H5N1 in turkey farms in Hungary and the United Kingdom (February 2007) proved that human activities, such as the movement of infected poultry and poultry products, remain a major cause of the spread of avian influenza world wide[6,7,8].

Humans [1,9,10]: Influenza virus subtypes that have become established in humans have been limited to H1, H2, H3, N1 and N2. Currently circulating strains are H1N1, H3N2 and H1N2. The H3N2 virus was introduced in 1968, when it caused the so-called "Hong Kong" pandemic and replaced the preceding H2N2 virus. The new pandemic virus was a reassortant between an avian influenza virus, providing the H3 HA and polymerase B1 genes, and the circulating human H2N2 strain, which was the donor of the N2 NA and five other genes. H1N1 circulated among humans from the early 20th century until 1950, when it temporarily disappeared, and was reintroduced in 1977. The current

theory is that the H1N1 virus escaped from a laboratory in 1977. After their introduction in the human population, influenza viruses usually undergo antigenic changes, and this phenomenon is responsible for the recurrent influenza epidemics that occur in between pandemics. Antigenic "drift" occurs more frequently with H3N2 than with H1N1, which is reflected in the number of changes recommended in vaccine composition. Since 2001, H1N2 influenza viruses have also been isolated from humans[10]. These viruses are reassortants between the circulating H1N1 and H3N2 viruses, but their prevalence and clinical importance are unclear. Novel influenza virus strains or variants that arise in one continent generally spread rapidly to other continents, where they cause outbreaks during the colder months of the year. Transmission of influenza between humans occurs via the respiratory route, mainly by direct person-to-person spread. Preschool and school age children, who are largely immunologically naive, play a major role in the transmission of influenza viruses in the community.

Pigs[11]: The same influenza subtypes as in humans – H1N1, H3N2 and H1N2 – are circulating in swine worldwide, but the swine influenza viruses (SIVs) are antigenically and genetically different from their human counterparts. The origin and nature of SIVs in different continents also differ, whereas there is a high degree of similarity among human influenza viruses worldwide. The predominant H1N1 SIVs in Europe, for example, are entirely of avian origin and they were introduced from wild ducks into the pig population in 1979. Two types of H1N1 SIV, in contrast, are circulating in the US: the so-called "classical" H1N1 viruses that have been present since the early 20th century and novel reassortants with the surface glycoproteins of the classical virus and internal proteins of more recently emerged H3N2 or H1N2 SIVs. Viruses of both other subtypes, H1N2 and H3N2, also have a different origin in Europe and in the US and were introduced at different times in the swine population. These differences between SIVs in different parts of the word also have implications for the diagnosis and control of SIV, and the strains used as antigens in vaccines and diagnostic tests also differ in Europe and in the US. A detailed overview of the genetic characteristics of SIVs worldwide is given in reference[11]. It is remarkable that most SIVs are reassortants with mixtures of human, avian and swine virus genes. This supports the classical notion that pigs are susceptible to both human and avian influenza viruses, and that they can serve as "mixing vessels" for those viruses. Antigenic drift within SIV lineages does occur, but it is less prominent than antigenic drift with human influenza viruses. The current European H1N1 and H3N2 SIVs, for example, still show some degree of cross-reactivity with SIVs from the 1980s, while replacement of human influenza virus variants occurs more quickly. SIVs are readily transmitted by contact with respiratory secretions from infected pigs as well as by the air. It is therefore impossible to prevent SIV infections by sanitary measures alone, and the virus is enzootic in most densely swine populated regions. In serological investigations in Flanders, Belgium, in 2001 and 2003, most individual sows had antibodies to a combination of two (48%) or to all three (31%) subtypes, indicating consecutive or simultaneous infections with several SIV subtypes during their lifetime. Similar findings have been made in Germany, Italy and Spain, where all three subtypes are also widespread.

The pathogenesis of influenza viruses in their native hosts

Wild and domestic birds[2]: The course of an infection with influenza virus in birds is extremely variable and it depends on the virus strain, host species and environmental factors. Most avian influenza viruses, which are designated as LP viruses, will cause an asymptomatic or mild infection. However, a few strains produce systemic infection with invasion of the central nervous system and high mortality. These HPAI viruses are invariably members of the H5 or H7 subtypes, but not all H5 or H7 strains are highly pathogenic. LP and HPAI viruses show structural differences at the so-called cleavage site of the precursor of the viral HA, which must be cleaved into HA1 and HA2 for the virus to become infectious. The LPAI viruses have only one or a few basic amino acids at this site and cleavage occurs exclusively by trypsin-like host proteases. Virus replication is therefore restricted to sites in the host where such enzymes are found, i.e. the epithelia of the respiratory and intestinal tracts. HPAI viruses, in contrast, posses multiple basic amino acids at their HA cleavage site and can be cleaved by a broad range of cellular proteases. Consequently, the tissue tropism of HPAI viruses is not restricted to the respiratory and intestinal tract. These viruses invade the submucosa and enter vascular or lymphatic systems to replicate and cause lesions in a variety of cell types in visceral organs, brain and skin. Mortality may reach 100% within one week and possible symptoms are cessation of egg laying, respiratory signs, sinusitis, oedema of the head and face, subcutaneous haemorrhages with cyanosis of the skin, particularly of the head and wattles, diarrhoea and neurological signs.

Still, the bird species also plays an important role in

the outcome of an infection with both LP and HPAI viruses. Turkeys are considered among the most susceptible birds and they may experience serious respiratory disease problems after infection with LPAI viruses. Turkeys and domestic chickens are also most susceptible to HPAI viruses. They usually show a fulminating disease or they die before clinical signs can be observed. In domestic ducks and geese and in wild birds, on the other hand, HPAI viruses either replicate poorly or to a limited degree and they produce few clinical signs. In addition, viruses of H5 or H7 subtype isolated from free-living birds are almost invariably of low pathogenicity for poultry. The rare isolations of HPAI viruses from feral birds were usually in the vicinity of outbreaks of HPAI in poultry. This is in line with the hypothesis that HP H5 or H7 viruses emerge after the introduction of a LPAI virus from wild ducks to poultry, in which such viruses can mutate to their pathogenic phenotype. While most HPAI viruses tend to be harmless in wild and domestic ducks, this is not always true for the currently circulating H5N1 viruses. These viruses have caused disease in waterfowl in nature and under experimental conditions and the increased virulence for ducks of some of the recent H5N1 isolates has been confirmed in experimental studies[12]. Nevertheless some recent H5N1 isolates still cause minimal disease in ducks and several bird species may be asymptomatic carriers of the virus.

Humans[1,13]: Influenza infections in humans can be asymptomatic or cause serious illness with both respiratory and general symptoms. Typical symptoms are an abrupt onset of fever, chills, headache, myalgia, loss of appetite, sore throat, cough and breathing difficulties. The immune status of the host is very important for the clinical outcome and influenza is one of the primary causes of lower respiratory tract disease in children. The infection is rarely fatal in young people, but mortality can occur in people older than 65 years and in those with underlying medical conditions. Influenza viruses of H1N1, H3N2 and H1N2 subtypes generally cause similar clinical manifestations.

The virus causes an acute infection of epithelial cells throughout the respiratory tract of humans and can be detected in both nasal and throat swabs. Intranasal inoculation of human volunteers with H1N1 or H3N2 influenza virus produces a characteristic virus shedding pattern and febrile upper respiratory illness. Virus is usually detected in nasal secretions within 24 h after inoculation, rapidly rises to a peak of $10^{3.0-7.0}$ 50% tissue culture infectious doses (TCID50)/ml on day 2, and is no longer detectable after 5-7 days of shedding. The amounts of virus shed and the severity of disease are positively correlated, which suggests that the extent of virus replication determines the clinical outcome. Nasal lavage fluids of experimentally inoculated humans contained elevated levels of several pro-inflammatory cytokines and chemokines within 2-3 days after inoculation[14]. Cytokines like interferon-a and interleukin-6 appear to be responsible for much of the systemic symptoms, such as chills, malaise, fever and muscular aches. It is often mentioned that the lungs are even more susceptible than the upper respiratory tract, but there are in fact no comparative studies of influenza viral loads in the upper and lower airways of humans. Nevertheless, high virus yields (up to 10^9 50% egg infectious doses (EID50)/g lung tissue) have been recovered from the lungs of patients with fatal influenza pneumonia. Viral antigen has been demonstrated in type 1 and 2 alveolar epithelial cells, as well as in intra-alveolar macrophages. The most significant pathology is also seen in the lower respiratory tract, where there is destruction and denudation of large areas of epithelial cells. Despite the systemic symptoms there are no indications for productive virus replication outside the respiratory tract. Viraemia is unusual and only rarely have low amounts of virus been recovered from human blood or extrapulmonary tissues. The presence of virus in the circulation most likely results from spillover from the lungs in those cases with severe lung tissue damage. Similarly, in the rare complication of influenza-associated encephalitis influenza viral RNA has been detected in cerebrospinal fluid with the polymerase chain reaction (PCR), but active viral replication in the central nervous system has not been shown. Unlike in birds, influenza viruses apparently do not replicate in the intestinal tract of humans. Also, inoculation of ducks with human influenza viruses does not produce an intestinal infection.

Swine[11,15]: Swine influenza virus is a major cause of acute respiratory disease in pigs. Typical swine "flu" outbreaks are characterised by a rapid onset of high fever, dullness, loss of appetite, laboured abdominal breathing and coughing. Morbidity may approach 100%, but mortality is usually less than 1% and recovery occurs within 7-10 days. Paradoxically, subclinical infections are also very common and many if not most pigs become infected with one or several SIVs without ever showing clinical signs. There are no indications for differences in virulence between SIV subtypes and strains.

The pathogenesis and pathology of influenza are strikingly similar in humans and in pigs. In experimental SIV infections, SIV has been demonstrated in epithelial cells of the nasal mucosa, tonsils, trachea and lungs. Nasal virus shedding usually begins on day 1 post

Table 1. Avian influenza viruses isolated from humans since 1996

Year	Subtype - pathotype	Location	Number infected	Number dead	Symptoms	Reference
1996	H7N7-LPAI	US	1	0	conjunctivitis	(13)
1997	H5N1-HPAI	Hong Kong	18	6	influenza-like	(14)
1998-99	H9N2-LPAI	Hong Kong/China	2	0	influenza-like	(15)
2003	H5N1- ?	Hong Kong	2	1	influenza-like	(16)
2003	H7N7-HPAI	The Netherlands	83	1	conjunctivitis	(17)
2004	H7N3-HPAI	Canada (BC)	2	0	conjunctivitis	(18)
2003-...[1]	H5N1-HPAI	Vietnam	93	42	influenza-like	(16)
		Thailand	25	17		
		Indonesia	100	80		
		Cambodia	7	7		
		China	25	16		
		Turkey	12	4		
		Iraq	3	2		
		Azerbaijan	8	5		
		Egypt	36	15		
		Djibouti	1	0		
		Azerbaijan	2	2		
		Nigeria	1	1		

[1] Situation on June 15 2007 (19)

inoculation and ceases within 7 days. Immunohistochemical studies demonstrate the virus' highly specific tropism for bronchiolar and alveolar epithelium and virus titres in the lungs may reach up to 10^8 EID50/g lung tissue. The characteristic microscopic lesions are necrosis and desquamation of lung epithelial cells and an influx of neutrophils into the airways. Virus clearance is extremely rapid and SIV is rarely isolated from the respiratory tract on or after day 7. As in humans, low virus titres have occasionally been detected in serum of experimentally infected pigs, but virus replication in extra-respiratory sites has never been demonstrated.

An infection with SIV can be easily reproduced experimentally by intranasal, aerosol or intratracheal inoculation, but the typical "flu" symptoms and severe pathology only result when pigs are inoculated with high virus doses directly into the trachea. The intratracheal inoculation produces a more fulminant virus replication in the lungs than other inoculation routes and a rapid and massive secretion of interferon-a, tumour necrosis factor-a, interleukin-1 and -6 locally in the lungs[15]. The same cytokines are thus probably responsible for the typical signs of influenza in both pigs and humans. Based on experimental data, it is plausible that the amount of virus that reaches the deeper airways and the resulting production of infectious virus determine the extent of cytokine production in the lungs, which in turn determines the severity of illness.

The fate of avian influenza viruses in mammals

Humans: Despite the avian origin of human influenza viruses, influenza viruses rarely "jump" directly from birds to humans. From 1959 to 1996 there had been only three reported human cases of AI and humans were considered to be at low risk of infection with AI viruses. During the last decade human infection with an AI virus has been reported on 7 occasions (Table 1) and all cases were due to direct contact with infected poultry. Only 4 AI virus subtypes - H5N1, H7N3, H7N7 and H9N2- have been found in humans and most strains were HP. The majority of AI viruses have spread to only one or a few people, but the HP H7N7 and H5N1 viruses have infected tens of humans and the latter virus is exceptionally virulent for humans.

The HP H7N7 outbreak in poultry in The Netherlands started in February 2003 and spread subsequently to 8 farms in Belgium and 1 farm in Germany. Almost 31 million poultry in The Netherlands were culled before the virus was contained in May 2003. At least 89 people, 86 of whom had close contact with infected poultry, contracted the H7N7 virus. Most of these people had conjunctivitis, but no influenza-like symptoms and the virus loads in throat/nose swabs were much lower than in conjunctival swabs, suggesting that the H7N7 virus had a predilection for the eye rather than for the respiratory tract[20]. For unknown reasons, one 57-year-old veterinarian died from a primary viral

Table 2. Avian influenza virus subtypes that have been isolated from pigs since 1979

Year	Location	Subtype	Source of infection	Extent of transmission in swine population	Reference
1979	Western Europe	H1N1	wild ducks	enzootic	(34)
1988	Taiwan, South China	H3N2	wild ducks	no data	(35)
1993-1994	South China	H1N1	wild ducks	low prevalence	(36)
1998-2000	South China	H9N2	wild ducks	low prevalence	(37)
1999	Canada	H4N6	wild ducks	single case	(38)
2001	Canada	H3N3	wild ducks	no data	(39)
2001, 2002	Canada	H1N1	wild ducks	no data	(39)
2001, 2003	China	H5N1	wild ducks	two cases	

pneumonia followed by acute respiratory distress syndrome and related complications[22]. Though serologic investigations suggest that about 250 humans may have become exposed, it is unlikely that the virus transmitted extensively from person-to-person[23].

The Asian HP H5N1 virus already infected poultry and 18 people in Hong Kong in 1997 and it continued to circulate in ducks and geese in Southern China after its eradication from Hong Kong. Since 2004, H5N1 has become endemic in poultry in several countries in Asia and the virus later spread to some parts of Europe and Africa. The H5N1 outbreaks in poultry are the largest and most devastating in history and more than 200 million birds have been slaughtered in largely unavailing efforts to contain the spread of the infection. Table 2 gives a chronological overview of the countries that have reported human infections with H5N1. Most cases had a history of very close contact with infected poultry, usually within a week before the onset of clinical signs[24,25]. Inhalation of infectious droplets and self-inoculation of the conjunctival or upper respiratory tract mucosa are probably the most common routes of infection. Some people may have become infected after consumption of poultry products or by oral contact with contaminated water. The first and most prominent clinical signs are a high fever (≥ 38°C) and influenza-like symptoms, but diarrhoea and gastro-intestinal signs are not uncommon. Symptoms of involvement of the lower respiratory tract – respiratory distress, difficulty in breathing and a crackling sound when inhaling – are seen in many patients. The pathogenesis of H5N1 in humans is not yet fully understood, but the lungs are clearly the major site of virus replication and viral antigen has been demonstrated in type 2 pneumocytes[26]. Almost all patients develop a primary viral pneumonia with diffuse alveolar damage, interstitial pneumonia, focal haemorrhages and bronchiolitis. There are indications that a prolonged overproduction of pro-inflammatory cytokines leads to the acute respiratory distress syndrome and multi-organ failure that are seen in so many patients,[27]. Recent virological and immunological investigations in H5N1 patients further support that the fatal outcome is associated with high virus replication in the respiratory tract and the resulting hypercytokinemia[28].

Unlike in poultry, there is no firm evidence for a systemic infection in humans. There are reports of two fatal H5N1 cases in Vietnam with severe diarrhoea and encephalitis without respiratory disease[25]. Only one of the patients was virologically examined and the virus was isolated from throat and rectal swabs, serum and cerebrospinal fluid. In another fatal case, in Thailand, viral RNA was detected in the lung as well as in the small and large intestines, but not in plasma, adrenal glands, brain, bone marrow, kidneys, liver and pancreas[26]. Viral antigen-positive cells, in contrast, have never been demonstrated in the intestines and viral RNA may have been the result of virus swallowed from throat infection rather than from intestinal production. Definitive evidence of H5N1 replication in the intestines of humans is thus lacking. On the other hand, it cannot be excluded that humans may become infected after oral uptake of H5N1 virus, but initiation of the infection in this case may result from infection of oropharyngeal tissue rather than from a primary gastro-intestinal infection.

At any rate, it must be stressed that H5N1 does not easily cross from birds to infect humans. Despite the infection of tens of millions of poultry over large geographical areas since mid-2003, only just over 300 human cases have been laboratory confirmed. Most of these cases have occurred in households with infected backyard poultry and very few cases have occurred in presumed high-risk groups, such as commercial poultry workers, cullers and veterinarians. This points to the importance of education and implementation of hygienic

measures in the prevention of human H5N1 infections. The H5N1 virus so far fails to spread from human-to-human and eliminating the source of infection, i.e. infected birds, remains the most effective control measure. Indonesia, for example, has already recorded more human deaths from bird flu than any other country and this is a direct result of its failure to control the virus in poultry. Some *in vitro* studies with respiratory tissues of humans have shown that H5N1 attaches preferably to epithelial cells in the lower respiratory tract, namely type 2 pneumocytes and epithelial cells in terminal bronchioles, whereas attachment becomes progressively more rare towards the trachea[30,31]. This was in line with the observation that the receptor for AI viruses occurs only in and around the alveoli of the human lung, but not in the nasal epithelium, trachea or bronchi, where the receptor for human influenza viruses predominates[31]. This led to the hypothesis that the H5N1 virus has difficulty in replicating in the upper region of the respiratory tract of humans, and that this hampers the virus to spread efficiently to and between humans. It is also feared that mutations in the receptor-binding site of the viral HA would enable the virus to bind to receptors in the upper respiratory tract and this could increase the risk of an H5N1 pandemic. In other in vitro studies, however, the H5N1 virus also readily infected epithelial tissues of the nasopharynx, adenoid and tonsils[32]. Most important, it remains uncertain to what extent the in vitro data reflect the in vivo situation and comparative, quantitative analyses of the replication of both human and avian influenza viruses in nasal tissues of humans are also lacking.

Pigs: For a long time, it was thought that transmission of AI viruses to humans does not occur directly, but via the pig as an intermediate host. However, several "old" theories obviously need to be revised and the recent HPAI outbreaks have also changed our understanding of the role of pigs.

A single experimental study in 1984 led to the conclusion that pigs readily become infected by a large variety of AI viruses[33]. In that study, pigs were inoculated intranasally with 38 LPAI viruses, mainly of duck origin, and 29 of them were excreted in nasal swabs and induced a serological response. The pathogenesis of such viruses, on the other hand, and their potential for transmission between pigs have never been examined. In the authors' lab we have been able to confirm that most LPAI viruses are able to infect pigs after experimental intranasal inoculation of a high virus dose, but we also found indications that AI viruses replicate much less efficiently than the typical SIVs. It is not so surprising, therefore, that the circulation of entirely AI viruses in pigs in nature is a rare event. Several AI viruses have been occasionally isolated from pigs as shown in Table 2. Serological studies have also shown evidence for infections of pigs in Asia with avian H4, H5 and H9 viruses[40]. However, only the H1N1 virus that crossed from wild birds to swine in Europe in 1979 has become established in pigs, whereas the other viruses have disappeared. Still, there is circumstantial evidence that the genes of avian viruses may persist after reassortment with one or more influenza viruses endemic in pigs. This probably means that genetic reassortment or mutations are needed for successful transmission of AI viruses between pigs.

One important observation was that the trachea of pigs contains receptors for both avian (sialic acids linked to galactose by an α-2,3 linkage) and human (sialic acids linked to galactose by an α-2,6 linkage) influenza viruses[41]. This was seen as an explanation for the fact that a) pigs are susceptible to both avian and human influenza viruses and b) reassortants with both avian and human virus genes frequently emerge in pigs. The pandemic human influenza viruses of 1957 and 1968 were also human-avian reassortants and one classical theory is that reassortment occurred in the pig, which served as an intermediate host to transfer the virus to humans. By now it has become clear that humans also have receptors for both human and avian influenza viruses, which predominate in the upper and lower respiratory tracts respectively[31]. In addition, we have learned that AI viruses can transmit to humans without the pig as an intermediary and that genetic reassortment can also occur in humans.

Though HPAI viruses can also infect pigs under natural and experimental conditions, it is doubtful whether such viruses replicate efficiently in pigs and whether they are readily transmitted among pigs. During the HP H7N7 outbreak in The Netherlands in 2003, H7N7 antibodies were temporarily found on some mixed farms with swine and infected poultry, but not on mixed farms with non-infected poultry or on farms with swine only[42]. After experimental intranasal inoculation with a high dose of the H7N7 virus, pigs shed virus in nasal swabs for one or a few days and showed seroconversion, but there was no disease or virus transmission to contact animals. Antibodies to the Asian H5N1 virus were found in only 0.25% of 3.175 pigs tested in Vietnam in 2004, where H5N1 has hit hardest[43]. There are also unpublished reports of occasional infection with H5N1 in pigs in China in 2001 and 2003, and more recently in Indonesia. In a limited experimental study, intranasal inoculation of pigs with the Vietnam/2004 H5N1 virus resulted in virus isolation

from the respiratory tract (tonsils, trachea and lungs), but not from the intestines, blood, spleen or kidney[43]. Though this experiment confirmed the susceptibility of pigs for H5N1, there was no virus transmission between experimentally inoculated and in-contact pigs. Unfortunately, detailed studies of the cell and organ tropism of H5N1 in pigs are still lacking.

Cats and other felidae: Cats are generally considered resistant to influenza[44]. However, during the 2003-2004 H5N1 outbreak in Asia, there have been occasional H5N1 infections in domestic cats and severe outbreaks in tigers and leopards in zoos in Thailand[45,46] after the animals had been fed virus-infected chickens. The tigers showed fever, respiratory distress and neurological signs and many of them died or were euthanatized. There was evidence of a generalized infection with lesions in the lungs, heart, thymus, stomach, intestines, liver, lymph nodes and brains. Virus replication was demonstrated in bronchiolar epithelium, hepatocytes and cerebral neurons. Additional natural cases of HP H5N1, presumably as a consequence of feeding on infected birds, have been reported in 3 cats and in a stone marten in Germany and another cat in Austria[19,47].

Under experimental conditions cats became infected with H5N1 after intratracheal inoculation or after feeding on H5N1-infected chicks[48]. All cats developed clinical signs, including fever, decreased activity, protrusion of the third eyelid, conjunctivitis and laboured breathing. Virological examinations suggest that cats may be more susceptible to H5N1 than humans or pigs. Like in the tigers, there was a generalised infection in the cats. High virus titres and lesions were not only found in the nasal conchae, tonsils, trachea and lungs, but also in multiple extra-respiratory tissues including the central nervous system. The cats fed with virus-infected chickens had viral antigen-positive cells and virus-associated ganglioneuritis in neural plexi in the small intestine, but not in intestinal epithelial cells. It was clearly proven that the infection can be established via oral uptake. However, this does not mean that the virus enters the body via the gastro-intestinal tract or that it replicates productively in intestinal cells, and the exact routes of virus entry and of spread to extra-respiratory tissues remain to be defined. Importantly, the cats excreted high amounts of H5N1 virus in nasal and throat swabs, and lower and more variable amounts in rectal swabs. Unlike in experiments with pigs, the H5N1 virus was transmitted from infected to sentinel cats in experimental conditions. However, data from naturally infected cats in Austria suggest that H5N1 AI infection in felines may be subclinical, and questions the horizontal transmission of the virus between cats[49].Thus, cats may be at risk of infection during H5N1 outbreaks in poultry, but we cannot be certain whether they will spread the virus to other cats. It needs to be assessed whether cats could spread the H5N1 virus between poultry farms or possibly from poultry to humans.

Mice, ferrets and macaques are also susceptible to H5N1 infection, but they will not be discussed in detail. In macaques, the infection causes acute respiratory distress and virus replication appears to be confined to the lungs and other respiratory tract tissues. In mice and ferrets, in contrast, some H5N1 strains cause a generalized infection with a strong neurotropic character and mortality[50]. There are several remarkable findings in mice and in ferrets: 1) H5N1 strains isolated from humans are much more pathogenic in ferrets than those isolated from birds. The latter viruses induced only mild disease in ferrets and they replicated only in the respiratory tract. 2) Pathogenicity in the ferret does not always correlate with pathogenicity in the mouse model. 3) Apart from the respiratory tract, the central nervous system is a major site of H5N1 replication in mice. According to some studies, virus spread to the central nervous system does not occur via the circulation, but via neural pathways following replication of the virus in the respiratory tract.

The major lesson from these studies with H5N1 is that the pathogenesis can strongly differ in different mammalian species and with different virus strains. Still, the ports of virus entry, the primary sites of virus replication and the routes of virus dissemination through the body remain to be studied for most mammals. Such studies are not only essential for the control of AI in domestic mammals, but they may also provide information that can be extrapolated to humans.

Transmission of influenza viruses between pigs and humans

Occasional zoonotic infections with SIVs have been confirmed by virus isolation from the respiratory secretions or lungs of humans in Europe, Asia and the US (reviewed in 9). In Europe, H1N1 and H3N2 SIVs have been isolated from a single child in Switzerland and in The Netherlands. Most human infections with SIV have been diagnosed in the US with the "classical" H1N1 SIV. In general, SIV infections in people are not clinically distinguishable from human influenza virus infections, but there have been a few fatalities in the immunosuppressed and in pregnant women. Serologic studies have suggested regular transmission of SIVs to people in contact with pigs. Researchers in the US found significant associations between seropositivity to

H1N1 SIV and being a farm owner or entering the swine barn for more than 4 days per week. A more recent study also suggested seropositivity to H1N2 SIV among swine farmers and veterinarians in Iowa (51). However, these studies must be interpreted with care, because some US SIVs show serological cross-reaction with current the human viruses. It is thus possible that SIV antibody titres in humans do not really correlate with infection with SIV. In every way, the total number of SIV isolations from humans is negligible compared to the number of people involved directly or indirectly in swine farming. Also, all confirmed cases had been in close contact with pigs and the SIVs appear to lack the critical property to spread further from human to human. The "New Jersey" incident in the US in 1976 may be a single exception. Here, an approximate 500 humans became infected with an H1N1 virus identical to viruses isolated from pigs at the time, and a national emergency immunization campaign was started. However, it has never been established that pigs were the source of the virus. Also, the virus strain caused little or no disease in infected people and in any case was not pandemic.

Wholly human influenza viruses are sometimes isolated from pigs, but sustained circulation of such viruses in pig populations seldom occurs. This contrasts with reassortant viruses carrying human virus genes, like the H3N2 viruses that have become widespread in swine in Europe and in the US. These viruses have mixtures of human genes, including the HA, and genes of swine and/or avian virus origin. In experimental infection studies in pigs, a wholly human H3N2 virus was excreted in much lower titres in nasal swabs and caused less severe lung lesions than a "triple reassortant" H3N2 virus[52]. In further studies, virus shedding and pathogenicity of the human virus could be increased by introduction of the HA and NA of the reassortant into the wholly human virus by reverse genetics[53]. This shows that the restricted infectivity of the human virus in pigs is at least in part dependent on the HA and NA gene. All these data support that efficient transmission of human viruses among pigs requires mutations and/or reassortment events, as appears to be the case for AI viruses in pigs.

General considerations on the species barrier and conclusions

There are many factors that limit the transmission of influenza viruses from one species to another and they are incompletely understood. An excellent summary of our current knowledge of the "host species barriers to influenza virus infections" has recently been published[54]. A first possible barrier is the lack of suitable receptors on the host cell, so that the HA of a virus from another species cannot attach. AI viruses prefer sialic acid receptors with an a2,3 linkage to galactose, while human viruses have a preference for sialic acids with an a2,6 linkage. The paucity of AI virus receptors in the nose and trachea of humans may hamper transmission of avian viruses to and between humans. However, this theory is still a mere hypothesis and it certainly needs further proof. Even if an influenza virus succeeds to enter the cell of a new host, it must successfully co-opt host cell processes to replicate there. The polymerases of the virus, which are responsible for the replication and transcription of viral RNA, play a key role at this stage. As an example, many AI viruses can infect mouse cells, but fail to replicate in them and this has been associated with specific amino acids in the polymerase B2 (PB2) protein. Finally, the influenza virus must escape from the cell it has infected. During this step, the viral HA tends to re-bind to receptors on the cell surface and the NA helps to break this binding. Like the HA, the NA also has a preference for one of both types of sialic acid linkages and thus for humans or birds. One reassurance is that a large number of viral mutations or genetic reassortment, are obviously needed for an influenza virus to break the species barrier. Also, most of such genetic changes will be deleterious for the virus. Other factors that will influence influenza virus transmission between species are non-specific immune mechanisms, the routes of virus dissemination/excretion by the "donor" host, the extent of contact between donor and "recipient" host and the influenza immune status of the new host. It cannot be excluded that people with a solid immunity to the N1 neuraminidase of a human H1N1 influenza virus may be partially protected against H5N1. Moreover, some experimental studies have shown short-term immunity between different influenza subtypes[55].

Many simple questions about the pathogenesis of influenza in natural and unnatural hosts also remain unanswered. It is still unclear whether human influenza viruses replicate better in the upper respiratory tract, as claimed by the authors of two recent *in vitro* studies[30,31,32], or in the lower respiratory tract, as mentioned in so many textbooks. There is still discussion about the tissue tropism of H5N1 in humans and the lack of virological examinations on excretions and tissues of H5N1 patients is regrettable. Most important, we do not know the portals of viral entry and primary replication sites of H5N1 in different mammals. The tissue tropism of H5N1 has been studied in cats, but not in other domestic mammals. Because of all these gaps in our knowledge, we cannot say which animal species could be the most

reliable model for human H5N1 infection.

The possible role of pigs in the generation of new pandemic influenza strains appears to be more limited than previously thought. The old theory is that pigs are more susceptible to AI viruses than humans and that they serve as intermediates for the transmission of AI viruses to humans. Still, this hypothesis has never been proven and it is questioned by some recent observations with HP H7N7 and H5N1 AI viruses. The current viewpoint is that a strong barrier exists to infection of pigs with influenza viruses from other species and that major genetic changes are required for consistent pig-to-pig transmission of such viruses. Experimental studies like that by Landolt and coworkers[53] have started to create insights into the nature of these genetic changes and must be continued. We also need detailed studies on the pathogenesis of influenza viruses from birds and other species in pigs. Only this way, speculations and hypotheses on the role of pigs will turn into knowledge.

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