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Emergence of influenza A viruses

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Pandemic influenza in humans is a zoonotic disease caused by the transfer of influenza A viruses or virus gene segments from animal reservoirs. Influenza A viruses have been isolated from avian and mammalian hosts, although the primary reservoirs are the aquatic bird populations of the world. In the aquatic birds, influenza is asymptomatic, and the viruses are in evolutionary stasis. The aquatic bird viruses do not replicate well in humans, and these viruses need to reassort or adapt in an intermediate host before they emerge in human populations. Pigs can serve as a host for avian and human viruses and are logical candidates for the role of intermediate host. The transmission of avian H5N1 and H9N2 viruses directly to humans during the late 1990s showed that land-based poultry also can serve between aquatic birds and humans as intermediate hosts of influenza viruses. That these transmission events took place in Hong Kong and China adds further support to the hypothesis that Asia is an epicentre for influenza and stresses the importance of surveillance of pigs and live-bird markets in this area.

Keywords: influenza; avian influenza; emerging disease; H5N1; H9N2

1. INTRODUCTION

On first consideration, it may appear paradoxical to describe influenza, a disease that was first clearly described in the late 1100s (reviewed in Potter 1998), as an emerging disease. The justification for this characterization comes from the capacity of the influenza A virus to undergo antigenic transformation and re-emerge within the human population. Through the genetic processes termed antigenic drift and antigenic shift, the virus has the ability to constantly sidestep the immune response and sporadically cause pandemic disease of noteworthy proportions. Antigenic drift, which is driven by the infidelity of the virally encoded polymerase (Parvin *et al.* 1986), results in point mutations in the viral haemagglutinin (HA) and neuraminidase (NA) glycoproteins. The HA molecule is the major viral antigenic determinant, and the selection applied by the host immune system constantly selects for drift variants that can no longer be neutralized by circulating antibodies. In this way, influenza emerges seasonally as an endemic disease and can re-emerge in populations that have considerable immunity from previous exposures. Less frequent, but potentially of far greater concern, is the process of antigenic shift. The influenza A genome is composed of eight single-stranded negative-sense RNA molecules. Infection of a single cell by two different influenza viruses can result in the production of progeny viruses containing a mixture of RNA segments from the parental viruses. Such reassortment has the potential to completely change the antigenic nature of the circulating virus and, as such, allow unimpeded spread through a host population. It is for these reasons that, although influenza has been circulating in human populations for at least 900 years, influenza can be considered an emerging disease.

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2. RESERVOIRS

Influenza A viruses have been isolated from a limited number of different animal hosts including humans, birds, horses, whales, seals, mink and swine. Many host populations are only transiently infected or harbour only a small number of antigenic subtypes. As will be discussed, various lines of evidence suggest that the primary reservoir of influenza A viruses is the aquatic birds of the world. In 1901, Centanni and Savunozzi demonstrated that a filterable agent was responsible for fowl plague, a disease of chickens. Some 50 years later, this filterable agent was shown to be an influenza virus (Schafer 1955). The first indications that aquatic bird species could be hosts for influenza came in 1972 when antibodies to human influenza NA were identified in Australian pelagic birds (Laver & Webster 1972). Shortly after, influenza viruses were isolated from a shearwater (Downie *et al.* 1973) and healthy wild ducks (Slemons *et al.* 1974). The site of viral replication in ducks and the disease symptoms contrast with those in humans. Influenza in wild ducks is an asymptomatic disease, with the virus replicating preferentially in the cells lining the gastro-intestinal tract (Webster *et al.* 1978). In humans, influenza often is associated with clinical symptoms, and the virus replicates primarily in the respiratory tract. The asymptomatic nature of the disease and the apparent evolutionary stasis of viruses in the aquatic bird reservoir are consistent with a stable host–parasite relationship developed during a prolonged period of coevolution. Upon interspecies transfer, the resultant infection often causes extensive disease, and an increased viral mutation

rate is observed as the virus adapts to the new host (Suarez 2000).

Probably all influenza A viruses of mammals have ancestral links to avian lineages. All 15 HA and nine NA influenza A subtypes have been identified in aquatic bird populations (Ito & Kawaoka 1998). In contrast, only viruses of HA subtypes 1, 2, and 3 and NA subtypes 1 and 2 have been identified as forming stable lineages in the human population. Out of the 15 HA subtypes, all but H13 (a recent surveillance of ducks in Siberia identified a single H13N6 isolate (Okazaki *et al.* 2000)) can be isolated from wild ducks, and this population has often been attributed with being the main reservoir of influenza viruses in nature (Hinshaw *et al.* 1983). Although this characterization may be true, there is no evidence to suggest other avian populations, such as migratory shorebirds, are of any less importance. In fact, most subtypes of HA and all NA subtypes of influenza A viruses also have been isolated from shorebirds in a single flyway in the Delaware Bay region of the USA (Webster *et al.* 1992). It is likely that both ducks and shorebirds are key reservoirs of influenza virus and that each may preferentially, although not exclusively, harbour distinct subtypes (Donis *et al.* 1989; Hinshaw *et al.* 1985; Kawaoka *et al.* 1988; Sharp *et al.* 1993). The separation of influenza reservoirs depends not just on the host species but also on geographical location. The separation of the different migratory routes of aquatic birds has resulted in the formation of geographically defined influenza gene pools of North American and Eurasian lineages (Gorman *et al.* 1992; Suarez *et al.* 1998). Recent data show, however, that within North American avian populations circulate at least two sub-lineages, one of which is more similar to the Eurasian lineage than to the other North American cluster (Makarova *et al.* 1999; Webby *et al.* 2000). Makarova *et al.* (1999) suggested that this similarity could be the result of some degree of contact between birds of the different flyways. Another possibility is that the phylogenetic separation of Eurasian and North American lineages is a result of sampling bias and the restricted data pool of avian virus gene sequence. It is logical to assume that the geographical separation of the avian reservoirs has led to distinct viral gene lineages, but sequencing genes of viruses isolated from other aquatic bird populations may reveal that more contiguity is occurring than is currently thought.

In comparison with influenza viruses of humans and swine, influenza viruses in aquatic bird reservoirs appear to be in evolutionary stasis (Kida *et al.* 1987; Gammelin *et al.* 1990; Gorman *et al.* 1990*a*,*b*; Suarez 2000; Webster *et al.* 1992). This apparent stasis in aquatic birds is further evidence that this population is the natural reservoir of influenza viruses. The direct comparison of evolutionary rates of viruses from mammalian and aquatic birds, however, should be approached with some degree of caution. The evolution of influenza in hosts such as chickens, humans and swine can be followed relatively easily because new introductions are easily identified and all subsequent viruses can be attributed to a single lineage. Also animal-to-animal transmission appears to maintain influenza in these alternative hosts. It is not as clear whether the same can be said for the aquatic bird reservoirs. Longitudinal studies of various duck and shorebird populations have shown that infection rates vary considerably depending on the time of year (Halvorson *et al.* 1983, 1985; Hinshaw *et al.* 1980, 1985; Stallknecht *et al.* 1990; Webster *et al.* 1976, 1992). The mechanisms for viral perpetuation in the aquatic bird population remain unclear, although numerous possibilities have been suggested (Webster *et al.* 1992). Two such suggestions reflect observations that viruses can be isolated from lake water near which infected ducks are nesting and that infected ducks can shed virus for extended periods (Hinshaw *et al.* 1980; Ito *et al.* 1995). Both scenarios would require limited passage in ducks for the maintenance of the virus within the population. Comparisons of evolutionary rates between viruses of ducks and those of populations in which virus perpetuation requires constant animal-to-animal transmission could be inappropriate. The sheer magnitude and diversity of viruses within the aquatic bird reservoir makes longitudinal studies very difficult and potentially misleading.

A plethora of other avian species also have been associated with influenza infection, although the importance of any one of these populations in the ecology and emergence of influenza is uncertain (reviewed in Alexander 2000). Turkeys seem to have a high susceptibility to influenza infection, although often the appearance of influenza in these birds is associated with contact with wild waterfowl (Halvorson *et al.* 1983; Karunakaran *et al.* 1983; Lang 1982). The close association of waterfowl with influenza in turkeys suggests that they are not a primary reservoir of influenza viruses but merely a transient host. The extent of influenza in passerine birds, ratites and other domestic poultry is far from understood and their contribution to the maintenance and emergence of influenza viruses remains unclear.

Additional non-human reservoirs of influenza A viruses are found in mammalian species such as swine (see § 3) and horses (Mumford & Chambers 1998). Only limited numbers of viral subtypes have been repeatedly isolated from swine (H1N1, H3N2 and H1N2) and horses (H7N7 and H3N8), although other subtypes can be isolated periodically. Marine and semi-aquatic mammals such as whales, seals and mink, also have yielded influenza viruses. Phylogenetically and antigenically the viruses of the marine mammals appear to be of avian origin and are probably the result of independent transmissions from aquatic birds rather than circulating lineages within these hosts (Hinshaw 1998).

3. ROLE OF INTERMEDIATE HOSTS IN HUMAN DISEASE

Clear lines of genetic and serological evidence support the hypothesis that human pandemic influenza can be a result of the reassortment between human and avian viruses (Kawaoka *et al.* 1989; Scholtissek *et al.* 1978*b*). This theory is certainly true for the Asian flu (H2N2) that emerged in 1957 and the Hong Kong flu (H3N2) of 1968. It may not, however, be true for the H1N1 virus responsible for the catastrophic 'Spanish flu' of 1918. Our current knowledge of the 1918 H1N1 virus comes from the analysis of fixed biopsy specimens and material from an Inuit woman frozen in the Alaskan permafrost. Sequence data

have been reported from the viral HA, NA, nucleoprotein (NP) and non-structural (NS) gene segments (Basler *et al.* 2001; Reid *et al.* 1999, 2000; Taubenberger *et al.* 1997). These sequence data place all of these genes at the root of the mammalian lineage, suggesting that the H1N1 virus may have crossed from the avian reservoir without reassortment. Regardless of whether they were reassortants, the 1918, 1957 and 1968 pandemic viruses all appear to contain gene segments recently acquired from avian reservoirs. Considering the widespread occurrence of influenza in avian species, one could speculate that the incidence of pandemic disease should be much higher than is observed. Fortuitously for humans, the limiting factor in the emergence of pandemic influenza is the inability of many viruses from aquatic birds to replicate effectively in the respiratory tract of primates (Beare & Webster 1991; Murphy *et al.* 1982). Likewise, human viruses inoculated using natural routes of infection replicate poorly in waterfowl (Hinshaw *et al.* 1983).

The receptor specificity of viruses from different hosts has been postulated to be a leading factor in the host restriction of influenza viruses. All influenza viruses attach to cell-surface oligosaccharides with terminal sialic acid, but distinct differences are apparent in which forms of these molecules are recognized.

Influenza viruses isolated from aquatic birds preferentially bind to NeuAα2,3Gal-terminated receptors whereas human viruses favour receptors terminated with NeuAα2,6Gal linkages. The relative abundance of these two sialic acid moieties in the human respiratory tract (high in α2-6 linkages) and the gastro-intestinal tract of ducks (high in α2-3 linkages) is considered a primary reason why avian viruses are not a more frequent problem in the human population (Couceiro *et al.* 1993; Ito *et al.* 1998; Murphy *et al.* 1982). However, the 1997 H5N1 Hong Kong bird-flu incident, in which multiple transfers of virus from chickens to humans occurred, demonstrated that receptor preference is not an absolute barrier to interspecies transmission. Analysis of the H5N1 viruses isolated from humans showed that these viruses retained their preference for NeuAα2,3Gal linkages despite their ability to replicate in the human respiratory tract (Matrosovich *et al.* 1999).

The subsequent identification of human infections with avian H9N2 viruses containing the same genes encoding internal proteins as those of the H5N1/97 viruses suggests that other gene segments are intricately involved in host range (Peiris *et al.* 1999). It has been very difficult to attribute interspecies transfer to any specific gene segment and it appears that all eight RNA segments have the capacity to attenuate virus growth in alternative hosts (Almond 1977; Buckler-White *et al.* 1986; Clements *et al.* 1986, 1992; Scholtissek *et al.* 1978*a*, 1985; Snyder *et al.* 1987, 1988; Subbarao *et al.* 1993; Tian *et al.* 1985). Host range is probably controlled by several factors; to cross the species barrier, a virus must have an optimal, but as yet undefined, complement of molecular features.

The recent development of reverse genetic techniques for the production of influenza virus may help to unravel these molecular features that confer the ability to cross the species barrier (Hoffmann *et al.* 2000*a*; Neumann *et al.* 1999; Pleschka *et al.* 1999).

4. PIGS AS INTERMEDIATE HOSTS

If aquatic avian and human viruses replicate poorly in their heterologous hosts, how do reassortant pandemic viruses emerge in the human population? The answer to this question may lie in the ability of other animal species to efficiently replicate viruses from both sources. Reassortant viruses containing genes from human and avian lineages appear to have caused at least two of the human pandemics of the 20th century (Kawaoka *et al.* 1989; Scholtissek *et al.* 1978*b*). These reassortant viruses retained some human virus gene segments, presumably imparting the ability of growth in humans, and gene segments from avian viruses facilitating the evasion from host immune defences. Considering the heterologous host restriction of avian and human viruses, one must ask 'Where does this reassortment occur?' By acting as the 'mixing vessel' for these events, pigs have been postulated to play an important role in the process of such genetic reassortment (Scholtissek 1990) and have been implicated in many viral interspecies transmission events between swine, avian and human hosts (reviewed in Brown 2000). Pigs seem to be readily infected by human viruses (Brown 2000; Chambers *et al.* 1991) and most, if not all, avian HA subtypes are capable of replicating to some extent in swine (Kida *et al.* 1994). Molecular analysis of the sialic acids of the pig trachea has revealed that NeuAα2,3Gal and NeuAα2,6Gal terminal linkages are present, a situation that may explain the permissive nature of the pig as a host of influenza virus (Ito *et al.* 1998). In addition, Ito *et al.* (1998) found that continued replication of avian-like swine viruses in pigs leads from a preference for dual receptor specificity to a preference for NeuAα2,6 linkages. Thus, not only are the appropriate repertoire of receptors available in the pig trachea, but continued passage of avian viruses in this host may lead to changes to create a more 'human-like' specificity (Suarez *et al.* 1998).

Endemic influenza in swine is restricted to three subtype combinations: H1N1, H3N2 and H1N2. Other subtypes, such as H9N2, H4N6 and H1N7, have been isolated sporadically from swine but have not become established (Brown *et al.* 1994; Karasin *et al.* 2000*a*,*c*; Peiris *et al.* 2001). Although only three established viral subtype combinations are found in swine, the different geographical populations are reservoirs for an increased number of distinct viral lineages. Classical-swine H1N1, which is phylogenetically related to the virus responsible for the 1918 human Spanish flu pandemic, circulates predominantly in North America and Asia (Chambers *et al.* 1991; Guan *et al.* 1996; Hinshaw *et al.* 1978; Webby *et al.* 2000). In Europe, H1N1 viruses also circulate, but this lineage is derived from a wholly avian-like virus that was first detected in the pig population in 1979 (Pansaert *et al.* 1981; Scholtissek *et al.* 1983; Schultz *et al.* 1991). This virus superseded the classical-swine viruses circulating at the time. In addition a distinct lineage of avian H1N1 virus has been reported in China (Guan *et al.* 1996). H3N2 viruses were first detected in swine in 1970, shortly after the emergence of similar viruses in humans (Kundin 1970). Since this time, human-like H3N2 viruses have been isolated from swine throughout Europe, Asia and the Americas and these viruses continue to co-circulate with H1N1 viruses. Further,

some H3N2 viruses isolated from swine in Asia have contained surface glycoproteins of avian origin (Kida *et al.* 1988). Reassortant H1N2 viruses of different lineages have been identified in various swine populations and are becoming more prominent. A reassortant H1N2 virus containing avian-like swine and human genes has become a significant problem in the UK, and this virus appears to have now spread to continental Europe (Brown *et al.* 1995, 1998; Van Reeth *et al.* 2000). Reassortant H1N2 viruses derived from classical H1N1 and various H3N2 viruses also have been isolated in Japan and the USA (Karasin *et al.* 2000*b*,*d*; Sugimura *et al.* 1980; Webby *et al.* 2001).

Although there are distinct geographical patterns of influenza in swine, presumably maintained through the limited intercontinental transfer of animals, common themes are apparent. One such theme is the maintenance of viruses with genes recently obtained from avian reservoirs. The avian-like H1N1 viruses distributed throughout Europe are entirely of recent avian descent; the prevailing H1N2 viruses in the UK contain the internal genes from the avian-like H1N1 (Brown *et al.* 1995); most human-like H3N2 viruses in Italy contain internal genes again from the avian-like H1N1 (Campitelli *et al.* 1997; Castrucci *et al.* 1993); and the dominant reassortant H3N2 viruses in the USA contain PA and PB2 (RNA polymerase subunits) of avian origin (Karasin *et al.* 2000*d*; Zhou *et al.* 1999). Other examples of avian viruses entering the swine population include H9N2 viruses in Hong Kong and H4N6 viruses in Canada (Karasin *et al.* 2000*a*,*c*; Peiris *et al.* 2001). Both of these latter events are of considerable concern because of the non-H1, H3 subtypes involved. Fortunately, the H4N6 outbreak seemed to be a confined event instigated by the drawing of drinking water from a nearby pond frequented by wild ducks. Likewise, with only small numbers of seropositive animals so far identified, there is no strong evidence that the H9N2 viruses are establishing in swine in south-eastern China. Although husbandry practices and animal movements probably play key roles, it is interesting to speculate that swine viruses with genes recently obtained from the avian reservoir have a competitive advantage.

After the avian-like H1N1 swine viruses entered the European swine population in 1979, their evolutionary rates were shown to be significantly higher than those of human and classical-swine viruses (Ludwig *et al.* 1995). Scholtissek *et al.* (1993) proposed that this difference was due to a mutator mutation in the viral polymerase complex. Such a mutator mutation could lead to an increase in viral variants and allow for the faster generation of viruses with advantageous mutations. Stech *et al.* (1999), however, showed that the mutational and evolutionary rates of the European avian-like swine viruses were independent of each other and that a mutator mutation was unlikely. These authors suggested that the increased heterogeneity of the avian-like swine viruses in culture may be due to the presence of partial heterozygotes. The presence of two gene segments with complementing functions may allow the growth of viruses in heterologous hosts. The state of partial heterozygosity would be lost upon further advantageous mutations in one of the duplicated gene segments.

During the emergence of H3N2 viruses in swine in the USA two genotypes of reassortant viruses were isolated. Both viruses had surface glycoproteins similar to those of human viruses circulating around 1995. One such reassortant (double reassortant) contained genes from the human (HA, NA and PB1) and classical-swine (PB2, PA, NP, matrix protein (M) and NS) lineages, and the other (triple reassortant) contained genes from the human (HA, NA and PB1), classical-swine (NP, M and NS), and avian (PB2 and PA) lineages. Both the double and triple reassortant viruses were capable of replicating and causing disease in swine (Zhou *et al.* 1999, 2000), but only the genotype containing the avian-like components became established (Webby *et al.* 2000). Certainly H3N2 viruses of human origin have been infecting swine in the USA for many years, albeit at low rates. This fact is demonstrated by the basal levels of H3N2 seroreactivity ($< 2\%$) seen in earlier studies (Chambers *et al.* 1991; Hinshaw *et al.* 1978). Why, therefore, has the triple reassortant lineage become established whereas other H3N2 viruses have not? One could speculate that the reassortment event(s) were the key steps.

Human H3N2 viruses are capable of replicating in swine, but to be transmitted and maintained they may require genetic changes in their internal proteins. The reassortant H3N2 viruses may have circumvented the need for such adaptation by associating with the classical-swine internal protein genes that are already well adapted to swine. Why the triple reassortant managed to spread and the double reassortant did not remains unknown, although it is tempting to speculate that the avian PA and PB2 genes may be of some relevance. Of some concern is the association of the triple reassortant internal gene complex with three phylogenetically distinct human H3 molecules (Webby *et al.* 2000). In addition, reassortant viruses with seven genes from the triple reassortant and the H1 gene from a classical-swine virus have been identified (Karasin *et al.* 2000*b*; Webby *et al.* 2001) (figure 1). The implication from these data is that the swine triple reassortant viruses have the capacity to produce further antigenically distinct lineages well capable of disseminating throughout the swine population. Certainly if another episode such as the H4N6 outbreak in Canada (Karasin *et al.* 2000*a*,*c*) were to happen in a swine population coinfected with the triple reassortant, one could predict potentially dire consequences for veterinary and human health.

What could be the advantage conferred to a swine virus by the acquisition of avian-like genes? One possibility is that these viruses simply are new and have an immunological advantage. This situation is certainly possible with the avian-like swine H1N1 viruses whose surface glycoproteins are antigenically distinct from those of classical-swine H1N1 (Pansaert *et al.* 1981; Scholtissek *et al.* 1983). This theory does not, however, readily explain the frequency with which swine-adapted viruses acquire internal genes of avian origin in reassortment events (e.g. H3N2 reassortant viruses in Italy and H1N2 in the UK). If viruses containing avian genes recently obtained from avian reservoirs have an advantage in swine, there is currently no satisfactory explanation for the phenomenon.

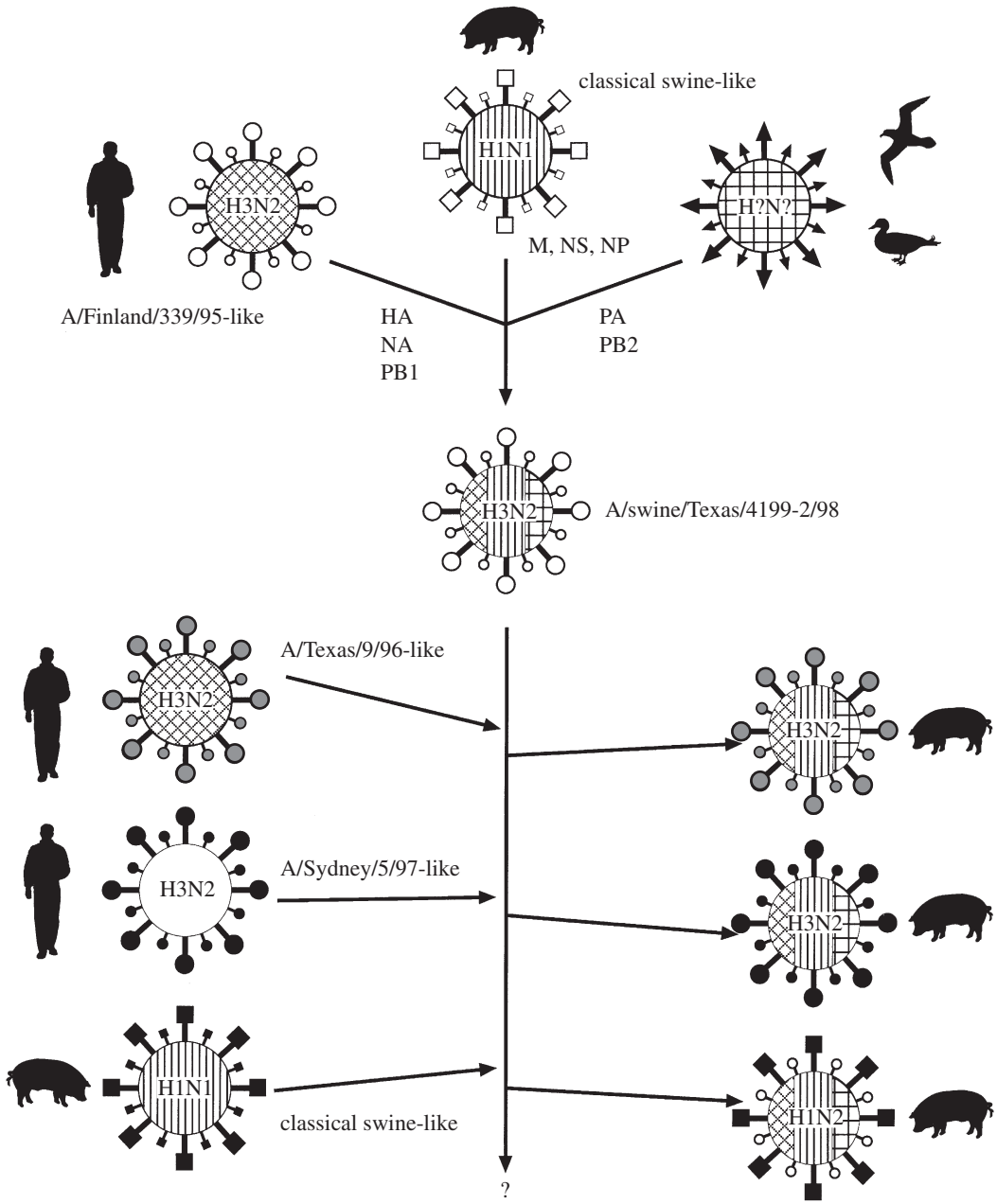

Figure 1. Schematic view of the reassortment events associated with the introduction of H3N2 viruses into the US swine population. During 1998, H3N2 influenza viruses (represented by A/swine/Texas/4199-2/98) were isolated from diseased swine herds. These viruses contained human-like HA, NA and PB1 genes, swine-like M, NS and NP genes, and avian-like PA and PB2 genes. A/swine/Texas/4199-2/98-like viruses spread throughout the country and have been endemic ever since. Further reassortment, on at least two occasions, has resulted in the production of viruses carrying six A/swine/Texas/4199-2/98-like genes and the HA and NA genes of recent H3N2 human viruses. In addition, H1N2 viruses containing the HA from classical swine-like viruses have been identified.

5. LAND-BASED POULTRY AS INTERMEDIATE HOSTS

The direct transmission of avian H5N1 and H9N2 viruses to humans in recent years has clearly demonstrated that reassortment or adaptation in an intermediate mammalian host is not necessary for infection of humans by an avian virus. These viruses did not, however, establish a lineage in humans, thereby suggesting that further adaptation or reassortment may be necessary for the successful transmission of avian viruses between humans. In such cases, humans themselves may act as the mixing vessel. One of the key lessons learned from the H5N1 and H9N2 events was the realization that domestic poultry may be intimately involved in the emergence of influenza in humans. This finding raises the possibility that pigs may not be the only host capable of serving as an intermediate between the aquatic bird virus reservoir and humans. If this situation is indeed true, then how does replication in poultry species allow for the adaptation of aquatic avian viruses? Recent studies by Matrosovich *et al.* (1999) may give some insight into the mechanisms involved.

By using H5N1 viruses as a model system, they showed that the HA and NA glycoproteins of chicken and duck

viruses have distinguishable properties. Specifically, the HA molecule from chicken isolates had increased glycosylation levels, and the NA contained deletions in the stalk regions, features that are in contrast to those of duck viruses. Detailed studies on the binding affinities of viruses from ducks, humans and chickens illustrated that chicken and human viruses have an increased affinity for gangliosides with long sugar chains. In contrast, duck viruses have an affinity for short-chain gangliosides (M. N. Matrosovich, personal communication). In the same study, human viruses also bound to the chicken epithelia, a finding suggesting that NeuAα2,6Gal-containing receptors are present, and that avian viruses could adapt to bind more strongly to these molecules. This possibility is highlighted by the human-like receptor specificity of H9N2 viruses isolated from land-based poultry species in the Hong Kong bird markets, including viruses similar to those that transmitted to humans (Matrosovich *et al.* 2001). These findings suggest that poultry species may be important intermediates in the emergence of influenza. Why then has it taken so long to identify this potentially important intermediate reservoir? One reason is that perhaps these birds are becoming more at risk of infection by influenza virus.

Influenza in poultry species manifests itself in one of two clinical outcomes. Highly pathogenic avian influenza (HPAI), as the name suggests, results in high mortality and morbidity. Conversely, low pathogenicity avian influenza (LPAI) tends to be subclinical, presenting as mild disease with symptoms such as respiratory distress and lowered egg production. HPAI generally is restricted to viruses of the H5 and H7 subtypes. HPAI viruses do not appear to be from single lineages but rather derived from non-pathogenic precursors through the accumulation of basic amino acids at the HA cleavage site. The accumulation of basic amino acids renders the HA molecule susceptible to an increased range of cellular proteases and leads to systemic spread and dissemination of the virus throughout the host (Bosch *et al.* 1981; Horimoto & Kawaoka 1994; Klenk *et al.* 1977). Throughout the 1980s and early 1990s, reports of influenza in land-based birds have been rare but regular, with many of the reported events occurring in turkey populations. In addition to the H5 and H7 subtypes, HA subtypes H1, H2, H3, H4, H6, H9 and H10 have been isolated from chickens throughout the world, a finding suggesting that they may be a permissive host for many different influenza viruses (Alexander 1993, 1998; Easterday & Hinshaw 1991; Pearson *et al.* 1993, 1998). The H5N1 episode in Hong Kong brought to our attention the possible role of domestic poultry in the emergence of human disease, and reports of LPAI infections in these hosts will probably increase as surveillance is intensified. It is also of interest to note that surveillance studies of Hong Kong bird markets before 1997 showed little influenza activity in chickens (Shortridge *et al.* 2000). In contrast, surveillance carried out in the markets in 1999 revealed that approximately 60% of chickens and quail were seropositive for H9N2 viruses. H9N2 viruses also were isolated from 4.7% of the faecal trays from cages containing chickens and 16% from cages containing quail (Guan *et al.* 2000). H9N2 viruses serologically related to those in Hong Kong have recently been isolated from poultry in Germany, Iran, Saudi Arabia and Pakistan. Genetic characterization of the isolate from Pakistan revealed that all gene segments were similar to those of the A/quail/HongKong/G1/97 lineage of viruses that appears to be the precursor of the H9N2 viruses transmitted to humans in Hong Kong (Cameron *et al.* 2000). Is it possible that we are now seeing the emergence of stable lineages of influenza in chicken and quail, species generally considered to be transient hosts?

6. ROLE OF ASIA AS A CENTRE OF PANDEMIC EMERGENCE

The hypothesis has been put forward that Asia is an influenza epicentre (Shortridge & Stuart-Harris 1982). This hypothesis reflects the emergence of the Asian/57(H2N2) and Hong Kong/68(H3N2) human pandemics from this region and the re-emergence of the Russian/77(H1N1) strain in northern China. The direct transmission of avian H5N1 and H9N2 viruses to humans in 1997 and 1999 (Peiris *et al.* 1999; Yuen *et al.* 1998) supports the notion that the conditions necessary for influenza viruses in avian species to be transmitted directly to humans are present in Hong Kong. Possible explanations as to why new influenza viruses arise in Asia include agricultural practices (Scholtissek & Naylor 1988) and the density of people, pigs and poultry.

At the beginning of the new century, China's economy is changing rapidly, bringing an increased standard of living and demand for more meat products. The economy is still primarily agriculturally based. The practice of each family raising their own ducks and pigs is being replaced by 'factory farming' as is practised in most Western countries. This practice is especially true for chickens and pigs, whose numbers continue to increase rapidly in response to the demand for meat. Although the methods of raising poultry are changing, the marketing practice in southern China remains traditional in that poultry largely are sold through live poultry markets. Agricultural authorities have been aware of the importance of live poultry markets in the emergence of influenza viruses for many years, particularly since the A/chicken/Pennsylvania/1370/83(H5N2) outbreak in the USA (Senne *et al.* 1993). Surveillance in poultry markets in Hong Kong from the early 1970s through to the late 1980s established that influenza A viruses of all known subtypes are prevalent in domestic ducks year-round, with a peak during the summer months (Shortridge 1992); during this study, influenza A viruses rarely were isolated from chickens and other land-based birds.

The importance of live poultry markets in the ecology of influenza was brought to the attention of those concerned with human influenza pandemic preparedness when this situation was shown to be the source of the H5N1/97 virus (Shortridge *et al.* 1998). Out of the 18 people infected with the H5N1/97 virus, six died. After the slaughter of all the poultry in the markets during December 1997, no more cases of bird flu occurred, thereby supporting the notion that the viruses came from the poultry markets. The importance of live poultry markets in the ecology of influenza is now under epidemiological investigation. The possibility exists that the poultry markets are breeding sites for influenza viruses

and that the H5N1/97 virus that emerged in a live poultry market in Hong Kong was derived from precursor viruses entering the markets from mainland China. The practice of housing many species of live birds in the same market provides near-optimal conditions for amplifying and perpetuating influenza viruses. We speculate that the H5N1/97 virus is a reassortant that emerged in a poultry market in Hong Kong and was perpetuated and spread through the markets by faecal contamination. We propose that the H5 gene was introduced by a goose virus (A/goose/Guangdong/1/96(H5N1)-like (Xu *et al.* 1999)); the internal gene segments by a quail virus (A/quail/Hong Kong/G1/97(H9N2)-like (Guan *et al.* 1999)); and the NA by a duck virus (A/teal/Hong Kong/W312/97(H6N1)-like (Hoffmann *et al.* 2000*b*)). In addition, we propose that the reassortment occurred in quail. Although, under experimental conditions, the H5N1/97 virus is lethal to chickens, no mortality was seen in chickens in the bird markets. It has been postulated that cross-protective cell-mediated immunity may have contributed to this protection of chickens in the bird markets (O'Neill *et al.* 2000; Seo & Webster 2001). Many birds entering the market in 1997 had been previously exposed to H9N2 viruses, and cell-mediated immunity to the G1/H9N2 virus may have afforded some cross-protection against the lethal effects of the highly pathogenic H5N1 reassortant virus (A/Hong Kong/156/97 (H5N1)-like) but allowed the spread of virus in the markets (figure 2).

Two distinguishable lineages of H5N1 co-circulated in the poultry markets in 1997 (Claas *et al.* 1998; Katz *et al.* 1999; Shortridge *et al.* 1998; Suarez *et al.* 1998; Subbarao *et al.* 1998); each was highly pathogenic in chickens but differed in pathogenicity in mice (Dybing *et al.* 2000; Gao *et al.* 1999; Gubareva *et al.* 1998; Lu *et al.* 1999). There is speculation that one lineage caused more of the fatal cases in humans than did the other. However, this hypothesis remains unresolved.

Before the H5N1/97 incident in Hong Kong, all types of live poultry for sale to the public were housed in about 1000 retail markets across the region (Shortridge 1999). Aquatic birds, including domestic ducks, geese, wild ducks and occasionally wild waterfowl, were kept in separate cages in the immediate vicinity of land-based birds that included chickens, silkie chickens, pigeons, quail, pheasant, chukka and guinea fowl. Lessons learned from the H5N1/97 incident have triggered changes in these bird-housing conditions. Aquatic birds are now slaughtered in a single central facility, and live aquatic birds are not available from retail outlets. Live land-based birds are still available for sale, although these birds are now tested for serological signs of H5N1 infection before transportation to a separate wholesale market. Approximately 120 000 chickens and more than 20 000 other land-based birds are imported each day. At the retail markets the majority of birds are sold each day, but the surplus, which often includes the more expensive birds such as pheasant, silkie chicken and guinea fowl, may still remain for many days.

After the poultry markets in Hong Kong were re-populated with land-based birds in early 1998, H9N2 influenza viruses became established and were perpetuated in the markets (Guan *et al.* 2000). Two lineages of H9N2 viruses co-circulate: one predominantly in chickens and represented by A/duck/Hong Kong/Y280/97-like viruses and the other predominantly in quail and represented by the A/quail/Hong Kong/G1/97(H9N2)-like virus. This latter virus possesses the same internal gene segments as found in human H5N1/97 viruses, and this quail H9N2 virus was transmitted to and caused mild influenza in two children in 1999 (Peiris *et al.* 1999). Viruses of the other H9N2 lineage (A/duck/Hong Kong/Y280/97-like) have been detected in pigs (Peiris *et al.* 2001) and there is speculation that the five human H9N2 cases reported in mainland China in 1999 (Guo *et al.* 1999) were caused by this sublineage. Each of the H9N2 lineages mentioned above continues to circulate in the poultry markets in Hong Kong, and H6N1 viruses represented by A/teal/Hong Kong/W312/97-like viruses (which have seven gene segments similar to those of H5N1/97) also are circulating.

In the separate aquatic bird market, in which ducks and geese are killed and sold chilled, H5N1 viruses similar to the A/goose/Guangdong/1/96-like virus continue to be isolated (Cauthen *et al.* 2000). Thus, each of the probable precursors of the H5N1/97 bird flu virus continues to circulate and the wisdom of separating aquatic birds from land-based birds in the live poultry market has been vindicated.

Despite the separation of poultry in Hong Kong, traditional poultry marketing continues in other areas of mainland China, where all kinds of poultry are sold together. Thus, live poultry markets will continue to be the breeding sites for influenza viruses of relevance to human and veterinary public health. The separate marketing of aquatic and land-based birds probably has already prevented the re-emergence of H5N1/97-like viruses in Hong Kong and emphasizes that changes in poultry marketing practices can play an important role in influencing the emergence of influenza viruses. Until the traditional practice of selling poultry in the live market changes, we will have to accept that live markets are breeding grounds for influenza viruses and that surveillance in the poultry markets is an essential part of an early-warning system for influenza viruses with zoonotic potential.

7. PANDEMIC PREPAREDNESS

There is general agreement that another pandemic of influenza will occur in humans and that future pandemics are inevitable (Leese & Tamblyn 1998; Patriarca & Cox 1997). Extensive planning to deal with the next pandemic has been done in many countries, and the World Health Organization has played an active role in the preparation of global plans. The six main areas summarized in the US plan are: '(i) improvements in ongoing virological and disease-based surveillance systems; (ii) vaccination of high-priority target groups, and, given sufficient vaccine supplies, the entire US population; (iii) liability programmes for vaccine manufacturers and health-care providers; (iv) research to improve detection of new variants and to accelerate the availability of existing and novel vaccines and antiviral agents; (v) integrated, multi-component communication systems for rapid information dissemination and exchange; and (vi) emergency preparedness plans to provide for adequate medical care and

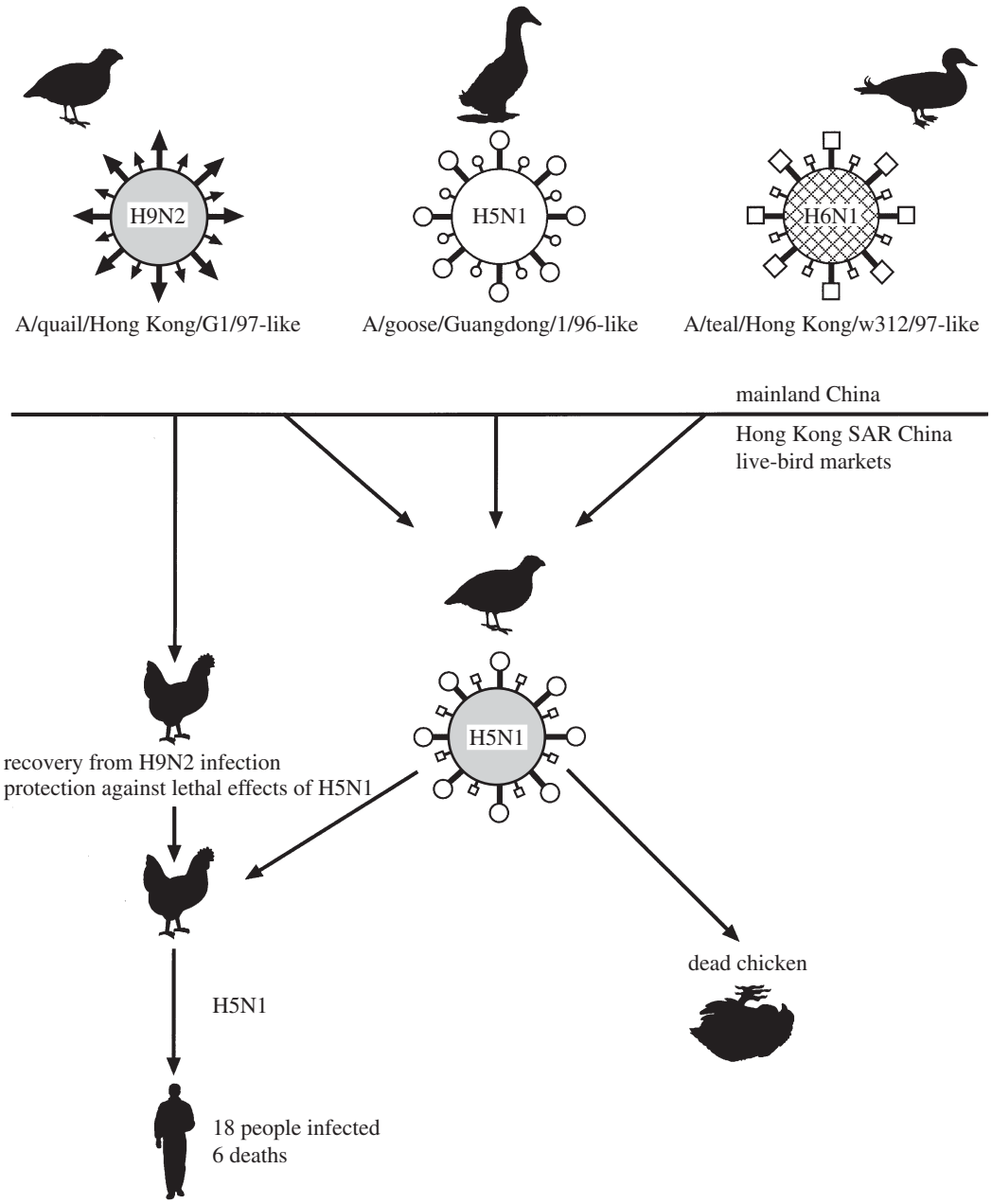

Figure 2. Postulated evolution of the 1997 Hong Kong 'bird-flu' incident. During 1997, 6 out of 18 people infected with an avian-like H5N1 influenza virus died. It is postulated that this virus was a reassortant that arose in the Hong Kong SAR bird markets. The possible precursors of the H5N1 virus were an H9N2 quail-like virus (source of the PB2, PB1, PA, NP, M and NS genes), an H5N1 goose-like virus (source of the HA gene), and an H6N1 teal-like virus (donor of the NA gene). These progenitor viruses were probably circulating in mainland China and were introduced into Hong Kong SAR through the live-bird markets. The practice of communal housing for many different bird species created a perfect environment for the reassortment event(s) to occur. Quail appear to be susceptible to a range of influenza viruses and we speculate that the reassortment happened in these birds. Although lethal to chickens (the predominant species in the market), the H5N1 virus was able to spread in these birds due in part to cell-mediated cross-protection afforded by previous exposure to the H9N2 quail-like viruses (many birds entering the market had H9N2 antibodies). All 18 human cases were from independent interspecies transmission events as opposed to human-to-human spread.

maintenance of essential community services' (Patriarca & Cox 1997). These plans were developed with input from the most-informed people in the world, and it is not the purpose here to criticize them. The issue we want to discuss is how many of the recommendations have been addressed or implemented. It is important that these recommendations are not left to gather dust until the inevitable occurs.

Several events including the Hong Kong bird-flu incident alert us to the need to keep the recommendations 'dusted off' and illustrate the importance of item (i), improved surveillance. We predict that improved surveillance at the human–animal interface will eventually show that interspecies transmission of influenza viruses from the avian reservoir through the live poultry markets to humans is not such a rare event. It will be important to

determine which of these transmitted viruses has pandemic potential in humans. The H5N1/97 bird-flu viruses will provide clues about which viral genes and genome sequences were important, and other reports (this issue) deal with this topic.

The modelling of influenza pandemics (Meltzer *et al.* 1999) alerts us to the reality that at this time probably no country in the world could adequately cope with a pandemic and that hospital facilities would be overwhelmed. The difficulties due to the vaccine shortage during the 2000–2001 influenza season in the Northern Hemisphere provide a reality check in an interpandemic year.

The availability of NA inhibitors for influenza (Gubareva *et al.* 2000) does provide hope that something could be done in the face of a new pandemic. However, it is necessary to immediately begin plans for stockpiling and to initiate studies on the stability of the NA inhibitors and how they should be stored. In the interim period, amantadine and rimantadine, which are stable for decades (Scholtissek & Webster 1998), should be stockpiled. Despite their side-effects and the rapid selection of resistant mutants, amantadine and rimantadine could be used in conjunction with the available NA inhibitors to reduce the amount of drug needed for treatment or prophylaxis.

Although planning for future pandemics has been a worthwhile exercise, the plan must be updated regularly. As technology advances so does the possibility of better planning with improved early warning, antiviral drugs that target other viral functions, more rapidly prepared vaccines, and domestic animals that are naturally resistant to influenza. An immediate practical approach is to close all live poultry markets, as the requirements that led to their development are no longer applicable (i.e. refrigeration systems are widely available, at least in Hong Kong, and keeping live birds is no longer a necessity). The reality is that traditions change very slowly; a new pandemic could accelerate this process.

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