Case 18

Influenza virus

A 59-year-old woman went to see her doctor, as she had been unwell for the past 3 days. She initially noticed a nonproductive cough, and then she became abruptly worse with a marked fever, headache, and shivering. Since then she had developed muscle aches all over her body, especially in the legs, and her eyes had become watery and painful to move. She was a nonsmoker, previously fit and well, and on no regular medication. On examination, she was **febrile** (38.2°C), and had difficulty in breathing through her nose, but there were no other abnormal physical signs.

A throat swab was taken, broken off into viral transport medium, and sent to the laboratory. Immunofluorescent staining with a **monoclonal antibody** against influenza A virus was positive, confirming a diagnosis of acute influenza virus infection.

1. What is the causative agent, how does it enter the body and how does it spread a) within the body and b) from person to person?

Causative agent

Influenza A virus belongs to the *Orthomyxoviridae* virus family (myxo = affinity for mucin). The viral genome consists of 8 segments of negative single-strand RNA (i.e. RNA that cannot be translated directly on the ribosome, but has to be first copied into its complementary, positive, strand), which collectively encode 10 (or possibly 11) viral proteins (Figure 1). Each RNA segment is closely associated with the nucleoprotein, to form a helical

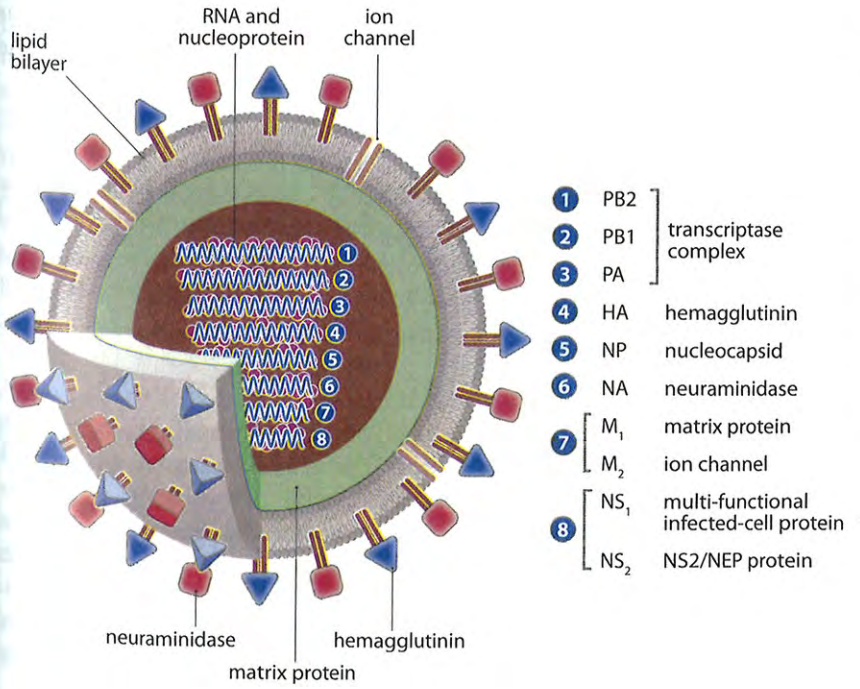

Figure 1. Schematic diagram of an influenza virus. The eight segments of RNA are enclosed within a nucleocapsid, which is in turn surrounded by a lipid envelope into which are inserted two surface glycoproteins, the hemagglutinin and neuraminidase. The helical nucleocapsid contains eight segments of ssRNA each coated with nucleoprotein. This is surrounded by a layer of M1 (membrane or matrix) protein, which in turn is surrounded by a lipid envelope into which are inserted two viral glycoproteins (hemagglutinin and neuraminidase) and a small amount of the M2 ion channel protein.

ribonucleoprotein (RNP), or **nucleocapsid**. The RNPs are in turn surrounded by a matrix protein and then a **lipid envelope**, which contains two viral glycoproteins, **hemagglutinin** (H or HA) and neuraminidase (N or NA), and also small amounts of the nonglycosylated M2 ion channel protein. Influenza viruses are grouped into types on the basis of the nature of the NP, which occurs in one of three antigenic forms, hence types A, B, or C influenza viruses. Influenza type A viruses are widespread in nature, infecting many avian species, but also humans, pigs, horses, and occasionally other species such as cats. Influenza B virus is an exclusively human pathogen, while influenza C viruses are not serious pathogens in humans. Influenza type A viruses are further subdivided into subtypes depending on the nature of their two external glycoproteins. Thus far, 16 distinct hemagglutinins and nine different neuraminidases have been identified, where each HA or NA molecule differs by at least 20% of its amino acid sequence from all other HA and NA molecules. When referring to an influenza A virus isolate, it is therefore necessary to specify precisely which subtype it is, for example influenza A/H1N1 or influenza A/H7N7.

Entry and spread within the body

Influenza virus enters via the nasal or oral mucosa. In humans and other mammalian species, the virus is **pneumotropic** (in avian species, the virus infects a variety of tissues and is primarily spread through the **fecal–oral** route), that is it preferentially binds to, and infects, respiratory epithelial cells, all the way from the **oropharynx** and nasopharynx right down to the alveolar walls. Influenza virus attaches to target cells via an interaction between the viral ligand, hemagglutinin, and a cellular receptor, comprising sialic acid residues, a component of the carbohydrate within glycoproteins, on the surface of respiratory epithelial cells. This can occur throughout the length of the respiratory tract. The virus enters the host cell via **vesicles** and uncoats. The virus then replicates and new virions are released by the infected cells by budding at the plasma membrane of the host cell. With infections of the lower respiratory tract, direct infection of **pneumocytes** and macrophages can occur. Given the systemic nature of the illness caused by influenza virus infection (see below), it is perhaps surprising that the virus itself does not usually spread beyond the respiratory tract.

Spread from person to person

Transmission of influenza viruses from person to person is believed to be via large droplets (≥5 μm diameter), which are generated from an infected respiratory tract during coughing, sneezing, or even talking. The droplets are deposited on the nasal or oral mucosa of a new susceptible host leading to infection.

Epidemiology

The epidemiology of influenza has several unusual characteristics (Figure 2). Annual outbreaks of infection are highly seasonal, arising each winter in temperate climates, with a considerable percentage (e.g. 10%) of the population acquiring infection, with concomitant increases in hospital admissions and influenza-related deaths. The size of these outbreaks varies from year to year. In the UK, an outbreak is referred to as an epidemic only when the consultation rate for 'influenza-like illness' recorded through the Royal College of General Practitioners surveillance scheme

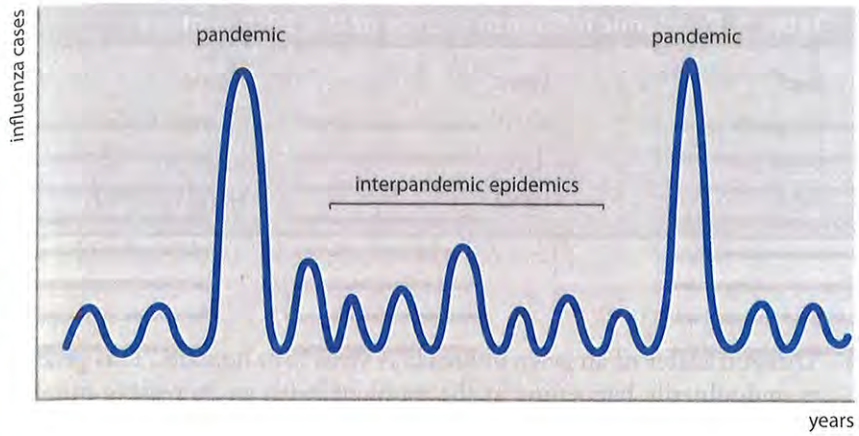

Figure 2. Epidemiology of influenza. This diagram shows the number of cases of influenza occurring over time. Each peak corresponds to a winter season, illustrating the annual epidemics. Superimposed on that, at irregular intervals averaging about once every 30–40 years, there is a massive peak corresponding to an influenza pandemic.

exceeds 400 per 100000 population. However, superimposed on this regular annual cyclical pattern, unpredictable global epidemics occur, on a scale much greater than the annual outbreaks, sweeping across the world with huge numbers of infections, and considerable morbidity and mortality. These latter phenomena are referred to as pandemics, and experience in the 20th century plus careful reading of historical records suggest that these have occurred about every 30 years or so. Influenza epidemics and pandemics arise from the processes of antigenic drift and antigenic shift, respectively.

Antigenic drift results in the emergence of new strains each year. It arises from random spontaneous mutation occurring within the influenza virus genome as it replicates. Virus causing an outbreak in a particular year will have up to 1% genome sequence difference from virus that caused the previous year's outbreak. Although this will occur across the whole viral genome, it is the mutations within the genes encoding the HA and NA surface glycoproteins that are important in this context. The HA protein contains five highly immunogenic regions (Figure 3) to which the antibody response to infection is directed. Mutations within these **epitopes** may therefore allow virus to escape the inhibitory effects of antibodies that would otherwise bind to these regions and prevent virus–cell interactions. The important amino acid differences that accumulate year-on-year within this protein are clustered precisely within these five epitopes. Thus, antigenic drift is an excellent example of Darwinian evolution – mutations occur randomly within the genome, but only those that confer a selective advantage to the virus emerge in the epidemic strain, the selective pressure being the population immune response generated by the previous year's epidemic. Drift occurs in both influenza A and B viruses.

Antigenic shift, which generates the new pandemic strains, is an altogether different process. The viruses causing the influenza pandemics of the 20th century are shown in Table 1. Each pandemic arose from the emergence of a new influenza A subtype into the human population. As the new pandemic strain appeared, so the old circulating strain disappeared – thus, in 1956–57, H2N2 completely replaced H1N1, only to be replaced itself by H3N2 virus in 1968 (an unusual exception to this, arising in 1976, is discussed later). There are two possible underlying mechanisms that can give rise to new pandemic strains, as described below.

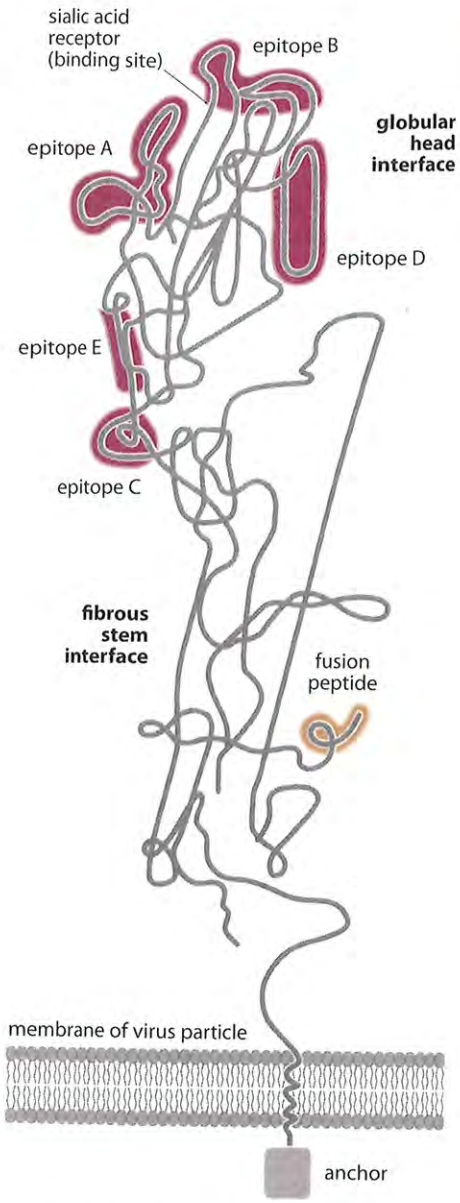

Figure 3. Hemagglutinin (HA) structure showing five key epitopes. This shows the protein chain of a single subunit of influenza A hemagglutinin trimer. Epitopes A, B, C, D, and E are positions where antibody molecules have been shown to bind to HA. The three dimensional structure was determined by X-ray diffraction of the crystalline protein.

Table 1. Pandemic influenza viruses of the 20th century

Year	*Virus*	*Name*
1918–19	H1N1	Spanish flu
1956–57	H2N2	Asian flu
1968	H3N2	Hong Kong flu

1. Direct transfer of an avian influenza A virus into humans. This process is undoubtedly happening at the moment, with an increasing number of human infections with the avian H5N1 virus (responsible for large avian epidemics, particularly among chickens) being reported worldwide. However, virus that crosses a species barrier in this way is often not well adjusted for replication in its new host. Currently, avian H5N1 virus does not replicate to high titer within infected humans. Person to person spread is therefore very inefficient, as infected individuals are not releasing large amounts of virus in their respiratory secretions. H5N1 virus has thus not emerged (yet) as a new human pandemic virus. There is a worry, however, that as it replicates within human cells, this virus may acquire mutations that could result in adaptation to efficient replication within human cells, at which point person to person spread will become more likely, and a true pandemic might eventuate. There is some evidence that the H1N1 virus that caused the 1918–19 pandemic was entirely avian in origin, and that it had been causing sporadic infections within humans for several years before its emergence as a pandemic virus in 1918. The presumption is that during those preceding years the virus acquired the necessary mutations to allow adaptation to increased replication within human cells.
2. Genetic reassortment of human and avian viruses within a co-infected host (Figure 4). Influenza viruses have a segmented genome. Thus, if a cell is infected with two different influenza viruses, it is possible that reassortment (mixing) of these gene segments can occur, such that progeny virus can contain gene segments derived from either one of the 'parent' viruses. In Figure 4, the emergent virus has six RNA segments derived from the human parent virus, plus segments 4 and 6 from the avian parent. Segments 4 and 6 encode the HA and NA proteins, respectively. Thus, the progeny virus will be one that is well adapted for growth in human cells (all its internal proteins are derived from the human parent), but has two entirely new proteins on its surface (each HA and NA protein differing by at least 20% amino acid sequence from all other HA and NA proteins). Such a virus would cause devastating infection across the whole human population, as no-one would have any immunity against these new surface proteins. The H2N2 and H3N2 pandemic viruses from 1956 and 1968 do indeed contain genes derived from both human and avian viruses. It is believed that the reassortment process that generated these viruses took place within pigs (hence referred to as the 'mixing vessel'), which are uniquely susceptible to infection with both human and avian viruses. However, it has become at least a theoretical possibility that humans themselves could act as the mixing vessel, for example if a human was co-infected simultaneously with an avian A/H5N1 and a human A/H1N1 or A/H3N2 virus. The

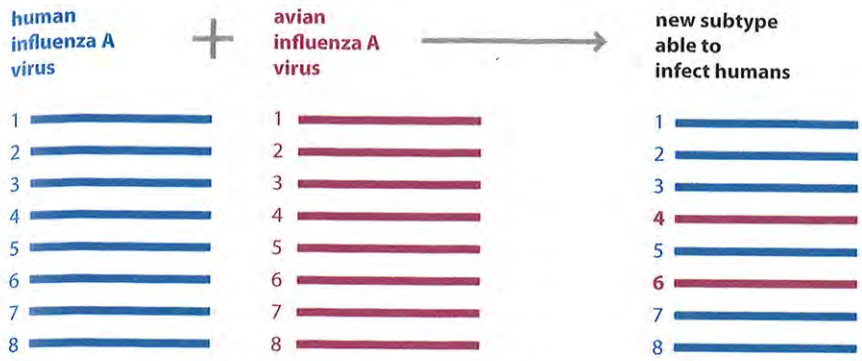

Figure 4. Genetic reassortment. Each RNA segment (numbered 1–8) is represented by a horizontal line. The human virus is blue, the avian virus is red. When co-infecting the same cell, emergent viruses may possess RNA segments from either 'parent' virus.

chances of the latter happening will clearly be increased the more humans become infected with avian A/H5N1 virus.

It is worth emphasizing the differences between antigenic drift and shift. The former occurs in both influenza A and B viruses, and is a result of random genetic mutation followed by Darwinian selection, resulting in up to 1% differences in amino acid sequences focused within key epitopes within the surface HA and NA molecules. The latter only occurs in influenza A viruses (presumably because influenza B viruses do not have an animal reservoir), and is a result of either direct cross-species transfer or of genetic reassortment, resulting in the generation of viruses with surface HA and NA proteins that differ by over 20% in amino acid sequence from those in previously circulating strains.

In 1976, Russian flu emerged, caused by an influenza A/H1N1 virus. This is not presented in Table 1 as a pandemic, because the mechanism of emergence of this virus is not believed to have been a natural occurrence. Instead, there is evidence that this virus emerged from a laboratory – its gene sequences were remarkably similar to those of the last previous isolates of H1N1 virus in 1957. Thus its reappearance in the human population was most likely due to human error. Interestingly, this virus did not displace the A/H3N2 virus and ever since 1976, both A/H1N1 and A/H3N2 influenza viruses have co-circulated among humans, together with influenza B viruses. Any one of the three circulating viruses can predominate in a particular year.

2. What is the host response to the infection and what is the disease pathogenesis?

Damage to the respiratory epithelial surface occurs due to the **cytolytic** interaction of the virus and the host cell, that is the infected host cells undergo acute cell death. In effect, the virus strips off the inner lining of the respiratory tract, and in so doing, removes two important innate immune defence mechanisms – mucus-secreting cells, and the **muco-ciliary escalator**. The production of mucus by cells within the respiratory epithelium allows entrapment of inhaled particulate matter (e.g. bacteria). The muco-ciliary escalator then transports any inhaled particulate matter towards the pharynx, to be coughed out in sputum or swallowed. Removal of these defenses results in potential exposure of the lower respiratory tract to inhaled particulate matter, such as bacteria.

The above processes result in impairment of lung function to a greater or lesser extent, so patients present with **rhinorrhea**, sore throat, cough, and shortness of breath. However, there is also an important systemic element to the disease influenza (see below). This arises because influenza viruses are potent inducers of **cytokines** such as **interferon-α** (**IFN-α**) and **interleukin** (**IL**)-6, and it is these cytokines, not the virus, that circulate in the bloodstream and give rise to the systemic manifestations of fever, headache, muscle aches and pains, and severe malaise. Administration of IFN, for example as treatment for chronic hepatitis C virus infection, reproduces this symptomatology. Note that the above applies strictly to infection with the currently circulating human influenza viruses – there is emerging evidence that infection of humans with avian influenza A/H5N1 may result in **viremia** and spread to other organs beyond the lungs.

In addition to this innate immune response to infection, adaptive humoral and cellular immune responses are also stimulated. Antibodies to the surface proteins, particularly hemagglutinin, may be neutralizing, that is they can prevent the interaction of the HA protein with cellular sialic acid residues and thereby prevent infection. However, antigenic drift results in the generation of strains of virus that can escape this protective immunity. T-cell responses to influenza virus are mostly directed against antigens derived from the internal viral proteins, for example the nucleoprotein. These proteins are much more conserved within influenza types than the surface proteins, so T-cell immunity may offer some protection each year to emerging drifted viruses.

3. What is the typical clinical presentation and what complications can occur?

There are two distinct components to the illness that arises following infection with influenza virus – a respiratory tract component, plus a marked systemic illness characterized by fever, headache, and **myalgia**. Infection does not necessarily result in clinical disease – this will be dependent on the pre-existing state of the patient's lung function, the infecting dose of virus, the presence of pre-existing immunity and the extent to which that immunity is able to cross-react with a new viral strain. However, symptomatic influenza virus infection is not a trivial illness. There is considerable morbidity, and it may take several days before patients are well enough to return to their normal daily activities.

The commonest life-threatening complication of influenza virus infection is **pneumonia**, of which there are two pathological types.

Primary influenzal pneumonia. The virus itself infects right down to the alveoli. There is a mononuclear cell infiltrate into the alveolar walls, and the airspaces become filled with fibrinous inflammatory exudates. This can occur in previously healthy individuals of any age.

Secondary bacterial pneumonia. In recent years, this has been considerably more common than viral pneumonia. Bacteria gain access to the lower respiratory tract for reasons explained above. There is a polymorphonuclear cell infiltrate into the alveoli. This complication is more common in the elderly and in those with pre-existing lung disease, of whatever etiology, for example chronic bronchitis.

There is some evidence that influenza infection can also result in a **myocarditis** – certainly patients with pre-existing cardiovascular disease are at increased risk of mortality should they acquire infection. An **encephalitis** (inflammation of the brain substance) is also well recognized. This is not due to the virus itself gaining access to the brain – as explained above, virus is restricted to the lungs. Thus, this is believed to be an immune-mediated phenomenon, or so-called post-infectious encephalitis. In certain individuals, the immune response generated to the influenza virus infection can cross-react with antigens present within the brain, resulting in an encephalitis.

Human infection with avian influenza A H5N1 carries a very high mortality (>50%), and yet, as outlined above, this virus does not replicate efficiently within human cells. This apparent paradox is explained by the fact that this virus induces an explosive acute inflammatory reaction within the lungs – referred to as a **cytokine storm**. The high mortality thus arises from the inappropriate hyperactivity of the host immune response to infection, as opposed to the cytolytic properties of the virus itself.

4. How is this disease diagnosed and what is the differential diagnosis?

Infections with a number of different agents (mostly viruses) can result in presentation with an 'influenza-like illness.' Infection with respiratory syncytial virus, especially in the elderly, is the most common mimic of influenza virus infection. Other possibilities include human metapneumovirus, adenoviruses, and *Mycoplasma pneumoniae*. Clinical 'end-of-the-bed' diagnosis is therefore neither sensitive nor specific enough for practical purposes – with the advent of antiviral drugs that are absolutely specific for influenza viruses, accurate diagnosis is necessary to ensure that these drugs are used appropriately and effectively. As the efficacy of these drugs is dependent on initiation of their use as soon as possible after infection, there is a need for rapidity as well as accuracy.

There are two main approaches to the rapid diagnosis of influenza virus infection. Historically, most laboratories relied on immunofluorescent (IF) antigen detection using monoclonal anti-influenza antibodies (Figure 5). This technique relies on the fact that cells in which a virus is replicating will express viral antigens somewhere within the cell. Thus, cells from the patient (e.g. from a throat swab, or ideally, a nasopharyngeal aspirate) are spotted down onto a glass slide, and a fluorescently tagged monoclonal antibody is added. The antibody will bind to cells infected with virus, but not to uninfected cells. After an incubation period, any unbound antibody is washed off, and the cells are examined using a fluorescence microscope. The presence of brightly fluorescent cells is a positive result indicating that the patient was infected with the virus to which the monoclonal antibody was raised. The whole process takes about 2 hours. More recently, some laboratories have adopted a genome detection technique such as the **polymerase chain reaction (PCR)** assay (with prior reverse transcription, as influenza virus carries an RNA genome, but PCR only amplifies DNA). PCR-based assays have a major advantage in terms of their incredible sensitivity, as theoretically they result in several logs of amplification of the targeted nucleic acid sequences. Real-time PCR assays are also rapid. In

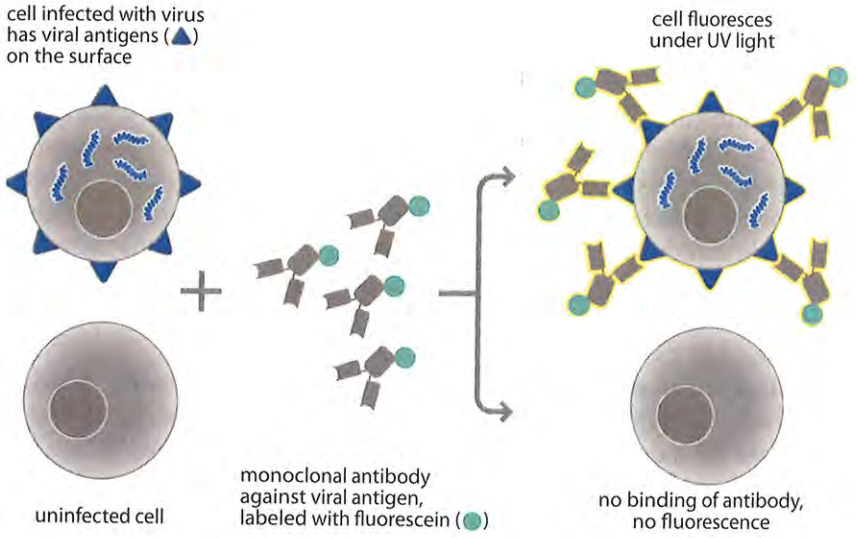

Figure 5. Detection of influenza virus by immunofluorescence. A throat swab (or ideally, a nasopharyngeal aspirate) is spotted onto a glass slide, and a fluorescently tagged monoclonal antibody is added. The antibody binds to cells infected with virus, but not to uninfected cells. After an incubation period, any unbound antibody is washed off, and the cells are examined under ultraviolet light. The presence of brightly fluorescent cells is a positive result indicating that the patient is infected with the virus to which the monoclonal antibody was raised. The whole process takes about 2 hours.

the context of anxieties about the spread of avian influenza H5N1 into humans, and the possible emergence of a new pandemic strain, most countries have adopted pandemic plans that rely on diagnosis of the first case (or cases) of infection as soon as possible, in order to institute emergency infection control procedures. Real-time PCR assays are recognized as the gold standard in this context.

If rapidity of diagnosis is not an issue (e.g. in epidemiological monitoring of infection), then alternative diagnostic approaches include isolation of virus in cell culture, and demonstration of a fourfold or greater rise in anti-influenza virus antibody titers in peripheral blood samples taken 7 days or more apart. Influenza viruses grow well in cell culture, but may take several days before their presence is revealed by the development of a **cytopathic** effect. **Serological** diagnosis (i.e. a rise in antibody titer) is also slow, since it requires, by definition, a second sample taken some days after the patient presents with acute illness.

5. How is the disease managed and prevented?

Management

Many cases of influenza virus infection require symptomatic relief only, for example with anti-pyretics and analgesics such as paracetamol, or other proprietary over-the-counter or home-grown remedies.

For the more seriously ill patients there are two classes of anti-influenzal agents shown to be effective. The first of these include amantadine and rimantadine. These drugs work by preventing the uncoating of influenza virions that have entered a target cell. They do this by binding to the viral matrix M2 protein and thereby blocking ion channels, whose function is essential for the pH-mediated dissolution of the viral capsid, that is uncoating (see Section 1). While appropriately conducted **clinical trials** of amantadine have provided clear evidence of efficacy, there are a number of drawbacks to the use of the agents. Firstly, they only work against influenza A viruses and therefore are of no benefit in a patient infected with influenza B virus. Secondly, these drugs are also dopamine agonists

and therefore have marked central nervous system stimulatory activity – in fact, amantadine was originally developed for the treatment of Parkinson's disease. Thus, it is very poorly tolerated in the elderly, the precise group of patients who are most likely to require antiviral therapy, giving rise to hallucinations, insomnia, and agitation. Thirdly, resistance to amantadine emerges within a few days of onset of therapy, due to point mutations in the M2 protein. Finally, many of the avian influenza viruses, including H5N1 strains, are inherently resistant to amantadine. For all of the above reasons, this class of drugs is therefore not widely recommended.

The second class of anti-influenzal drugs comprise the neuraminidase inhibitors (Figure 6). The development of these inhibitors was a purposeful effort to design effective antiviral drugs through a logical process – these molecules can therefore be regarded as 'designer drugs.' The influenza neuraminidase was purified, crystallized, and its three-dimensional structure was elucidated. Small molecules were then designed to bind to the active site of the enzyme. One advantage of this class of drugs is that they have activity against all known influenza neuraminidase subtypes. Both of the currently licensed members of this family, zanamavir and oseltamivir, are effective in inhibiting production of infectious viral particles, and are effective in randomized clinical trials. Currently, their use in the UK is reserved for the treatment of seriously ill patients admitted to hospital, although in the USA they may be prescribed by primary care physicians. The importance of these drugs is illustrated by the decision of several governments to stockpile millions of doses as part of their influenza pandemic preparedness plans. Unsurprisingly, however, as these drugs are more widely used, there are increasing reports of viral variants

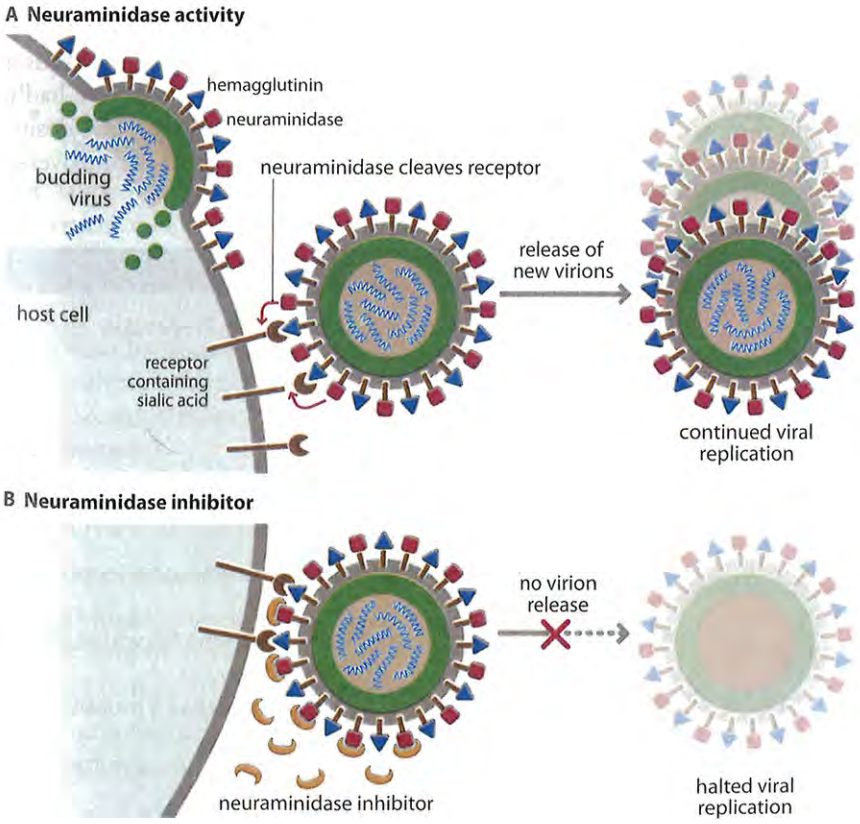

Figure 6. Neuraminidase on the surface of the virus fulfills an essential role in the life cycle of the virus. As newly formed viral particles bud out of an infected cell (A), the hemagglutinin on the viral surface would naturally bind to sialic acid receptors on the surface of the cell. Thus, it would not be possible for these new virus particles to move away from the cell and infect other cells, were it not for the fact that the neuraminidase is there to remove the sialic acid residues and release the viral particles. Thus, inhibition of the viral neuraminidase by small molecule inhibitors (B) prevents virus release from the cell and therefore also prevents any downstream viral infection of and replication within other cells.

emerging with point mutations within the neuraminidase gene that confer drug resistance. Worryingly, oseltamivir-resistant avian H5N1 strains have now been documented to occur in infected humans.

Prevention

Vaccines

Prevention of influenza virus infection is possible through the use of active vaccination. There are two different types of vaccine currently in use. Inactivated vaccines contain either whole virus grown in embryonic hens' eggs and chemically inactivated, or just the surface HA and NA proteins purified from whole virus – the latter are less reactogenic. Live attenuated vaccines are a relatively recent development, containing cold-adapted viruses, and administered by intranasal inoculation. Preliminary data from the use of these novel vaccines suggest that they induce a more robust protection from influenza infection, possibly even from antigenic drift variants. It remains to be seen whether these live vaccines will ultimately replace the use of the inactivated ones. The propensity for influenza viruses to undergo antigenic drift and shift creates major problems for the vaccine manufacturers, as essentially the viruses are moving targets. In practice, the World Health Organization (WHO) monitors circulating influenza viruses through the collaborative activities of a large number of reference laboratories around the world. Each year, the WHO (based on monitoring current strains in its reference laboratories) announces which particular A/H1N1, A/H3N2, and B viral strains should be used for vaccine being manufactured for the following influenza season. The protection offered by these vaccines depends to a large extent on the degree of antigenic match between the vaccine strains and the strains actually circulating during the season – some years this is better than others!

The availability of a vaccine then begs the question of who should be vaccinated. Most countries adopt a selective policy, that is the recommendation is to vaccinate those subgroups within the population who will fare badly should they acquire infection. Table 2 lists those groups in the UK designated in the Department of Health guidelines. There is some controversy

Table 2. Target groups for influenza vaccination

UK – Department of Health recommendations

- Patients aged 6 months or older with underlying:
  - chronic respiratory disease (including asthma)
  - chronic heart disease
  - diabetes requiring insulin or oral hypoglycemic drugs
  - chronic renal disease
  - immunosuppression
  - chronic liver disease
- Individuals over the age of 65
- Health and social care staff directly involved in patient care
- People who live in nursing homes and other long-term care facilities

USA – Centers for Disease Control and Prevention recommendations

- Children aged 6 months to 5 years
- Pregnant women
- Individuals over the age of 50
- Patients with certain chronic medical conditions (see list above)
- People who live in nursing homes and other long-term care facilities
- Household contacts of individuals who fall within the groups above
- Household contacts of children less than 6 months of age
- Health-care workers

as to whether children should also be vaccinated. There are also some important contraindications to the use of influenza vaccine. Given the nature of the inactivated vaccine, patients with allergy to egg proteins should not be vaccinated.

Prophylaxis with anti-influenzal drugs is also effective at preventing infection, although clearly the protection mediated by this approach only lasts as long as the drugs are administered. However, this will be important if/when the next pandemic emerges. It will take some months before an effective vaccine against the pandemic strain of virus is developed and manufactured in enough doses to offer realistic protection on a population basis. Thus, initial prevention measures once a new pandemic strain is identified may well include the use of prophylaxis with the neuraminidase inhibitors.

SUMMARY

1. What is the causative agent, how does it enter the body and how does it spread a) within the body and b) from person to person?

- Influenza A, B, and C viruses, carry a segmented (n = 8) negative single-stranded RNA genome, are enveloped, and belong to the family *Orthomyxoviridae*.
- Typing into A, B, C is according to the nature of the internal proteins.
- Type A is subtyped according to the nature of the surface H and N proteins.
- Entry is via inhalation, or droplet inoculation onto oropharyngeal mucous membranes.
- Viral tropism is for respiratory epithelial cells, with no evidence of spread beyond the lungs (except perhaps for avian H5N1 infection of humans).
- The viral ligand hemagglutinin binds to cell surface sialic acid receptors.
- On entry the virus uncoats and replication begins.
- Epidemiology is characterized by pandemics arising from antigenic shift, and interpandemic epidemics, arising from antigenic drift.
- Antigenic drift is due to Darwinian selection of variants with mutations in key neutralizing epitopes within the surface glycoproteins in the face of immune selection pressure.
- Antigenic shift is due to the emergence of a new influenza A subtype.
- Possible mechanisms include genetic reassortment of human and avian influenza viruses within a mixing vessel, either pigs or humans, or direct trans-species transfer of avian viruses to humans, with subsequent adaptive mutations.
- The last pandemic, due to A/H3N2 virus, was in 1968.
- Currently, A/H1N1, A/H3N2, and B viruses co-circulate, giving rise to annual interpandemic epidemics.

2. What is the host response to the infection and what is the disease pathogenesis?

- Viral infection results in **lysis** or **apoptosis** of the cell.
- Influenza virus infection therefore effectively strips off the inner lining of the respiratory tract, including mucus-secreting and ciliated epithelial cells.
- This predisposes to inhalation of particulate matter, including bacteria, into the lower respiratory tract.

- Innate immune responses include potent induction of interferon and other cytokines.
- Adaptive immune responses include neutralizing antibody production and T-cell responses.

3. What is the typical clinical presentation and what complications can occur?

- There are two components to the clinical manifestations of influenza virus infection.
- Respiratory tract symptomatology (e.g. rhinorrhea, cough) arises from local cellular damage and inflammation.
- Systemic manifestations (fever, headache, pronounced myalgia) arise from the effects of circulating cytokines.
- Life-threatening complications include primary influenzal pneumonia, secondary bacterial pneumonia, myocarditis, and post-infectious encephalitis.
- Avian H5N1 infection of humans has a high mortality, due to an intense acute inflammatory reaction within the lungs (a cytokine storm).

4. How is this disease diagnosed and what is the differential diagnosis?

- Diagnosis is by demonstration of influenza virus in a respiratory sample – for example nasopharyngeal aspirate, throat swab.
- Immunofluorescent antigen detection with labeled monoclonal anti-influenza antibodies is a rapid, specific, and reasonably sensitive approach.
- Influenza virus RNA detection by genome amplification (e.g. real-time reverse transcriptase polymerase chain reaction assay) is also rapid and much more sensitive. This is the approach used for early diagnosis of human infections with avian A/H5N1 virus.
- Retrospective diagnosis can be made by isolation of virus in cell culture (takes several days), or by demonstration of a rise in specific antibody titers (requires a blood sample taken several days after onset of illness).
- Many other infections may present with an 'influenza-like illness' – the most common is with respiratory syncytial virus.

5. How is the disease managed and prevented?

- The majority of patients infected with influenza virus infection can be managed symptomatically, for example with appropriate analgesia and antipyretics.
- Amantadine has activity against some (not all) influenza A viruses. It works by binding to the M2 protein and blocking an ion channel necessary for uncoating of the virus.
- It has central nervous system stimulatory side effects and is not well tolerated, particularly in the elderly.
- Resistance emerges rapidly due to mutations in the M2 protein.
- The neuraminidase inhibitors (zanamavir, oseltamivir) have activity against all known influenza virus neuraminidase enzymes. In the UK, their use is focused on seriously ill hospitalized patients.
- Resistance to the neuraminidase inhibitors has been reported, due to point mutations within the NA gene.
- Vaccination is with either inactivated whole virus, a subunit derivative containing only purified hemagglutinin and neuraminidase, or live attenuated vaccines.
- The vaccines are trivalent (i.e. contain antigens from all three co-circulating viruses).
- Vaccine composition is adjusted annually to take account of antigenic drift.
- Vaccine targeting may differ in different countries. In the UK, recommendations are to vaccinate high-risk subgroups within the general population, that is those with pre-existing respiratory, cardiac, renal, endocrine, or liver disease, immunodeficiency, or those over the age of 65.
- Targeted individuals require annual vaccination.
- An alternative to vaccination is prophylactic use of neuraminidase inhibitors.

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MULTIPLE CHOICE QUESTIONS

The questions should be answered either by selecting True (T) or False (F) for each answer statement, or by selecting the answer statements which best answer the question. Answers can be found in the back of the book.

1. **Which of the following statements regarding influenza viruses are true?**
   - A. Their genome consists of eight segments of double-stranded RNA.
   - B. They are classified into types A, B, and C on the basis of the nature of their internal proteins, particularly the nucleoprotein.
   - C. Type A influenza viruses are further subdivided into subtypes on the basis of the nature of their matrix proteins.
   - D. Type B influenza viruses are further subdivided into subtypes on the basis of the nature of their surface proteins.
   - E. There is no animal reservoir of type B influenza viruses.

2. **The emergence of new pandemic strains of influenza virus may arise from which of the following processes?**
   - A. Trans-species transfer of an avian influenza virus directly to humans.
   - B. Spontaneous mutations in the genes encoding the surface glycoproteins.
   - C. Reassortment of avian and human influenza viruses within a single host.
   - D. Use of neuraminidase inhibitors resulting in mutations in the neuraminidase gene.
   - E. Natural selection of viral variants in an immunized host population.

MULTIPLE CHOICE QUESTIONS (continued)

3. **Which of the following statements regarding the epidemiology of influenza viruses is/are correct?**
   A. Influenza B viruses undergo antigenic shift but not antigenic drift.
   B. Antigenic shift in influenza viruses gives rise to global pandemics of influenza.
   C. Antigenic drift in influenza viruses gives rise to interpandemic epidemics of influenza.
   D. Antigenic shift describes the emergence of new influenza A virus subtypes.
   E. Antigenic drift results in amino acid changes clustered within key epitopes of the viral nucleoprotein.

4. **Which of the following statements regarding disease associated with influenza virus infection is/are true?**
   A. Influenza virus infection of respiratory epithelial cells results in transformation of those cells.
   B. Systemic manifestations of influenza virus infection (e.g. fever, myalgia, headache) arise from the presence of virus circulating in the bloodstream.
   C. Pneumonia arising as a complication of influenza virus infection is usually due to secondary bacterial invasion.
   D. Influenza-related mortality is higher in patients with pre-existing cardiac disease.
   E. Influenza-related encephalitis arises through cross-reactivity of the immune response to infection with the patient's brain tissue.

5. **Which of the following statements regarding the avian H5N1 influenza virus are true?**
   A. Infection of humans results in death in 10–20% of cases.
   B. The pathogenesis of disease arises from explosive release of cytokines within the respiratory tract.
   C. Infection can be prevented by vaccination with vaccines containing antigens derived from A/H1N1 and A/H3N2 viruses.
   D. This virus is always sensitive to oseltamivir.
   E. This virus is a possible candidate for the next influenza pandemic.

6. **Which of the following statements regarding diagnostic tests is/are true?**
   A. Virus isolation in cell culture is a rapid diagnostic technique.
   B. Antigen detection techniques are dependent on the presence of viable virus in the sample sent to the laboratory.
   C. Genome detection techniques are the most sensitive assays for diagnosis of virus infections.
   D. Genome amplification assays cannot be used for RNA viruses.
   E. Demonstration of high antibody titers to the H5 hemagglutinin in serum samples taken from acutely ill patients will be the mainstay of diagnosis of human infection with avian H5N1 influenza virus.

7. **Which of the following drugs has proven efficacy against influenza A viruses?**
   A. Aciclovir.
   B. Foscarnet.
   C. Indinavir.
   D. Zanamavir.
   E. Zidovudine.

8. **With regard to anti-influenza drugs, which of the following statements are true?**
   A. Amantadine is effective as a prophylactic agent against influenza B virus.
   B. The mode of action of amantadine involves blockage of an ion channel and prevention of viral uncoating.
   C. Neuraminidase inhibitors have no activity against influenza B virus.
   D. Resistance to oseltamivir has not been described in influenza A viruses.
   E. Zanamavir should not be used in patients with a history of egg allergy.

9. **Which of the following statements regarding influenza are true?**
   A. Influenza re-infections occur despite the presence of high levels of serum antibodies.
   B. Influenza pandemics have only occurred since the beginning of the 20th century.
   C. Human influenza viruses only infect humans.
   D. Epidemics of influenza occur in the winter months in the northern hemisphere.
   E. Infections with influenza B virus are less severe than those with influenza C virus.

10. **With regard to influenza vaccines, which of the following statements are true?**
    A. Vaccine-induced immunity is clinically useful for at least 10 years.
    B. Universal vaccination against influenza is currently recommended.
    C. Inactivated influenza vaccines are contraindicated in immunosuppressed individuals.
    D. Influenza vaccines contain antigens derived from A/H1N1, A/H3N2, and B viruses.
    E. Live attenuated influenza vaccines are administered by subcutaneous injection.

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Case-control study of risk factors for human infection with avian influenza A(H7N9) virus in Shanghai, China, 2013

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SUMMARY

The first human infection with avian influenza A(H7N9) virus was reported in Shanghai, China in March 2013. An additional 32 cases of human H7N9 infection were identified in the following months from March to April 2013 in Shanghai. Here we conducted a case-control study of the patients with H7N9 infection ($n = 25$) using controls matched by age, sex, and residence to determine risk factors for H7N9 infection. Our findings suggest that chronic disease and frequency of visiting a live poultry market (>10 times, or 1–9 times during the 2 weeks before illness onset) were likely to be significantly associated with H7N9 infection, with the odds ratios being 4·07 [95% confidence interval (CI) 1·32–12·56], 10·61 (95% CI 1·85–60·74), and 3·76 (95% CI 1·31–10·79), respectively. Effective strategies for live poultry market control should be reinforced and ongoing education of the public is warranted to promote behavioural changes that can help to eliminate direct or indirect contact with influenza A(H7N9) virus.

Key words: Avian influenza A(H7N9) virus, case-control study, human infection, risk factor.

INTRODUCTION

Avian influenza A(H7N9) infections are normally seen in animals and are mostly asymptomatic [1]. Human infections with H7N9 are uncommon [2]. The first human case of H7N9 infection was reported on 31 March 2013 in Shanghai, China [3]. As of 31 December 2013, the China National Health and Family Planning Commission has reported 144 laboratory-confirmed case of human H7N9 infection in mainland China, with 46 (31·94%) deaths. This rapid expansion of H7N9 infections has raised concerns regarding the pandemic potential of H7N9 virus. However, investigations of risk factors for human H7N9 infection are rare. Although an analytical study conducted in Jiangsu province identified chronic illness and environment-related exposure as risk factors for human infection with H7N9 [4], current H7N9 outbreaks in China suggest different geographical, sociodemographical, or behavioural contexts might be involved in virus transmission.

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Therefore, further studies are needed to clarify the mode of transmission of H7N9 viruses from animals to humans. Here we conducted a case-control study to identify potential risk factors for H7N9 infection in Shanghai, where the first case of human H7N9 infection occurred, and to guide the strategy for control and prevention of H7N9 infection.

METHODS

Subjects

As of December 2013, 33 laboratory-confirmed cases of human infection with H7N9 have been reported in Shanghai, China, resulting in 18 (54·5%) deaths. All confirmed cases of human H7N9 infection in Shanghai were encouraged to enrol into this study. Of the 33 H7N9 cases, 25, including 11 deceased cases that were confirmed positive for H7N9 using validated real-time RT–PCR TaqMan® assay [5] were finally included in the study. The remaining eight cases (seven fatal cases) were excluded from the study owing to refusal to participate by the cases or their proxies. Cases were defined following the Diagnosis and Treatment Guideline of Human Infection with Avian Influenza A (H7N9) Virus issued by the Chinese National Health and Family Planning Commission [6]. Specifically, all cases of human H7N9 infection in this study had symptoms of fever (oral temperature $\geqslant$38 °C), cough, headache or severe pneumonia. All cases had a history of poultry exposure or close contact with H7N9 patients during the 2 weeks prior to their illness onset and were seropositive for H7N9 virus. Each case was matched with three controls (75 controls in total) that were of the same gender, had less than 3 years' age difference, and had lived in the same community or village for more than 6 months. All of the controls were seronegative for H7N9, and had no respiratory symptoms and fever ($\geqslant$38 °C) in the 2 weeks prior to illness onset of the matched cases. If there were not enough eligible controls, the closest neighbours were recruited instead. For example, for H7N9 cases in the urban area, controls were first recruited from the same unit of the block where the cases lived, this was then expanded to the adjacent unit of this block if necessary. For H7N9 cases in the rural area, controls were recruited from the nearest neighbours in the village where the cases lived. The interviewing staff went from door-to-door asking for volunteer controls. Of a total of 80 controls invited to participate, 75 were finally enrolled in this study.

Data and sample collection

Data were collected from 27 May to 7 June 2013. All of the participants were interviewed by the trained employees of the local district Center for Disease Control and Prevention using interviewer-administered questionnaires. The questionnaire was self-developed and a pre-test was performed prior to the official investigation. The questionnaires consisted of demographical characteristics, health status, daily habits, and other related potential risk behaviours including infrequent hand-washing before meals or after using the bathroom and smoking. The questionnaires also included environment-related exposure variables including visiting a live poultry market, visiting a temporary roadside poultry vendor, raising chickens or pigeons in the neighbourhood, or other activities involving direct and indirect contact with live poultry during the 2 weeks before illness onset of the cases. Direct contact was referred to as touching live poultry with bare hands in a live poultry market (slaughtering or purchasing poultry), at home (raising, cleaning or processing poultry), or occupational exposure to live poultry without protection (poultry transportation, restaurant poultry preparation and cooking). Indirect contact was defined as being in close proximity (<1 m away from poultry) at home without direct physical contact. All questions were close-ended. Proxies were interviewed for the deceased patients (n = 11), severe H7N9 patients who were too sick to respond to the interviewers (n = 3), or subjects aged <6 years (n = 4). To ensure accuracy of proxy data, spouses or parents who lived together with the patients for more than 2 weeks before illness onset were interviewed and hospital medical records of the patients were reviewed as well. Data from the medical records were used if there was a discrepancy between the proxy description and the medical records. Following the interview, 5 ml of venous blood from each control was collected for laboratory testing of H7N9 to exclude asymptomatic or past H7N9 infection.

Laboratory analysis

Serum samples from the controls were tested using haemagglutination inhibition (HI) assay with turkey red blood cells against avian influenza A(H7N9) virus strain (A/Shanghai/2/2013). The HI was performed following the Diagnosis and Treatment Guideline of Human Infection with Avian Influenza

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A(H7N9) Virus [5]. The serum from a confirmed H7N9 case was used as a positive reference.

Statistics

All tests were performed two-sided at the 5% significance level. The Wilcoxon rank sum test, Pearson's χ^2 test and Fisher's exact test were performed to analyse the difference of general characteristics between cases and controls. Potential risk factors were compared between cases and controls, using univariate logistic regression. Given human H7N9 infection is uncommon and the studies of risk factors for human H7N9 infection are rare, we treated all the variables in this study as potential significant factors. Therefore, we further conducted a backward stepwise (entry and removal probability were 0·05 and 0·10, respectively) multivariate logistic regression analysis including all variables in the univariate analysis to correct possible confounding factors. All statistical analyses were performed using SAS v. 9·2 (SAS Institute Inc., USA).

Ethical approval

The objectives and methods of the study were clearly explained to all participants. Informed written consent from participants or their proxies was obtained before data collection. The ethical approval for the study was obtained from the Ethics Committee of Shanghai Municipal Centre for Disease Control and Prevention and the study was conducted in full compliance with the principles of the Declaration of Helsinki.

RESULTS

General characteristics

Of the 25 cases of human H7N9 infection, only two cases had occupational exposure to live poultry. One was engaged in poultry transportation, and the other worked in a restaurant preparing and cooking poultry. Data for 15 cases (11 fatal and four discharged) and three controls (all aged <6 years) were obtained from their proxies. Data for the remaining 10 cases and 72 controls were provided by the subjects themselves. All of the enrolled controls were seronegative for H7N9.

The demographical and social characteristics of subjects are shown in Table 1. The age of the cases varied from 2·5 to 89 years, with a median age of 69 years. The age of the controls varied from 2 to 92 years (median 67 years). All subjects were aged >25 years except for one case (2·5 years) and three controls (aged 2–5 years). Eighteen (72·0%) cases were aged >60 years and 21 (84·0%) cases were male. Twenty-one cases lived in an urban area. Eighteen cases and 36 controls had been diagnosed with chronic diseases, including chronic bronchitis, hypertension, diabetes, pulmonary disease, or heart disease. The percentage of chronic medical conditions in cases was significantly higher than that in

Table 1. *Demographic and social characteristics of participants in a case-control study of avian influenza A(H7N9) in Shanghai, China*

Variables	Cases ($n = 25$) n (%)	Controls ($n = 75$) n (%)	P value
Age, yr (median, range)	69 (2·5–89·0)	67 (2·0–92·0)	
<60	7 (28·0)	23 (30·7)	0·464*
⩾60	18 (72·0)	52 (69·3)	
Male	21 (84·0)	63 (84·0)	
Location			
Urban	21 (84·0)	63 (84·0)	
Rural	4 (16·0)	12 (16·0)	
Body mass index			0·557†
<20	3 (12·0)	8 (10·7)	
20–25	12 (48·0)	45 (60·0)	
⩾25	10 (40·0)	22 (29·7)	
Diagnosed chronic diseases			
No	7 (28·0)	39 (52·0)	0·037†
Yes	18 (72·0)	36 (48·0)	
Education			
Primary school and below	7 (28·0)	17 (22·7)	0·635†
Junior middle school	8 (32·0)	24 (32·0)	
Senior middle school	5 (20·0)	24 (32·0)	
College and higher	5 (20·0)	10 (13·3)	
Household income *per capita*			0·542‡
<5000 RMB§	4 (16·0)	12 (16·0)	
5000–10 000 RMB	1 (4·0)	7 (9·3)	
10 000–20 000 RMB	3 (12·0)	17 (22·7)	
>20 000 RMB	17 (68·0)	39 (52·0)	

* Wilcoxon rank sum test.
† Pearson's χ^2 test.
‡ Fisher's exact test.
§ 10 RMB = ~1 GBP.

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controls ($P < 0{\cdot}05$). However, there was no statistically significant difference in terms of characteristics including body mass index (BMI), education level and *per capita* household income between cases and controls ($P > 0{\cdot}05$).

Univariate analysis of risk factors for human H7N9 infection

The univariate analysis of possible risk factors for human H7N9 infection is shown in Table 2. Persons with chronic medical conditions appeared to be susceptible to H7N9 infection as 72% of the cases had chronic diseases compared to 48% of the controls [unadjusted odds ratio (OR) 2·79, $P = 0{\cdot}037$]. Indirect contact with poultry at home ($P = 0{\cdot}038$), and environment-related exposures including visiting a live poultry market and visiting a temporary roadside poultry vendor during the 2 weeks before illness onset ($P < 0{\cdot}05$) tended to be associated with H7N9 infection (Table 2). A trend χ^2 analysis ($\chi^2 = 8{\cdot}25$, $P = 0{\cdot}004$) suggested greater frequency of visiting a live poultry market posed a greater risk of H7N9 infection. By contrast, BMI, frequent hand-washing, smoking, direct contact with poultry at a live poultry market, preparing or cooking poultry at home, raising poultry or pigeons at home or in the neighbourhood, occupational contact with poultry and travel history were not significantly different between cases and controls ($P > 0{\cdot}05$).

Multivariate analysis of risk factors for human H7N9 infection

The backward stepwise logistic regression model was fitted to analyse potential risk factors for human H7N9 infection. All variables in univariate analysis were incorporated into a model fitting with multinomial variables converted into dummy variables (Table 3). Two variables, chronic disease and visiting a live poultry market during the 2 weeks before illness onset, were found to be significantly associated with human H7N9 infection. Visiting a live poultry market during the 2 weeks before illness onset was more likely to cause H7N9 infection with odds ratios of 10·61 and 3·76 for frequencies of >10 times and between 1 and 9 times, respectively. Notably, chronic disease appeared to be an independent risk factor for H7N9 infection. Persons with chronic disease were about four times more likely to be infected with H7N9 compared to those without chronic disease (Table 3). Although indirect contact with poultry at home and visiting a temporary roadside poultry vendor in the 2 weeks before illness onset were statistically significant in univariate analysis, they did not enter the ultimate multivariate logistic model. By contrast, other factors had no significant influence on H7N9 infection after adjusting for potential confounding factors.

DISCUSSION

Although poultry infected with avian influenza A (H7N9) virus are usually asymptomatic, H7N9 virus is highly pathogenic in humans. Here we conducted a case-control study to identify risk factors in cases of human H7N9 infection during the first reported outbreak of human H7N9 infections in 2013. To date, it still remains inconclusive whether age, sex and residence are risk factors for human H7N9 infection. In order to minimize possible impact of differences in age, sex and residence on the association of potential risk factors with H7N9 infection, the cases and the controls were matched by these factors. Our study identified that visiting a live poultry market during the 2 weeks prior to illness onset and chronic disease were likely associated independently with human H7N9 infection found in Shanghai, China. Our findings reinforce the hypothesis that visiting a live poultry market and chronic disease are risk factors for H7N9 infection [4], and further prove the consistency of these risk factors in different geographical areas.

Our finding that visiting a live poultry market was probably an independent risk factor for H7N9 infection is also consistent with previous studies on human H5N1 cases [7–9]. Notably, the risk of H7N9 infection appears to increase with increasing frequency of visiting a live poultry market, which implies that the frequency of exposures might have played an important role. The surroundings of live poultry markets are easily contaminated by poultry body secretions, faeces, or processed organs of poultry. In addition, multiple species of live poultry and birds are concentrated at a high density in live poultry markets, which could facilitate viral spread and interspecies transmission [10–12]. The live poultry market is hence considered as a reservoir and amplifier of H7N9 viruses. People in this environment are more likely to be exposed to pathogens including H7N9 carried by live poultry. According to our investigation, cases visiting a live poultry market might just pass by the retail poultry stall, or just observe the live

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Table 2. *Univariate analysis of risk factors for human infection with avian influenza A(H7N9) virus in Shanghai, China, 2013*

Variables	Variable level	Cases *n* (%)	Controls *n* (%)	OR (95% CI)	*P* value
Chronic disease	Yes	18 (72·0)	36 (48·0)	2·7 (1·04–7·45)	0·037*
Body mass index	<20	3 (12·0)	8 (10·7)	Reference	
	20–25	12 (48·0)	45 (60·0)	0·7 (0·16–3·10)	0·650
	⩾25	10 (40·0)	22 (29·7)	1·2 (0·26–5·56)	0·804
Frequent hand-washing	No	5 (20·0)	9 (12·0)	1·8 (0·55–6·10)	0·323
Having ever smoked	Yes	10 (40·0)	33 (44·0)	0·8 (0·34–2·13)	0·727
Direct contact with poultry in the live poultry market	Yes	3 (12·0)	11 (14·7)	1·1 (0·57–2·22)	0·740
Preparing or cooking at home†	Yes	4 (16·0)	9 (12·0)	1·4 (0·39–5·00)	0·608
Occupational contact with poultry†	Yes	2 (8·0)	1 (1·3)	6·4 (0·56–74·24)	0·136
Raising poultry or pigeons at home†	Yes	3 (12·0)	3 (4·0)	3·2 (0·62–17·24)	0·160
Indirect contact with poultry at home	Yes	5 (20·0)	4 (5·3)	4·4 (1·09–18·18)	0·038*
Raising poultry or pigeons in the neighbourhood‡	Yes	11(44·0)	31 (41·3)	0·9 (0·36–2·24)	0·815
Visiting a live poultry market‡	No	9 (36·0)	51 (68·0)	Reference	
	1–9 times	12 (48·0)	20 (26·7)	3·4 (1·24–9·31)	0·017*
	⩾10 times	4 (16·0)	4 (5·3)	5·6 (1·20–26·87)	0·029*
Visiting a temporary roadside poultry vendor‡	Yes	4 (16·0)	2 (2·7)	6·95 (1·19–40·63)	0·031*
Travel history§	Yes	6 (24·0)	9 (12·0)	2·32 (0·73–7·33)	0·153

OR, Odds ratio; CI, confidence interval.
† Direct contact with poultry.
‡ Environment-related exposure.
§ Travel to another city where H7N9 cases were reported.
* $P < 0·05$

Table 3. *Multivariate logistic regression analysis of risk factors for human infection with avian influenza A (H7N9) virus infection in Shanghai, China, 2013*

Factors	Categories	β	*P* value	OR	95% CI
Constant		−2·69	<0·001	0·07	
Visiting a live poultry market	1–9 times	1·32	0·014	3·76	1·31–10·79
	⩾10 times	2·36	0·008	10·61	1·85–60·74
Chronic disease	Yes	1·40	0·015	4·07	1·32–12·56

OR, Odds ratio; CI, confidence interval.

poultry at close quarters, or they may simply purchase eggs from the egg stall adjacent to the live poultry stall. Evaluation of the airborne transmissibility of the human H7N9 isolates A/Shanghai/2/2013 and A/Anhui/1/2013 suggests the H7N9 viruses could infect ferrets via airborne exposure, albeit the transmission is not as effective as intranasal inoculation of the viruses [13–14]. Transmission of H7N9 virus in animals could select and enrich some mutations similarly seen in influenza A/H5N1 virus that can gain the capacity for airborne transmission between mammals [15]. Although no sustained human-to-human transmission of H7N9 viruses has been confirmed to date, identification of some family clusters of H7N9 infection raised concerns of human-to-human transmission via the aerosol route [16]. Visiting a live poultry market, even for a short period of time, is thus likely to result in contracting H7N9 virus through contaminated aerosols. Consistent with this speculation, H7N9 virus was detected from an environmental specimen collected from the poultry cage at a live poultry market in the epidemic region [17]. This finding suggests that transmission of H7N9 virus via environmental contamination may occur in China. Therefore, effective live poultry market control strategies should be developed and implemented. These strategies include segregating bird species, improving biosecurity, establishing central poultry

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slaughtering facilities, conducting regular disinfection, and having a periodic rest day [18–20].

Studies have shown that persons with chronic pulmonary disease, renal dysfunction, or haemoglobinopathies are at increased risk of development of complications from influenza infection [21]. Our findings show that having chronic disease(s) is likely to be significantly associated with H7N9 infection. Eighteen (72%) of the 25 cases of human H7N9 infection had pertinent chronic diseases before illness onset, which was higher than that of controls (48%) ($P = 0{\cdot}037$). Persons with chronic disease had compromised immune function, which might have contributed to the increased risk of H7N9 infection. Due to the small numbers of each type of chronic disease, we were not able to further analyse the association of specific underlying medical conditions with H7N9 infection in our study. However, our data suggest that at least some of these medical conditions might be independent risk factors for H7N9 infection. The individuals, especially those with underlying chronic diseases such as chronic bronchitis, hypertension, diabetes, pulmonary disease, or heart disease, should reduce exposure to possibly contaminated environments to minimize the risk of H7N9 infection.

There are several potential limitations to our findings. First, the study may be underpowered to detect the risk factors. In this case-control study, the matched elements including age, gender and residence were excluded from analysis as risk factors. The lack of a statistically significant association between H7N9 infection and direct contact with live poultry in this study may result from the relatively small number of cases ($n = 25$). Further studies to include more cases are warranted to determine whether the frequency and duration of direct contact with poultry are associated with H7N9 infection. Second, data collection bias was likely to have occurred. Although a standardized questionnaire and trained staff were deployed for interview to minimize interviewer bias, masking case-control status from the interviewers was not possible in this study. In addition, a larger proportion of interviews in the case group (15/25) than in the control group (3/75) were completed by proxies. Although the proxies (spouse or parents) lived closely with the cases, it is likely that the proxies might not be aware of some of the activities and poultry exposure history of the cases. The substantial delay (>1 month, range 2–3 months) between illness onset and the interviews could be another potential source of recall bias or inaccuracy both for living and deceased cases. Finally, it is possible that we did not identify all H7N9 cases that occurred in Shanghai during the study period, especially the cases with mild symptoms.

Although our findings indicate that visiting a live poultry market and chronic disease are major risk factors for human H7N9 infection, the exact mechanism of virus transmission is uncertain. Avian influenza viruses have the potential to either reassort with human influenza strains or to undergo genetic mutations and might consequently become more transmissible among humans. In conclusion, interventions based upon our findings may help prevent further avian influenza A(H7N9) transmission to humans. Ongoing education of the public, especially those with chronic medical conditions, is warranted to promote behavioural changes that can help to avoid direct or indirect contact with H7N9 virus. Last, but by no means least, effective strategies for live poultry market control should be reinforced. In addition, the feasibility of wearing protective masks for workers and visitors to live poultry markets could also be considered.

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DECLARATION OF INTEREST

None.

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MAJOR ARTICLE

Risk Factors for Human Illness with Avian Influenza A (H5N1) Virus Infection in China

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(See the article by Tiensin et al. on pages 1735–43, the article by Vong et al. on pages 1744–52, and the editorial commentary by Briand and Fukuda on pages 1717–9)

Background. In China, 30 human cases of avian influenza A (H5N1) virus infection were identified through July 2008. We conducted a retrospective case-control study to identify risk factors for influenza H5N1 disease in China.

Methods. A questionnaire about potential influenza H5N1 exposures was administered to 28 patients with influenza H5N1 and to 134 randomly selected control subjects matched by age, sex, and location or to proxies. Conditional logistic regression analyses were performed.

Results. Before their illness, patients living in urban areas had visited wet poultry markets, and patients living in rural areas had exposure to sick or dead backyard poultry. In multivariable analyses, independent risk factors for influenza H5N1 were direct contact with sick or dead poultry (odds ratio [OR], 506.6 [95% confidence interval {CI}, 15.7–16319.6]; $P < .001$), indirect exposure to sick or dead poultry (OR, 56.9 [95% CI, 4.3–745.6]; $P = .002$), and visiting a wet poultry market (OR, 15.4 [95% CI, 3.0–80.2]; $P = .001$).

Conclusions. To prevent human influenza H5N1 in China, the level of education about avoiding direct or close exposures to sick or dead poultry should be increased, and interventions to prevent the spread of influenza H5N1 at live poultry markets should be implemented.

In parallel with the unprecedented epizootic of highly pathogenic avian influenza A (H5N1) viruses among poultry and migratory birds [1], 418 confirmed human cases of influenza H5N1 with 257 deaths were reported in 15 countries from November 2003 through 17 April 2009 [2]. Despite widespread human exposure to influenza H5N1 virus–infected poultry [3, 4], human influenza H5N1 disease remains rare, and avian-to-human transmission of influenza H5N1 virus is believed to have occurred in most human cases [5], with rare instances of limited, nonsustained human-to-human influenza H5N1 virus transmission [6–8]. Environment-to-human transmission remains a possibility [5, 9] for some human influenza H5N1 cases without an identified exposure source. Although influenza H5N1 virus has infected multiple species of animals [10, 11], to date, only poultry and wild birds have been implicated in transmission to humans.

Only limited data are available on risk factors associated with illness caused by human infection with influenza H5N1 viruses. A case-control study conducted during the 1997 outbreak of influenza H5N1 in Hong Kong Special Administrative Region, China, found that hav-

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ing visited a live poultry market the week before illness onset was the only significant risk factor for influenza H5N1 [12]. Studies conducted during 2004 in rural Thailand [13] and Vietnam [14] found that the most significant risk factor for influenza H5N1 was recent direct contact with sick or dead poultry.

Of the 38 confirmed human influenza H5N1 cases reported to date in China, 30 had occurred as of July 2008. Of these, 29 were identified through surveillance from October 2005 [15] through July 2008 [2]. These 29 cases occurred sporadically and were distributed across 18 counties and 11 districts of 13 provinces, with no obvious geographic clustering. One additional influenza H5N1 case occurred during 2003 [16]. To inform prevention efforts, we conducted a retrospective matched case-control study to determine risk factors for human influenza H5N1 illness in China.

SUBJECTS AND METHODS

Patients. In China, all suspected influenza H5N1 cases are reported to the Chinese Center for Disease Control and Prevention (China CDC) through a national surveillance system. A confirmed case of influenza H5N1 was defined as pneumonia or influenza-like illness (marked by fever [temperature, $\geq$38°C] and cough or sore throat, with no other confirmed diagnosis), with laboratory evidence of influenza H5N1 virus infection with use of viral isolation or reverse-transcription polymerase chain reaction of respiratory specimens or with a $\geq$4-fold increase in influenza H5N1 antibody titer in paired acute- and convalescent-phase serum samples. All 29 patients with influenza H5N1 who were identified by surveillance from October 2005 through July 2008 were eligible to participate in the study. Exclusion criteria for patients included insufficient epidemiological data or inability to recruit matched control subjects. A rural patient was defined as a village resident, and an urban patient was defined as a city resident.

Control subject selection. Up to 5 randomly selected control subjects were matched with each patient by sex, age ($\pm$1 year for patients aged $<$18 years and $\pm$5 years for patients aged $\geq$18 years), and location. Eligible control subjects were persons who lived in the same location as the matched patient for at least 3 months before the date of illness onset in the patient.

Two methods were used for random selection of potential control subjects. For rural patients, population registries from each patient's village were used to identify eligible age- and sex-matched residents at the time of symptom onset in the patient. Five potential control subjects were selected using randomly generated numbers from the list of eligible control subjects. For urban patients, 1 apartment building immediately adjacent to the patient's home was selected randomly. One floor in this building was selected randomly, and all apartments on the floor were visited to recruit 5 control subjects. Additional control subjects were recruited from adjacent floors if needed. Inclusion criteria for eligible control subjects were absence of fever (temperature, $>$37.5°C), feverishness, and respiratory illness during the 7 days before and after the matched patient's illness onset date and having a specimen test seronegative for influenza H5N1 antibodies.

Data collection. After trained investigators from the China CDC described the purpose of the study to eligible patients and control subjects or their proxies and obtained written informed consent, participants were enrolled. A standardized questionnaire was used to collect information about demographic characteristics, underlying medical conditions, backyard poultry raising, poultry H5 vaccination coverage levels, type of contact with sick and/or dead or healthy-appearing poultry, visits to places where live poultry were kept (e.g., wet poultry markets or poultry farms and/or factories), eating habits, exposure to other animals (including wild birds), and exposure to other humans with acute respiratory illnesses or confirmed influenza H5N1. Interviews were conducted a median of 360 days (range, 11–486 days) after the date of onset of illness in the patient. A wet poultry market was defined as a place where small animals and poultry may be purchased live or slaughtered [17]. Contact with sick and/or dead or healthy-appearing poultry was defined as direct contact (e.g., touching) and indirect contact (defined as no physical contact but being within 1 m of poultry, poultry products, or poultry feces).

An adult household member (e.g., parent or legal guardian) who was closely familiar with the participants was interviewed as a proxy for any patient who died, was severely ill and unable to respond, or was aged $<$10 years and for control subjects aged $<$10 years. For questions posed to patients about activities and exposures that occurred during the 2 weeks before their illness onset, control subjects were asked about the same activities and exposures during the same reference period.

Epidemiological and clinical data for 20 (71%) of the 28 patients enrolled in the study were previously collected during field investigations by China CDC staff as a public health response. These data were compared with the data collected from patients in our case-control study. Discrepancies were resolved in favor of the data obtained during the earlier field investigations.

If a proxy for any patient or control subject was unable to provide sufficient information for the study or refused to participate or if no suitable proxy could be identified, the patient or control subject was excluded from the study. Up to 2 visits were made in 1 week to recruit eligible persons to participate in the study. If selected control subjects were unavailable or declined participation, the next eligible control subject was recruited to participate in the study.

Serological testing. A single blood specimen was collected from surviving patients with influenza H5N1 and from matched control subjects at enrollment for influenza H5N1 serological testing, which was performed at the National Influenza Center (China CDC; Beijing) with use of a microneutralization assay

[18] in a biosafety level 3 enhanced laboratory and with use of a modified hemagglutinin-inhibition assay with horse red blood cells under biosafety level 2 conditions, as described elsewhere [19]. Antigens for the assays were selected to match the genetic and antigenic characteristics of the influenza H5N1 virus strains that infected the matched patients, if available, or that were known to be circulating at the same times when and locations where the cases occurred. Serum samples were tested in duplicate by 2 separate microneutralization assays conducted on different days. A serum specimen with an influenza H5N1 neutralizing antibody titer of $\geqslant$1:80 was considered to be positive, with confirmation by the hemagglutinin-inhibition assay with horse red blood cells [20, 21]. Control subjects whose specimens tested seropositive for influenza H5N1 antibodies were excluded from the final analyses.

Statistical analyses. Questionnaire data from patients and control subjects were entered in duplicate and were verified using EpiData software. Data were analyzed using SAS, version 9.13 (SAS Institute). Median and range values were calculated for continuous variables and were compared between urban and rural patients with use of the Wilcoxon rank sum test. For categorical variables, frequencies of urban cases and rural cases were compared using Fisher's exact test. Baseline characteristics of patients and control subjects and independent associations between exposures and influenza H5N1 disease were compared using exact conditional logistic regression. Matched odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for potential influenza H5N1 risk factors. For multivariable exact conditional logistic analyses, we included variables with $P \leqslant .10$ in univariate matched analyses for the initial model. Backward conditional logistic regression was performed by excluding variables with $P > .10$. In matched analyses, if any patient was missing exposure data, the data for all matching control subjects were excluded. However, if any control subject was missing exposure data, only the data for that control subject were excluded. All statistical tests were 2-sided, with a significance level of $\alpha = .05$.

Study approval. The study protocol was approved by the Institutional Review Board of the China CDC. Written informed consent to participate in the study was obtained from adult participants or, for deceased patients, from family member proxies. A parent or legal guardian provided written consent for participants aged <18 years; participants aged 10–17 years also provided written informed assent.

RESULTS

Twenty-eight patients (97%) with influenza H5N1 were enrolled in the study. We excluded 1 case of influenza H5N1 that occurred in military personnel with insufficient data: 1 from 2003 [16] and 1 from 2007 [22]. Among the 28 enrolled patients, influenza H5N1 virus was detected by isolation for 23 (82%), by reverse-transcriptase polymerase chain reaction and serologic testing for 3 (11%), and by serologic testing only for 2 (7%). All recruited control subjects agreed to participate, and none withdrew from the study. Four patients (3 rural and 1 urban) were matched to <5 control subjects because of unavailability of eligible control subjects. Samples from all control subjects tested seronegative for influenza H5N1 antibodies. The final study population included 28 patients with influenza H5N1 and 134 matched control subjects. Data for patients (18 died, 1 was severely ill, and 3 were aged <10 years) were obtained by proxy interviews more often than were data for control subjects (22 [79%] vs. 24 [18%]). The baseline characteristics of patients and control subjects were similar for highest education level attained, annual household income, and smoking history (table 1).

Table 1. Baseline characteristics of participants in a case-control study of influenza H5N1 in China.

Characteristic	Patients (n = 28)	Control subjects (n = 134)	P[a]
Age, median (range), years	29 (6–62)	29 (5–66)	
Female sex	15 (54)	74 (55)	
Location			
Urban area	10 (36)	49 (37)	
Rural area	18 (64)	85 (63)	
Han ethnicity	25 (89)	118 (88)	NA
Interviewed by proxy	22 (79)	24 (18)	NA
Highest level of education			
Illiterate	3 (11)	7 (5)	.485
Primary school	8 (29)	50 (37)	
Junior high school	9 (32)	40 (30)	
High school	5 (18)	19 (14)	
College or higher	3 (11)	18 (13)	
Annual household income, RMB[b]			
<2000	9 (33)	42 (33)	.978
2000–4999	8 (30)	35 (27)	
5000–10,000	4 (15)	19 (15)	
>10,000	6 (22)	33 (26)	
Current smoker	6 (21)	25 (19)	.835
Seasonal influenza vaccination within past year	0 (0)	2 (2)[c]	NA

NOTE. Data are no. (%) of participants, unless otherwise indicated. NA, not available (because of small sample size or because data distribution was not suitable for conditional logistic regression model).

[a] Comparison of frequencies between patients and control subjects were analyzed by exact conditional logistic regression. Matched factors (age, sex, and location) were excluded from analyses. When the P value was calculated, if any patient was missing exposure data, the data of all matching control subjects were excluded. If any control subject was missing exposure data, only the data from that control subject were excluded.

[b] Data are for 27 patients and 129 control subjects. Exchange rate: US$1 is equal to ~7.1 RMB.

[c] Data are for 131 control subjects.

A descriptive analysis was performed to compare exposures between urban and rural patients (table 2). Urban patients (n = 10) had a higher level of education and a higher annual household income and were significantly more likely to have

Table 2. Demographic characteristics and exposures of 28 urban and rural patients with human influenza A (H5N1) in China.

Characteristic	Urban patients (n = 10)	Rural patients (n = 18)	P[a]
Age, years			
Median (range)	30 (15–52)	25 (6–62)	.443
6–14	0 (0)	5 (28)	.132
15–59	10 (100)	12 (67)	
⩾60	0 (0)	1 (5)	
Female sex	3 (30)	12 (67)	.114
Highest level of education			
Illiterate	0 (0)	3 (17)	**.006**
Primary school	0 (0)	8 (44)	
Junior high school	5 (50)	4 (22)	
High school	2 (20)	3 (17)	
College or higher	3 (30)	0 (0)	
Annual household income, RMB[b]			
<2000	0 (0)	9 (53)	**<.001**
2000–4999	1 (10)	7 (41)	
5000–10,000	3 (30)	1 (6)	
>10,000	6 (60)	0 (0)	
Travel history[c]	3 (30)	1 (6)	.116
Occupational poultry exposure[d]	1 (10)	3 (17)	>.99
Household with backyard poultry	0 (0)	15 (83)	**<.001**
Exposure to healthy-appearing poultry[e]	10 (100)	17 (94)	>.99
Exposure to sick and/or dead poultry[f]	1 (10)	14 (78)	**.001**
Visited a wet poultry market	10 (100)	7 (39)	**.002**
Raised animals in home[g]	1 (10)	14 (78)	**.001**
Lack of indoor water supply	0 (0)	14 (78)	**<.001**
Exposed to persons with fever and respiratory symptoms	0 (0)	1 (6)[h]	>.99
Exposed to a person with confirmed influenza H5N1	1 (10)[i]	0 (0)	.357

NOTE. Data are no. (%) of patients. Boldface indicates statistical significance.

[a] Comparison of frequencies between urban and rural patients were analyzed by Fisher's exact test; median age was compared with the Wilcoxon rank sum test.

[b] Data are for 17 control subjects. Exchange rate: US$1 is equal to ~7.1 RMB.

[c] Travel outside home township (for rural patients) or outside home city (for urban patients) for >24 h during the 2 weeks prior to the patient's illness onset.

[d] Defined as workplace exposure to live poultry (e.g., poultry farm and/or factory or wet poultry market), not including backyard poultry exposure.

[e] Includes direct and indirect contact with apparently healthy poultry.

[f] Includes direct and indirect contact with sick and/or dead poultry.

[g] Includes cats, pigs, dogs, cows, and goats.

[h] A family cluster was reported in Yu et al. [15].

[i] A family cluster consisting of confirmed son and his father was reported in Wang et al. [8].

visited a live poultry market, compared with rural patients (n = 18; 10 [100%] vs. 7 [39%]; P = .002). Rural patients were significantly more likely than urban patients to raise backyard poultry (15 [83%] vs. 0 [0%]; P < .001) or other animals (14 [78%] vs. 1 [10%]; P = .001), to have had exposure to sick or dead poultry (14 [78%] vs. 1 [10%]; P = .001), and to lack an indoor water supply (14 [78%] vs. 0 [0%]; P = .001). One urban patient was exposed to a person with confirmed influenza H5N1 before illness onset [8]. One rural pediatric patient was exposed to an ill sister with fever and respiratory illness 2 days before illness onset [15].

In univariate analyses including all participants, the most significant risk factor was direct contact with sick or dead poultry (OR, 34.7 [95% CI, 4.3–276.9]; P = .001). Visiting a wet poultry market (OR, 3.1 [95% CI, 1.2–7.9]; P = .019) and having an underlying medical condition (OR, 5.2 [95% CI, 1.3–19.9]; P = .018) were also statistically significant. Other significant risk factors are listed in table 3. In univariate analyses restricted to rural participants, the most significant risk factors were direct contact with sick or dead poultry (OR, 29.8 [95% CI, 3.7–241.5]; P = .001) and indirect contact only (OR, 11.3 [95% CI, 2.2–58.5]; P = .004). Although a higher proportion of urban patients than control subjects visited a wet poultry market during the 2 weeks before illness onset (100% vs. 45%), these proportions could not be compared statistically (table 3).

Among the participants, 5 patients (18%) and 6 control subjects (4%) had pertinent underlying medical conditions. Of the 3 female patients, all were adults, including 2 who were pregnant and 1 who had a 10-year history of chronic bronchitis. Of the 2 male patients, 1 was aged 15 years and had a 10-year history of minimal-change glomerulopathy that required treatment at the time of illness onset, and 1 was aged 24 years, had *Salmonella* bacteremia identified at the time of onset of respiratory symptoms, and had intermittent fevers during the previous 3 months [8]. Of the 6 adult control subjects, 4 were pregnant women, 1 was a woman who reported anemia, and 1 was a man with chronic bronchitis.

In multivariable analyses including all participants, significant independent H5N1 risk factors were direct contact with sick or dead poultry (OR, 506.6 [95% CI, 15.7–16319.6]; P < .001), indirect exposure to sick or dead poultry (OR, 56.9 [95% CI, 4.3–745.6]; P = .002), and visiting a wet poultry market (OR, 15.4 [95% CI, 3.0–80.2]; P = .001). Direct contact (OR, 67.3 [95% CI, 5.8–783.8]; P < .001) and indirect exposure to sick or dead poultry (OR, 25.4 [95% CI, 2.4–274.3]; P = .008) remained independent risk factors for influenza H5N1 when multivariable analyses were restricted to rural participants.

DISCUSSION

We identified 3 independent risk factors for human influenza H5N1 disease in China, including direct contact with sick or dead poultry, indirect exposure (being within 1 m without direct contact) to sick and/or dead poultry, and visiting a wet poultry market. Direct contact with sick or dead poultry was the most

Table 3. Univariate matched-pair analyses of potential risk factors for influenza H5N1, overall and stratified by urban and rural groups, in China.

	Participants											
	All				Rural				Urban			
Potential risk factor	Patients (*n*= 28)	Control subjects (*n* = 134)	OR (95% CI)	*P*[a]	Patients (*n* = 18)	Control subjects (*n* = 85)	OR (95% CI)	*P*[a]	Patients (*n* = 10)	Control subjects (*n* = 49)	OR (95% CI)	*P*[a]
Underlying medical condition	5/28 (18)	6/134 (4)	5.2 (1.3–19.9)	**.018**	3/18 (17)	4/85 (5)	5.6 (0.9–36.3)	**.073**	2/10 (20)	2/49 (4)	4.7 (0.7–33.6)	**.121**
Travel history[b]	4/28 (14)	20/134 (15)	1.0 (0.3–3.6)	.964	1/18 (6)	13/85 (15)	0.2 (0.0–2.4)	.208	3/10 (30)	7/49 (14)	2.8 (0.5–15.2)	.222
Occupational poultry exposure[c]	4/28 (14)	5/134 (4)	13.1 (1.4–125.4)	**.026**	3/18 (17)	5/85 (6)	8.3 (0.8–90.1)	**.081**	1/10 (10)	0/49 (0)	NA	
Raise backyard poultry	15/28 (54)	48/134 (36)	4.5 (1.1–17.5)	**.031**	15/18 (83)	48/85 (56)	4.5 (1.1–17.5)	**.031**	0/10 (0)	0/49 (0)	. . .	
Location of backyard poultry cage												
No backyard poultry	13/28 (46)	86/134 (64)	Ref		3/18 (17)	37/85 (44)	Ref		. . .	. . .	. . .	
Present outside house	9/28 (32)	37/134 (28)	3.7 (0.9–15.3)	**.071**	9/18 (50)	37/85 (44)	3.7 (0.9–15.3)	**.071**	. . .	. . .	. . .	
Present inside house	6/28 (22)	11/134 (8)	9.7 (1.8–53.3)	**.009**	6/18 (33)	11/85 (12)	9.7 (1.8–53.3)	**.009**	. . .	. . .	. . .	
Raise domestic waterfowl[d] or chickens												
No backyard poultry	13/28 (46)	86/134 (64)	Ref		3/18 (17)	37/85 (44)	Ref		. . .	. . .	. . .	
Only raise chickens	7/28 (25)	34/134 (25)	2.6 (0.6–12.1)	.226	7/18 (39)	34/85 (40)	2.6 (0.6–12.1)	.226	. . .	. . .	. . .	
Raise waterfowl	8/28 (29)	14/134 (11)	6.4 (1.6–26.3)	**.010**	8/18 (44)	14/85 (16)	6.4 (1.6–26.3)	**.010**	. . .	. . .	. . .	
Backyard poultry H5 vaccination												
No backyard poultry	13/28 (46)	86/124 (70)	Ref		3/18 (17)	37/75 (50)	Ref		. . .	. . .	. . .	
Vaccination coverage ⩾80%	6/28 (22)	19/124 (15)	4.0 (0.9–17.9)	**.070**	6/18 (33)	19/75 (25)	4.0 (0.9–17.9)	**.070**	. . .	. . .	. . .	
Vaccination coverage <80%	9/28 (32)	19/124 (15)	7.1 (1.6–31.6)	**.010**	9/18 (50)	19/75 (25)	7.1 (1.6–31.6)	**.010**	. . .	. . .	. . .	
Domestic waterfowl H5 vaccination												
No domestic waterfowl	20/28 (71)	120/132 (91)	Ref		10/18 (55)	71/83 (86)	Ref		. . .	. . .	. . .	
Vaccination coverage ⩾80%	3/28 (11)	7/132 (5)	2.4 (0.5–11.2)	.257	3/18 (17)	7/83 (8)	2.4 (0.5–11.2)	.257	. . .	. . .	. . .	
Vaccination coverage <80%	5/28 (18)	5/132 (4)	8.4 (1.6–45.1)	**.013**	5/18 (28)	5/83 (6)	8.4 (1.6–45.1)	**.013**	. . .	. . .	. . .	
Exposures to healthy-appearing poultry												
Direct contact	11/27 (41)	31/133 (23)	3.3 (1.0–10.4)	**.043**	9/17 (53)	27/85 (32)	2.9 (0.8–10.4)	**.099**	2/10 (20)	4/48 (8)	5.3 (0.4–70.8)	.206
Only indirect contact (within 1 m)	8/26 (31)	43/133 (32)	0.8 (0.3–2.4)	.724	7/17 (41)	40/84 (48)	0.7 (0.2–2.3)	.594	1/9 (11)	3/49 (6)	1.6 (0.1–19.4)	.713
Consumed healthy-appearing poultry	22/28 (79)	99/134 (74)	1.3 (0.4–4.2)	.610	12/18 (67)	59/85 (69)	0.8 (0.2–2.8)	.689	10/10 (100)	40/49 (82)	NA	
Exposures to sick and/or dead poultry												
Direct contact	9/28 (32)	4/133 (3)	34.7 (4.3–276.9)	**.001**	8/18 (44)	4/84 (5)	29.8 (3.7–241.5)	**.001**	1/10 (10)	0/49 (0)	NA	
Only indirect contact (within 1 m)	6/28 (21)	4/132 (3)	11.3 (2.2–58.5)	**.004**	6/18 (33)	4/83 (5)	11.3 (2.2–58.5)	**.004**	. . .	. . .	. . .	
Consumed	11/28 (39)	1/134 (1)	NA		10/18 (56)	1/85 (1)	NA		1/10 (10)	0/49 (0)	NA	
Wet poultry market exposure												
Visited wet poultry market	17/28 (61)	51/133 (38)	3.1 (1.2–7.9)	**.019**	7/18 (39)	29/84 (35)	1.2 (0.4–3.8)	.725	10/10 (100)	22/49 (45)	NA	
Visited wet poultry market and witnessed poultry slaughtering at market	15/28 (54)	35/129 (27)	5.0 (1.7–14.9)	**.004**	6/18 (33)	17/83 (20)	2.2 (0.6–7.7)	.224	9/10 (90)	18/46 (39)	NA	

Frequency of visits to wet poultry market within 2 weeks before illness onset												
Never	11/27 (41)	82/131 (63)	Ref		11/17 (65)	55/82 (67)	Ref		0/10 (0)	27/49 (55)	Ref	
1–5 times	8/27 (30)	27/131 (20)	2.8 (0.9–8.1)	**.062**	4/17 (23)	17/82 (21)	NA		4/10 (40)	10/49 (21)	NA	
6–10 times	3/27 (11)	8/131 (6)	7.6 (1.1–53.7)	**.043**	2/17 (12)	2/82 (2)	NA		1/10 (10)	6/49 (12)	NA	
>10 times	5/27 (18)	14/131 (11)	5.8 (1.2–28.6)	**.031**	0/17 (0)	8/82 (10)	NA		5/10 (50)	6/49 (12)	NA	
Contact with live poultry at the market												
No contact	22/27 (82)	120/133 (90)	Ref		14/17 (82)	78/84 (92)	Ref		8/10 (80)	42/49 (86)	Ref	
Only indirect contact (within 1 m)	3/27 (11)	9/133 (7)	1.9 (0.4–8.1)	.411	2/17 (12)	3/84 (4)	3.0 (0.5–19.2)	.247	1/10 (10)	6/49 (12)	NA	
Direct contact	2/27 (7)	4/133 (3)	4.6 (0.4–51.9)	.222	1/17 (6)	3/84 (4)	2.4 (0.1–41.3)	.534	1/10 (10)	1/49 (2)	NA	
Exposure to animals [e]												
Raise backyard animals	15/28 (54)	61/134 (46)	1.4 (0.6–3.7)	.459	14/18 (78)	50/85 (59)	2.5 (0.7–8.9)	.145	1/10 (10)	11/49 (22)	0.4 (0.0–3.2)	.367
Direct contact with backyard animals	8/28 (29)	38/134 (28)	1.0 (0.4–2.6)	.987	7/18 (39)	27/85 (32)	1.4 (0.5–4.0)	.548	1/10 (10)	11/49 (22)	0.4 (0.0–3.4)	.368
Lack of indoor water supply	14/28 (50)	68/134 (51)	0.7 (0.1–4.3)	.726	14/18 (78)	68/85 (80)	0.7 (0.1–4.3)	.726	0/10 (0)	0/49 (0)	. . .	
Exposed to persons with fever and respiratory symptoms	1/28 (4)[f]	0/134 (0)	. . .		1/18 (6)[f]	0/85 (0)	NA		0/10 (0)	0/49 (0)	. . .	
Exposed to persons with confirmed influenza H5N1	1/28 (4) [g]	0/134 (0)	. . .		0/18 (0)	0/85 (0)	. . .		1/10 (10)[g]	0/49 (0)	NA	

NOTE. Data are proportion (%) of participants, unless otherwise indicated. Boldface indicates $P \leq .10$, and those variables with $P \leq .10$ were included in univariate matched analyses for the initial model. CI, confidence interval; NA, not available (because of small sample size or because data distribution could not be analyzed by conditional logistic regression); OR, odds ratio; Ref, reference.

[a] Comparison of frequencies between patients and control subjects were analyzed by exact conditional logistic regression. When matched OR and *P* values were calculated, data for matched control patients were excluded for patients with missing exposure data, and control subjects with missing data were excluded from analyses; however, matched patients or other control subjects with available data were included.

[b] Travel outside home township (for rural patients) or outside home city (for urban patients) for >24 h during the 2 weeks prior to the patient's illness onset.

[c] Defined as workplace exposure to live poultry (e.g., poultry farm and/or factory or wet poultry market), not including backyard poultry exposure.

[d] Includes ducks and geese.

[e] Includes cats, pigs, dogs, cows, and goats.

[f] A family cluster was reported in Yu et al. [15].

[g] A family cluster consisting of confirmed son and his father was reported in Wang et al. [8].

significant risk factor for influenza H5N1, consistent with previous studies [13, 14]. Close indirect exposure to sick and/or dead poultry was also reported in a descriptive study of Indonesian influenza H5N1 [9]. This could reflect inhalation of aerosolized material contaminated with influenza H5N1 viruses or contact with surfaces or fomites contaminated with virus or with fertilizer containing fresh poultry feces, followed by self-inoculation of the respiratory tract [5]; however, our study design did not address these mechanisms.

Our finding that visiting a wet poultry market during the 2 weeks before illness onset was a significant risk factor for influenza H5N1 is consistent with findings from a case-control study conducted during the outbreak of influenza H5N1 in Hong Kong in 1997 [12]. Although widespread poultry deaths from influenza H5N1 were noted in wet markets during the outbreak in Hong Kong, this has rarely been observed in urban China. Wet poultry markets are considered to be a reservoir and amplifier of avian influenza A viruses, because avian host species are present together in a high-density setting that can facilitate viral persistence, cross-species infection, and genetic reassortment [23, 24]. Influenza H5N1 viral RNA was detected in an environmental specimen collected from a goose cage at a market that an urban patient with influenza H5N1 had visited before illness onset [25], which suggests that influenza H5N1 virus transmission through environmental contamination may occur in urban areas of China.

Most patients with influenza H5N1 virus infection had previously been healthy [5, 26]. However, 5 (18%) of the 28 patients with influenza H5N1 had a pertinent underlying medical condition before illness onset, which was a significant risk factor for influenza H5N1 in univariate analysis in our study. Although studies have shown that pregnant women and persons with chronic pulmonary disease, renal dysfunction, hemoglobinopathies, or immunodeficiencies are at increased risk of complications of influenza [27], they may not necessarily be at increased risk of influenza H5N1 virus infection. We were not able to further analyze the specific medical conditions in our study because of the small numbers, but our data suggest that at least some of these conditions may be risk factors for influenza H5N1 disease. Additional factors, including pre-existing immunity or host genetic factors [28], might also contribute to the development of influenza H5N1 disease, particularly for persons with underlying medical conditions. Additional research is needed to understand the association between underlying medical conditions and influenza H5N1 disease that we observed.

Chinese patients with influenza H5N1 comprised 2 distinct populations with respect to poultry exposures. Most rural Chinese persons raise backyard poultry for food production and income. In contrast, wet poultry markets are sustained by the demand for freshly slaughtered poultry in urban areas of China. Not surprisingly, exposures to poultry varied depending on where the patients lived. Most urban patients had not been exposed to sick or dead poultry or to backyard poultry before illness onset, but all had visited wet poultry markets, whereas most rural cases had been exposed to backyard poultry and to sick or dead poultry. This suggests that public education and interventions to control disease should target different settings. Rural patients were less educated, poorer, and more likely to lack an indoor water supply, compared with urban patients—similar to risk factors identified in Vietnam [14]. Because of the exposure differences between rural and urban patients, we performed analyses stratified by patient location in addition to including all participants. The overall results were similar to the analyses restricted to rural participants alone.

Our study suggests that exposure to domestic waterfowl may be a greater risk to public health, compared with contact with chickens. Studies from Vietnam, Thailand, and southern China have documented that domestic ducks and geese can be infected with highly pathogenic avian influenza H5N1 viruses without apparent symptoms [29–31]. Earlier studies, conducted during 1997–2004, suggested that most influenza H5N1 viral shedding by domestic ducks was in feces, but more recently, a great amount of influenza H5N1 viral shedding has been detected in the upper respiratory tract of waterfowl for up to 17 days [31, 32]. Both respiratory and fecal shedding of influenza H5N1 viruses can cause contamination of the environment and water sources used by birds and humans [5]. In univariate analyses, raising waterfowl, such as ducks or geese, was a risk factor for human influenza H5N1 disease, but raising only backyard chickens was not a risk factor. This finding suggests that domestically raised waterfowl exposure may pose a greater risk of avian-to-human transmission, compared with exposure to backyard chickens in rural areas.

In China, a national influenza H5 poultry vaccination program was implemented in 2005 [33]; after that, subsequently documented decreases in outbreaks of influenza H5 among poultry were noted [1]. However, the effectiveness of poultry H5 vaccination to reduce the risk of influenza H5N1 virus transmission to humans is unknown. H5-vaccinated poultry that are infected with H5N1 viruses may shed fewer viruses or may not display clinical signs of disease but could still be a risk to other poultry and to humans [34, 35]. Our findings suggest that very high H5 poultry vaccine coverage may be needed to reduce the risk of avian-to-human transmission of H5N1 viruses. Universal influenza H5 vaccination of poultry, including domestic waterfowl, in conjunction with other control measures, is recommended as an important control strategy by the World Animal Health Organization and the United Nations Food and Agriculture Organization [36]. The possibility that H5-vaccinated poultry may be infected with H5N1 viruses but may not shed enough H5N1 virus for transmission to humans was suggested by recent field evidence [37–39]. However, cases of influenza H5N1 continued to occur in China during 2006–2008, despite the national poultry H5 vaccination program. A simulation study revealed

that "silent spread" of influenza H5N1 can occur among poultry as a result of incomplete immunity at the flock level, even if a poultry vaccine is effective in individual birds [40]. Poultry H5 vaccine effectiveness studies are needed to examine outcomes, such as influenza H5N1 virus infection, as well as duration and quantitative viral shedding among vaccinated poultry, to assess the public health risk, particularly in urban wet poultry markets.

There are a number of limitations to our findings. Because 20 patients (71%) and 98 matched control subjects (73%) were asked in 2007 about exposures that may have occurred much earlier, recall bias may have occurred if patients or their proxies were more likely than control subjects to recall poultry exposures. Although we interviewed patients with influenza H5N1 or their proxies long after the patients' illnesses occurred, nearly all of the patient data collected in our study were concordant with data collected during the earlier field investigations. However, because no exposure data for control subjects were collected when cases occurred, the potential for differential recall and potential misclassification of some exposures could have introduced bias. A much higher proportion of patients' responses than control subjects' responses were provided by proxy interviews because of high mortality among patients, and these proxies may not have known all of the respective patient's exposures. We could not verify the poultry H5 vaccination coverage reported by participants who raised backyard poultry. Although urban control subjects were selected by a method different from that used for selection of rural control subjects, it is unlikely that selection bias was a significant limitation. All 28 patients had laboratory-confirmed influenza H5N1 virus infection, and all control subjects were seronegative for H5N1 neutralizing antibodies. Therefore, there was no misclassification of patients or control subjects on the basis of H5N1 virus infection status. A few collinear variables were included in the multivariable analysis, but this did not influence the final results. Although our study included a greater number of participants than in previous case-control studies [12–14], the most important limitation was the small number of patients that precluded precise estimation of the magnitude of risk factors; our study was underpowered to detect risk factors among urban patients with influenza H5N1, because nearly twice as many cases occurred in rural areas. Finally, it is possible that we did not identify all cases of influenza H5N1 that may have occurred in China during the study period.

Although human influenza H5N1 disease is very rare and persons with the risk factors that we identified seldom develop influenza H5N1 virus infection [41], interventions based on our findings may help prevent further influenza H5N1 virus transmission to humans in China. Ongoing education is needed that results in behavioral change to avoid direct or indirect contact with sick or dead poultry, which should be removed and disposed of promptly using appropriate protective equipment. In rural areas, ongoing efforts to achieve and maintain universal poultry H5 vaccination should be a high priority, especially among domestic waterfowl, and poultry should be raised outside the home. In urban areas, consideration should be given to implementing control strategies in wet poultry markets that have been instituted in Hong Kong Special Administrative Region, such as only selling H5-vaccinated poultry, segregating bird species, improving biosecurity, and having central poultry slaughtering locations, regular disinfection, and a monthly rest day [42–44]. In addition, the feasibility of the wearing of protective masks or respirators by workers and visitors to wet poultry markets could be considered.

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