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Influenza research at the human and animal interface

Report of a WHO working group

Geneva, Switzerland
21–22 September 2006

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From 21 to 22 September 2006, WHO convened a working group of 22 laboratory directors and senior scientists leading research on influenza at the human and animal interface. The researchers included directors from some of the laboratories in the WHO H5 reference network, scientists from veterinary medical institutes, and virologists and microbiologists in countries affected by outbreaks. The research discussed represented the work of scores of scientists, students and technicians in the various laboratories over the last few years. The attending scientists were specifically asked to interpret their latest research in terms of its implications for public health policy. Although several avian influenza viruses were considered, emphasis was firmly placed on what is currently known about human infections with the H5N1 virus and the presence of this virus in poultry, wild migratory birds, and other animals. At the same time, however, participants recognized that the next pandemic might well arise from another virus subtype; surveillance at the animal and human interface should not be restricted to H5N1 viruses.

Discussion focused on four main topics: methods for the detection and diagnosis of human infections, the use of vaccines and antiviral drugs to protect humans, current findings from animal surveillance in countries and regions with recent outbreaks, and factors governing the virulence and pathogenicity of H5N1 viruses. Issues explored ranged from explanations for the severity of this disease and its tendency to affect younger people, through the role of migratory birds in virus spread, to the possibility that genetic factors might influence transmissibility of the virus among humans. Issues relating to control, including diagnostic limitations in the detection of human cases, vaccination policies in poultry, and the identification of avian species that act as vectors for maintaining virus transmission, were also critically assessed. Throughout the meeting, repeated reference was made to the added complexities arising from the recent divergence of H5N1 viruses into several distinct genetic groups that are now circulating in different parts of the world.

Discussions took place within the context of knowledge about the epidemiology and ecology of influenza A viruses in avian and other animal species that has been accumulating for more than 40 years. Some of the scientists who pioneered this research were present. Their perspective on developments over the past decades helped the group to pinpoint unusual or unprecedented features of the current disease situation. In the past, research at the human and animal interface has nearly always been crisis driven; the present severe crisis with H5N1 infections similarly brings a need for cohesion and urgency in collaborative research efforts. Information was generously exchanged during the meeting. Evidence presented indicates that the H5N1 virus is still evolving in animals and humans; much about the disease it causes remains poorly understood. Nonetheless, the group had little difficulty in agreeing on the most pressing research needs. It was further acknowledged that the seriousness of the present situation, including the risk that a pandemic virus might emerge, is not likely to diminish in the near future.

Executive summary

The technology for diagnosing human H5N1 infections is mature, but many tests are complex, some are liable to error, and some can be performed safely only in biosafety level 3 facilities. A simple, rapid, robust and reliable test, suitable for use in the field or at the patient's bedside, is urgently needed.

In humans, much recent research has focused on the factors responsible for the pathogenicity and transmissibility of the H5N1 virus. Several lines of evidence suggest important roles for the polymerase genes, though no single gene has yet been implicated and several genes may be working in tandem. Nor can the distinctive age profile of this disease be adequately explained at present. A genetic predisposition for infection is suspected based on data from rare instances of human-to-human transmission in genetically-related persons. This possibility, if more fully explored, might help explain why human cases are comparatively rare and why the virus is not spreading easily from animals to humans or from human to human.

The development of a pandemic vaccine has become more difficult following the divergence of circulating viruses into distinct genetic and antigenic groups. To date, results from clinical trials of candidate pandemic vaccines have not been promising, as these vaccines confer little protection across the different genetic groups. International standards, or "benchmarks", for evaluating the efficacy of vaccines are urgently needed. Integrated studies of sera from individuals being vaccinated in the various clinical trials would be equally useful – for industry as well as for national authorities.

Monitoring for virus resistance to antiviral drugs needs to continue. Although resistance to amantadine is now widespread, the possibility exists that these resistant strains may be replaced by fully susceptible strains as the virus continues to evolve. Innovative work on novel strategies for drug development was welcomed by the participants, but new drugs will not be on the market for some time to come.

The global picture of influenza viruses in the avian world has changed significantly since 2002. The massive die-off of migratory birds at Qinghai Lake in mid-2005 was unprecedented, and migratory birds now appear to be contributing to geographical spread of highly pathogenic virus. Importantly, evidence was presented for a change in virus shedding patterns, with increased shedding from the respiratory tract rather than the cloaca. Thus, for surveillance purposes, a corresponding change in sampling strategies – including both cloacal and pharyngeal swabs – is called for to get a true picture of the situation. Furthermore, domestic ducks and geese – and not chickens – have been identified as the true vectors of disease transmission in poultry. Recently, studies have demonstrated that the virus is now moving both ways in relay transmission, from poultry to migratory birds and back again. This finding might help explain some of the continuing geographical spread.

Continued widespread infections in poultry were viewed as an important on-going risk for human cases and the related risk of a pandemic. Participants agreed that more needs to be done in the animal sector to control this virus. Countries with adequate resources should continue to make culling their first-choice control strategy; experiences in Japan and Korea have shown that such an approach, though costly and disruptive, can ultimately be successful. In countries with limited resources, however, the group strongly recommended use of widespread poultry vaccination with appropriate, high quality vaccines accompanied by appropriate surveillance to detect possible asymptomatic virus circulation. In a related recommendation, the group suggested that countries with outbreaks should look at the factors driving continued circulation of the virus, and then use that knowledge to develop tailored interventions. Baseline data could be used, for example, to identify seasons of peak virus activity and this information, too, can guide intervention strategies. Hong Kong used this approach following the 1997 outbreak and found that live animal markets were maintaining, amplifying, and disseminating the virus. Intervention at this critical point eventually freed Hong Kong from the virus. More recently, Viet Nam introduced a policy of mass poultry vaccination; human cases subsequently ceased. Poultry vaccination is, however, recognized as having some limitations as a control strategy, and these limitations need to be addressed on an urgent basis: chicken immunology is much better understood than duck immunology; ducks react differently to poultry vaccines, yet vaccines tend to be approved based on protection in chickens only; production quality control and antigen content of vaccines are not standardized worldwide and sub-optimal vaccines have been used; in some countries, not all vaccine manufacturing takes place under the control of national authorities and vaccine efficacy is not always monitored.

Recommendations

1. Make the development of a simple, robust, and reliable diagnostic test for use in the field and at the patient’s bedside a high priority. Facilitate industry’s development of such a test by providing representative panels of viruses and addressing relevant issues of intellectual property rights.

2. Publish recommended diagnostic tests and methods for their accurate performance, including an alert to common pitfalls, on the WHO web site, and develop a schedule and system for regularly updating tests and kits with appropriate reagents.

3. Investigate the sensitivity with which currently available diagnostic tests are capable of detecting mild or asymptomatic infections.

4. Establish benchmarks for evaluating the effectiveness of candidate pandemic vaccines.

5. Integrate data on antibody responses in persons participating in the various clinical trials of candidate pandemic vaccines.

6. Determine which (if any) animal model provides the best information on cross-clade protection among H5N1 variants.

7. Continue to monitor H5N1 virus strains, in humans and avian species, to determine changing patterns of resistance to antiviral drugs.

8. Investigate factors that may make children and young adults especially vulnerable to infection.

9. Conduct studies to determine whether a genetic predisposition increases the likelihood of human infection or of human-to-human transmission among genetically-related persons.

10. Address and resolve the ethical issues that arise when DNA banks are established using specimens from deceased patients, family members, close contacts, and controls.

11. Develop a single, agreed upon system of nomenclature to describe different phylogenetic, genetic, and antigenic groups of H5N1 viruses globally.

12. In countries experiencing continuing outbreaks in poultry, conduct studies to identify the factors driving continued transmission of the virus, and plan interventions accordingly.

13. When culling is impracticable as a control strategy, introduce a policy of poultry vaccination, accompanied by systematic monitoring, in the interest of reducing opportunities for human exposures and infections to occur.

14. Standardize antigen content in poultry vaccines and insist on rigorous quality control worldwide in line with OIE standards.

15. Monitor virus activity in backyard flocks and live animal markets as well as at commercial farms.

16. Adjust sampling procedures for ducks in line with changes in the currently recognized pattern of virus excretion, whereby more virus is now being shed via the respiratory tract than via faeces.

17. Continue to recognize, for the purposes of surveillance and research on pathogenesis, the potential role of pigs (or other species) as intermediate hosts in the generation of pandemic viruses.

18. Enhance international collaboration in the surveillance of wild birds and in the sharing of data from such surveillance efforts.

19. Improve understanding of migratory routes for wild waterfowl and strengthen collaborative interactions with ornithologists.

I. Detection and diagnosis of human H5N1 infections

Serological methods. A presentation from the US Centers for Disease Control and Prevention (CDC) described the state-of-the-art in diagnostic testing, including laboratory confirmation of H5N1 infections based on serological tests. Such testing becomes important when poor sampling technique, poor specimen quality, or other problems rule out isolation of the virus or preclude the use of PCR-based tests. For example, two cases from the 1997 outbreak in Hong Kong were confirmed on the basis of serological results alone. Serological tests are also used in studies aimed at screening for mild or asymptomatic illness in population groups with high potential exposures to the virus, including family members and contacts of patients, poultry workers and cullers, and workers in live animal markets.

Serodiagnosis depends on the use of paired sera collected at specified dates during the course of a patient's illness, as dictated by the kinetics of the antibody response. Antibody can usually be detected 10 days post-infection; detection of antibody 9 days post-infection is rare. When the collection of paired sera is not possible, diagnosis can be made using results from a single serum sample evaluated together with clinical and epidemiological data.

Four methods for serodiagnosis were discussed. Microneutralization is considered to be the gold standard, but this assay requires use of biosafety level 3 facilities, which can be an important limiting factor. A second method, horse red cell haemagglutination inhibition, performs well for H5N1 and can be conducted in a biosafety level 2 facility. Western blot can be used as a confirmatory assay, but is not suitable for screening purposes, as it produces too many false-positive results. Serological methods based on the detection of IgG and IgM have specificity issues that depend on the patient's age, thus necessitating the use of age-matched controls. This method can, however, be useful in children.

Seroprevalence studies. Results of seroprevalence surveys conducted in Hong Kong in 1997 were briefly reviewed. Seroprevalence in sampled poultry workers was significant, at 10%, but was less in cullers (3%), and was 0% in the general population. More recently, serological testing, using paired sera, of 2,109 cullers in the Republic of Korea detected H5 antibodies in 9 persons (4 in CDC tests and 5 in Korean tests); infections were associated with mild or no illness and were acquired prior to the use of personal protective equipment by cullers. In Thailand, all health care workers studied were negative. In Viet Nam, all contacts of patients were negative, but some family members had positive results. All sera submitted from Djibouti, Nigeria, Kazakhstan, and Mongolia for testing at CDC were negative.

RT-PCR. RT-PCR tests that amplify gene fragments are a universal assay. They are highly sensitive and specific, and follow a fairly simple protocol. The risk of cross-contamination is, however, an important problem. To ensure accurate results, it is important to run quality controls, to validate primers and probes, and to validate the many different machines and platforms now in use. False-negative test results can arise from poor sample quality and inefficient extraction, pointing to the need to control the quality of samples to determine whether degradation had occurred. The interpretation of test results must always be made with caution; even with PCR, a test result might be negative for influenza A virus, but positive for the H5 virus subtype, or vice versa.

In the US, plans to stockpile PCR diagnostic kits, as part of pandemic preparedness aimed at increasing laboratory surge capacity, encountered some initial difficulties in securing regulatory approval to distribute the kits. Other countries planning to strengthen laboratory surge capacity might want to anticipate similar regulatory constraints. Countries planning to stockpile PCR diagnostic kits must also anticipate the need to update primers in line with the continuing evolution of the virus.

Development of a rapid field test. A presentation from the University of Hong described an ongoing joint effort with Xiamen University to develop a rapid test, based on panels of monoclonal antibodies, capable of detecting H5 antigen under field conditions. Such a test is urgently needed, as the virus is presently having its greatest impact in developing countries which must often rely on external diagnostic verification. The assay aims to detect H5 antibody within 20 minutes and to achieve reliable results without the need for extensive training. A major problem in the development of such a test arises from the significant antigenic and genetic diversity now being observed in circulating H5N1 viruses. Research has, however, identified some monoclonal antibodies with broad cross-reactivity across many of the genetic groups. The test shows greater sensitivity when compared with commercial tests and is achieving good specificity as well.

Detection and diagnosis of human infections in Indonesia. A presentation from the Ministry of Health's National Institute of Health Research and Development in Jakarta gave a comprehensive account of research challenges and plans in Indonesia, offering insight into many unresolved questions that continue to cloud understanding of this disease. The Institute is responsible for coordinating all avian influenza research relevant to humans within the country, and welcomes outside collaboration, particularly when such collaboration can result in capacity building and technology transfer.

In Indonesia, the H5N1 virus has been circulating in poultry since mid-2003. Outbreaks have occurred in 29 of the country's 33 provinces. Research is focusing on the extent of the problem in animals, the epidemiology of human cases, and the epidemiology of clusters of human cases. During 2006, Indonesia had (by end-September) confirmed 46 human cases, of which 37 were fatal, resulting in a case fatality of 80.4%. Most patients were under the age of 30 years. For unknown reasons, fatality was higher in females. One third of the cases were part of clusters.

Human cases have been reported from 8 provinces. It is not presently understood why human cases have occurred in only 8 out of the 29 provinces affected by animal outbreaks or why – given the widespread nature of poultry outbreaks – so comparatively few human cases have occurred. As in many other countries, Indonesia sees a need for much greater collaboration between the human health and veterinary sectors.

Efforts are under way to achieve a better picture of the size of the problem in humans. As a detection strategy, the country is conducting systematic surveillance for influenza-like illness. In collaboration with the Netherlands, seroprevalence studies are being conducted in poultry farmers and cullers. Hospital records will be examined in a search for retrospective cases of acute respiratory illness. Some 44 hospitals have been appointed for this study and 8 laboratories have been designated, but these are not yet fully functional.

Testing of suspected human H5N1 infections is performed rapidly in the national laboratory, with parallel confirmatory testing performed in the NAMRU-3 laboratory. Three tests are used in Indonesia: RT-PCR, haemagglutinin inhibition with horse red blood cells as a confirmatory assay, and DNA sequencing. Some 34 viruses have been characterized, and none has shown evidence of reassortment. The country does, however, face a backlog in the testing of samples arising from active surveillance for influenza-like illness. Around 1000 samples are now being submitted for testing each week; testing can face a backlog of as much as 3 months, occasionally resulting in the delayed confirmation of a human case.

Indonesia sees a clear need to conduct more research, yet lacks the capacity to undertake all studies on its own and welcomes collaborative efforts.

Discussion

Some participants expressed surprise that seroprevalence studies were detecting so few cases, especially in close contacts of confirmed cases. Moreover, recent surveillance studies in poultry continue to find a high prevalence of H5N1 viruses in live animal markets. Are tests sufficiently sensitive to pick these up, especially if infections are mild or asymptomatic? Is there something inherently different about this virus that complicates the detection of antibodies in human sera? Additionally, in some birds fully protected by vaccination, tests have been unable to detect antibodies, suggesting that immune mechanisms other than antibodies may be important. Several participants agreed that careful interpretation of results is needed.

A caution was raised concerning use of the horse red blood cell test as a confirmatory assay: while this test performs well with the H5 virus subtype, it is not suitable for detection of human infections with the H7 virus subtype.

Given the widespread presence of the virus in poultry in Indonesia, a question was raised about the use of poultry vaccination as a control strategy. It was felt that the public health advantages of doing so could be considerable. Viet Nam, for example, introduced such a control strategy and subsequently experienced no further human cases. The meeting was informed that the Indonesian government was presently weighing the advantages and disadvantages of large-scale poultry vaccination, but has not yet reached a decision. Such decisions understandably have a high political profile.

Also, concerning the situation in Indonesia, participants cited suggestive evidence, largely from the Karo family cluster in North Sumatra, that genetic factors might influence human susceptibility to infection, as only blood relatives were infected in that cluster, despite multiple opportunities for the virus to spread to spouses or into the general community. Indonesia welcomed collaboration in exploring this possibility, which has been included among the country’s research plans. The banking of DNA from deceased patients, family members, contacts, and controls would, however, raise important ethical issues. It was suggested that WHO could play a leading role in proactively addressing these issues.

II. Protecting humans: vaccines and antiviral drugs

Vaccines. A presentation from the WHO collaborating centre in the United Kingdom reviewed the status of vaccine development. Results to date have not been promising. Efforts to develop a vaccine that confers adequate protection have been greatly complicated by the emergence of genetically and antigenically diverse viruses that are now simultaneously circulating in different geographical areas. Vaccines that protected against viruses from clade one demonstrated poor cross-reactivity for virus subgroups in the second clade.

Distinct genetic groups of circulating viruses. In August 2006, WHO published its recommendations for H5N1 candidate vaccine viruses in the Weekly Epidemiological Record[1]. This publication also draws attention to the problem caused by the diversity of viruses currently in circulation. Viruses have recently evolved into two distinct genetic groups, or phylogenetic clades. Clade 1 viruses circulated in Cambodia, Thailand, and Viet Nam during 2004 and 2005 and were responsible for human cases reported in those countries. Clade 2 viruses, which are genetically and antigenically distinct, initially circulated in poultry in China and Indonesia during 2004 and the first half of 2005, without causing human cases. In mid-2005, the epidemiology of clade 2 viruses shifted, circulation of the viruses increased, and westward spread began, initially in wild birds, then in poultry, then in sporadic human cases in Turkey, Azerbaijan, Iraq, Egypt, and Djibouti. Beginning in the second half of 2005, these viruses also caused human cases in Indonesia and China.

Six distinct subgroups within clade 2 were subsequently distinguished, of which three also differ in geographical distribution. Of these, one subgroup has continued to circulate in Indonesia, a second subgroup of the so-called Qinghai Lake-like viruses has caused outbreaks in Europe, the Middle East, and Africa, and the third subgroup is circulating mainly in China and, to a lesser extent, in Viet Nam. Haemagglutination inhibition tests using ferret antisera have, unfortunately, demonstrated poor cross-reactivity among the different clades and subgroups, thus complicating efforts to produce a fully protective vaccine.

WHO has also published a phylogenetic tree[2] showing how the various human and animal viruses from different geographical areas cluster together in genetic groups. An exception occurs with the viruses from the Karo family cluster in North Sumatra; in inhibition tests, these viruses did not react well with other viruses isolated in Indonesia and showed greater similarity to viruses circulating in China.

Status of vaccine development. The results of several vaccine trials were reviewed. In the USA, Sanofi-Pasteur's candidate vaccine uses a split virus and 90 micrograms of antigen in a two-dose schedule. In France, trials with a Sanofi-Pasteur alum-adjuvanted split virus vaccine using 30 micrograms of antigen in a two-dose schedule suggested that the adjuvant was not having a substantial effect. In Australia, CSL is developing a split virus vaccine using 15 micrograms of antigen in a two-dose schedule. Sinovac in China has shown good results with an alum-adjuvanted whole virus vaccine using 10 micrograms of antigen in a two-dose schedule. In

[1] http://www.who.int/wer/2006/wer8134_35/en/index.html
[2] http://www.who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf

Belgium, GlaxoSmithKline has reported results with a candidate vaccine using 3.8 micrograms of virus in a two-dose schedule with AS03 adjuvant. It was also reported that Omnivest in Hungary is developing a vaccine and evaluating a one-dose schedule.

Clinical trials. Several clinical trials of candidate vaccines are under way in Europe, two are being conducted in the USA, and four are ongoing in Japan. In the USA, phase I clinical trials, have, unfortunately, demonstrated low antibody response to viruses outside the same clade as the candidate vaccine virus. In Japan, phase I clinical trials produced data similar to that in the USA, showing a lack of cross-protection among the two clades and subgroups within clade two.

Other antigen-sparing strategies. Intradermal injection as an antigen-sparing strategy does not look promising and is not likely to be suitable for worldwide use.

Use of pre-pandemic vaccines. Participants noted that many fundamental questions underlying the development of an effective pandemic vaccine have not yet been answered. What is the best adjuvant for boosting the response? Does one antigen dominate in influencing antigen recognition? What are the benchmarks for assessing an adequate level of protection? In the absence of scientific answers to these questions, concern was expressed that national policy decisions about which vaccines to stockpile may be premature, despite the understandable desire of governments to invest now in some means of protecting their populations in the event of an influenza pandemic.

Resistance of H5N1 viruses to antiviral drugs. A presentation from the Hong Kong University summarized the results of surveillance for drug-resistant strains of H5N1 virus. For amantadine, which is the second-choice antiviral drug, clade 2 viruses are more sensitive than clade 1 viruses. In Indonesia, however, (where clade 2 viruses are circulating) the prevalence of resistance to amantadine is approximately 50%. For 2006, Hong Kong data showed 28.8% (17/15) amantadine resistance among Indonesian isolates and 20% (15/75) for Chinese isolates. For the most part, the subgroup of Qinghai Lake-like viruses showed susceptibility to amantadine. In Viet Nam, from 50% to 100% of viruses studied in 2003 and 2004 showed a mutation associated with amantadine resistance, but this figure dropped to 10% in 2005. Amantadine resistance is also being observed in viruses responsible for seasonal influenza. It is not known if resistant strains of these viruses will persist or be replaced by fully susceptible strains. Resistance to the neuraminidase inhibitor, oseltamivir – presently the first-choice antiviral drug – has been observed in a few patients, and that finding is of concern. Further, surveillance studies also indicate a low prevalence of resistance mutations to oseltamivir in avian isolates, especially in 2005 and 2006.

The quest for new drugs. The worrisome H5N1 situation has greatly increased interest in the development of antiviral drugs, as it would be unwise to rely on the present limited range of therapeutic and prophylactic options. Clinical trials of additional neuraminidase inhibitors, including zanamivir and peramivir, are presently under way. In Japan, trials are under way for so-called “long-lasting” neuraminidase inhibitors capable of achieving very high blood plasma levels after a single-dose treatment via inhalation. Recent research on the structure of neuraminidase has found a slightly different catalytic site among different virus subtypes including the N1, pointing the way towards the structure-based design of new drugs that might provide broader-based therapy.

Research from the University of Wisconsin described the use of a novel peptide to inhibit virus attachment as a new approach to drug development. In *in vitro* studies, the peptide, which is not specific to virus subtype, inhibits virus binding to cells in a dose-dependent manner. In mice, the peptide prolonged survival time when challenged with H5N1 viruses. The potential for side effects of such peptides in humans needs to be addressed.

Discussion

Concerning pandemic vaccines, participants saw a great need for benchmarks for the evaluation of candidate vaccines. On such an important matter, it is unwise to leave assessments of appropriate vaccines to competing manufacturers. Could WHO advise the world on norms and standards for good vaccines? Would broader cross-protection conferred by a candidate vaccine make it the superior product? What is the most appropriate animal model for assessing protection? Participants also saw a need for integrated studies of sera from vaccinated individuals arising from the various clinical trials, and agreed that governments should not rush to place orders for pre-pandemic vaccines when so many fundamental scientific questions are still outstanding.

III. Surveillance in birds and other animals: assessing the coming risks

H5N1 surveillance in Europe. A presentation from the National Influenza Centre in the Netherlands summarized surveillance efforts in Europe for avian influenza viruses in poultry, wild birds, and other animals. For wild birds, collaboration with ornithologists has provided a wealth of useful information; flyways and migration patterns are extremely complex. Different wild and domestic species are now known to respond to the virus in different ways. These differences are clearly important for the identification of sentinel and reservoir species and the performance of risk assessment, particularly concerning the potential for a recurrent westward spread of the virus. In addition, patterns of virus excretion are known to vary according to the species, and this, too, can affect modes and risks of transmission, particularly as transmission occurs via water sources.

From 1959 to 2002, surveillance of influenza A viruses in avian species revealed few major ecological changes. The situation is now very different. The Qinghai Lake incident, which began in China in late April 2005 and resulted in the death of some 6000 migratory birds, was followed by a progressively westward spread of highly pathogenic H5N1 virus. In terms of geographical spread of the virus, mallard ducks are now regarded as the “champion” vectors; mute swans are highly susceptible birds that are thought to serve as sentinels, but probably not as vectors of virus transmission. The prevalence of highly pathogenic H5N1 infection is now known to be higher in dabbling ducks than in diving ducks. Studies have shown that ducks shed more H5N1 virus via the respiratory tract than via faeces; as a sampling technique, there is growing evidence to suggest that pharyngeal swabs are more important than cloacal swabs, which might fail to detect infections.

The surveillance network in Europe includes parts of Africa and Asia. Studies of viruses isolated from poultry in Nigeria have found evidence for three separate introductions of the virus; this finding argues against spread of the virus through trade or smuggling from a single source. In other situations, both illegal and normal trade in birds can play a role in virus spread.

For wild birds, the risk of infection is governed by such factors as the number of animals, their geographical origin, and their behaviour, including their contact with free-ranging domestic birds. Relay transmission, in which the virus moves both ways between wild and domestic birds, may also be an important mechanism for geographical dispersion of the virus, as it allows, via sequential series of infections, for virus transmission to occur over long distances. Surveillance has further determined that highly pathogenic H5N1 viruses show a high rate of mutation; reassortment with other avian influenza viruses has also been documented.

Other animal species that have been infected with the H5N1 virus include zoo tigers in Thailand, domestic cats in Europe and elsewhere, and a stone marten and a mink in Europe. Studies show that cat-to-cat transmission can occur in both domestic cats and tigers. While the participants considered that avian species remain by far the most important source of human infections, they also felt that the role of domestic cats needs to be further investigated, given their very close association with humans. In experimentally infected non-human primates or macaques, no evidence of disseminated disease has been found – a finding that appears to be similar to what is seen in human infections.

Laboratory testing for European outbreaks. These findings were further elaborated by a second presentation on surveillance in Europe, provided by the UK’s Central Veterinary Laboratory Agency at Weybridge. This laboratory has done much of the confirmatory testing for avian outbreaks in Europe. Test results further confirm the significance of mute swans as a sentinel species. Surveillance has found some highly pathogenic H5N1 virus in live, but healthy, wild birds, including gulls. Again, findings indicate that relay transmission is now occurring between wild and domestic birds. Rural areas, where wild and domestic birds can easily mingle, are considered to be of particular concern.

Europe experienced its first outbreaks during late 2005 and early 2006, with mainly wild birds affected and only a few, mostly well-contained, poultry outbreaks documented. The winter season at that time was especially severe, resulting in more frozen water areas than usual, which might explain the high concentrations of birds in some areas, such as northern Germany. Animal vaccination policy in Europe has become more open, and some countries are now using vaccination as a control strategy. Experimental work with sub-lethal doses of H5N1 virus shows that birds can still shed virus in the absence of signs of illness. Experimental work in avian species has further shown that highly pathogenic H5 viruses are more lethal than viruses of the H7 subtype. An analysis of diagnostic proficiency in European laboratories was undertaken. Overall, performance was good; poor performance was often due to the use of commercial diagnostic kits, which did not always produce reliable results.

Recent evolution of the H5N1 virus in poultry in China. A presentation from the University of Hong Kong reviewed findings from a surveillance network that covers some 4 billion birds in China. These findings indicate that the virus has become endemic and is continuing to evolve. The Z genotype of the virus remains dominant. In migratory birds, the Qinghai Lake outbreak changed the epidemiology in mid-2005, and this change signalled the progressive westward spread of Qinghai Lake viruses and their descendants. Surveillance over the past 24 months showed a peak in virus activity during the month of January, followed by a decline in virus activity in April. The situation is severe and not yet fully under control. During the past year, more than 1,300 H5N1 isolates have been obtained from poultry in southern China. Prevalence is higher in domestic ducks and geese than in chickens, and highly pathogenic virus was being found in apparently healthy birds. Most of these isolates belonged to the dominant Z genotype. In southern China, viruses related to the Fujian-like lineage of the Z genotype were found in 80% of the isolates and that figure has recently risen to 95%, indicating that, in southern China, Fujian-like viruses are replacing other virus lineages and becoming the dominant lineage within the genotype. To manage this situation, the whole poultry population will need to be vaccinated, accompanied by monitoring of effectiveness of the vaccination programs.

Lessons from recent poultry outbreaks. Japan experienced an outbreak, beginning in January 2004, of highly pathogenic H5N1 avian influenza in poultry in three areas, and this outbreak – which was controlled – has been well documented. In June 2005, a second outbreak, caused by a low pathogenic H5N2 virus, began and continued through April 2006, resulting in the culling of some 5.68 million birds. Interestingly, the closest virus on the phylogenetic tree was from Nicaragua, a finding that could not be readily explained. Studies demonstrated that the H5N2 virus is highly adapted to chickens; it grows very well in chick embryos. Serosurveillance of poultry has continued in Japan, and the virus is no longer being detected. Japan has also been working region-wide to improve diagnostic capacity in Asian countries, as many have no biosafety level 3 facilities or sequencing capability. A panel of viruses for diagnostic testing has been developed and training courses in animal influenza have been conducted. A new website cataloguing Japan's collection of low pathogenic avian influenza strains available to researchers was presented and discussed.

In Kazakhstan – a large country with many different ecological zones and wetlands criss-crossed by migratory routes – a system for surveillance, mainly in wild birds, has been in place since 1979. In 2005, the country experienced an outbreak of highly pathogenic H5N1 avian influenza on a goose farm, an open facility allowing opportunities for wild and domestic birds to mingle. In March and April of 2006, the surveillance system detected highly pathogenic H5N1 virus in 80 wild birds found dead. All viruses isolated from these birds were highly pathogenic, resulting in 100% mortality. Characterization of the viruses showed their similarity to Qinghai Lake-like strains isolated from birds from Angola, Nigeria, and the Russian Federation. Work was also described regarding Kazakhstan’s efforts to develop an ISCOM-based poultry vaccine.

The Republic of Korea was the first country, in the current wave of outbreaks, to report highly pathogenic H5N1 in poultry. That outbreak began on 10 December 2003 and continued for 14 weeks. Since 21 March 2004, no H5N1 virus has been detected in the country. The outbreak was costly and highly disruptive. At that time, Korean animal authorities implemented strong control measures without vaccination, including culling within a 3 km radius, strict movement controls, disinfection activities, intensive surveillance, and biosecurity and public awareness activities in line with the contingency plan. The government adopted a policy of 100% compensation for lost birds. Altogether, some 5.6 million birds were destroyed at a cost of more than US$ 150 million. As part of risk assessment for a resurgence or reintroduction of highly pathogenic avian influenza, active surveillance has focused on duck farms and wild migratory birds, including both winter and summer migrations. Serological surveillance is used on duck farms. In addition, passive surveillance is conducted for wild birds found dead.

Discussion

Participants expressed concern about the detection of H5N1 birds this spring in Kazakhstan at the Caspian Sea and reports of dead birds in recent weeks, as that finding suggests a possible repeat of the pattern of westward spread of the virus that caused so much alarm in late 2005 and early 2006. As outbreaks at Qinghai Lake have been reported again in 2006, countries located along the autumn migratory routes will need to be vigilant.

Questions were raised about the unusual outbreak of H5N2 avian influenza in Japan. As the H5N2 strain was used in poultry vaccines, could the illegal use of possibly sub-standard vaccine have been a source of the outbreak? The group was informed that Japanese authorities investigated that possibility, but were unable to reach a conclusion. Similar to results with H5N1 virus infections in previous studies, the Japanese H5N2 virus was found to infect pigs (miniature pigs) in experimental studies, but virus shedding was limited and of short duration.

As Korea was the first country to report a poultry outbreak during the most recent H5N1 outbreak, questions were also raised about the source of that outbreak. The meeting was informed that the Korean virus is very similar to H5N1 viruses that circulated in southern China during 2002.

Participants emphasized that the potential role of pigs in the emergence of a pandemic virus should not be forgotten. Surveillance for influenza viruses in pigs can yield important information about the prevalence and ecology of these viruses; such an approach can also provide early evidence of a reassortant virus with pandemic potential.

IV. Deciphering the virulence and pathogenicity of H5N1 infections in humans

H5N1 pathogenesis. A presentation from the University of Hong Kong considered how the virus infects humans and why the resulting disease can be so severe. The situation with human infections was described as paradoxical: thousands upon thousands of people are likely to have been exposed through contact with poultry, yet fewer than 300 human infections have been detected; conversely, 30% of the initially infected human patients in Hong Kong in 1997 had no apparent contact with birds. Overall, the virus continues to show inefficient spread, both from animals to humans and from human to human. Though the virus replicates in humans, infected people do not transmit it easily to others. Clusters of cases have occurred, but transmission has not been sustained and appears to be confined to genetically-related persons.

What factors might be at work? Genetic susceptibility of the host? Host resistance factors? The significant species barrier observed clearly involves receptor specificity; however, other genes unrelated to receptor interactions might be involved. Equally possible, multiple genes might be working in tandem to govern human susceptibility to infection.

Avian-like receptors in the human lung. Recent research on receptor expression in the human respiratory tract was reviewed. Work has shown that receptors for avian-like viruses can be found in the epithelia deep in the human lung. This finding helps explain several features of human infections – if the virus lodges deep in the lung, that would explain the severity of the disease, the inefficient spread from animals to humans, and the virtual absence of human-to-human transmission. The paradox was not, however, fully resolved, since data were also presented showing that the H5N1 virus can also cause infection in cells from nasopharyngeal biopsies of the upper respiratory tract, like normal influenza. It is furthermore known that normal human H3N2 influenza viruses can cause infection deep in the lungs. In H5N1 infection, higher viral loads have been detected in the pharynx than seen with normal influenza, but the high prolonged virus replication observed in H5N1 infection might simply reflect a lack of pre-existing immunity.

The question remains open: what is needed for the H5N1 virus to transmit efficiently from one human to another? Overall, the genetic controls of cross-species infectivity and transmissibility of influenza viruses are complex and not yet fully understood. In experimentally infected ferrets, very inefficient animal-to-animal transmission was shown to occur. In further ferret experiments using artificially reassorted viruses, the efficiency of animal-to-animal transmission improved somewhat. These experiments suggest that a reassortment of genes between mammalian and avian viruses may not alone have a great impact on transmissibility with the viruses tested to dated. This finding does not, however, exclude the possibility that adaptive mutation alone or reassortment together with adaptive mutation may give rise to a pandemic virus.

Disease severity. The disease caused in humans by the H5N1 virus was described as fundamentally different from that caused by normal influenza. In H5N1 infection, the disease syndrome typically shows progressive primary viral pneumonia, acute respiratory distress, marked leukopenia and lymphopenia, and (in some cases) diarrhoea and liver or renal dysfunction. What might explain this severity? Some limited findings suggest that the virus might cause disseminated infection, affecting multiple organs. In some patients with a fatal outcome, virus has been detected in faeces, serum, and blood plasma. However, respiratory pathology remains the primary cause of death. Additional data presented support the hypothesis that severe disease is based on induction of a "cytokine storm"; it was pointed out, however, that this remains a "chicken-and-egg" dilemma – does an overwhelming level of cytokinemia result in, or from, extensive tissue damage and disease?

Determinants of virulence and transmissibility. A mutation in the PB2 gene, at position 627, has been shown to influence pathogenicity in mice, but that finding has not consistently correlated with severity of infection among viruses isolated from patients in Indonesia and Viet Nam. Investigation of the role of internal polymerase genes is continuing. It appears clear, however, that the external HA and NA genes are not the sole drivers of disease severity. Likewise, transmissibility of the virus may ultimately prove to be a genetically complex trait. One especially important question that was discussed is whether the H5N1 virus is likely to retain its present high lethality should it acquire an ability to spread easily from person to person, and thus start a pandemic. Should the virus improve its transmissibility by acquiring, through a reassortment event, internal human genes, then the lethality of the virus would most likely be reduced. However, should the virus improve its transmissibility through adaptation as a wholly avian virus, then the present high lethality could be maintained during a pandemic.

Host range of avian influenza viruses. A presentation from the WHO collaborating centre in the United Kingdom explained ongoing research aimed at identifying specific genes in influenza viruses that influence their ability to infect different species. These experiments, which used a chloramphenicol acetyltransferase reporter system to map the contribution of individual amino acids to polymerase function, had confirmed the role of a Lys mutation at position 627 on the PB2 gene in regulating the species-specific activity of internal polymerase genes. In these experiments, neither the HA nor NA external genes showed a role in determining the ability of influenza viruses to infect human cells. Interferon induction and response were shown to contribute to the host range of avian influenza viruses; however, the control of these effects depends on genes other than just the NS1. Additional studies described the creation of chicken/human chimeric cell lines with variable amounts of chicken genetic components, which may be found to be useful in understanding the role of host genetic factors in species specificity.

Studies of animal H5N1 viruses in Australia. A presentation from the Australian Animal Health Laboratory reviewed a range of recent studies. Concerning avian influenza in birds, in the north, surveillance has found Newcastle disease virus but no avian influenza viruses whatsoever. It is somewhat puzzling that Qinghai Lake viruses have migrated north and westwards but not towards the south. The laboratory has also conducted work on experimentally infected birds showing that the severity of disease is dose-dependent. In ducks, studies indicate that the inoculation dose of H5N1 virus could be titrated down to a point where birds become infected and seroconvert, but do not develop clinical illness. In ferrets, experimental infection at low doses induced pulmonary disease; higher doses induced systemic disease. Ferrets have also been used to test vaccines and have demonstrated improved antibody response with an adjuvanted vaccine. Isolates from Cambodia, Indonesia, and Malaysia have been characterized; some Indonesian viruses have shown mutations associated with resistance to oseltamavir.

Pathogenesis in the duck. A presentation from the WHO collaborating centre for animal influenza in Memphis, Tennessee concentrated on pathogenicity in ducks. As mentioned previously, mallard ducks are thought to be a major force in maintenance of viruses in the wild, but the highly pathogenic phenotype does not remain stable; instead, there appears to be rapid selection for a virus that is non-pathogenic for ducks, but still highly pathogenic for chickens and, presumably, for humans as well. In both Viet Nam and Thailand, ducks were infected but appeared to remain healthy. From 1997 to 2002, most virus shedding in ducks occurred via faeces. More recently, however, most virus shedding has occurred via the respiratory tract; this route of virus shedding does, however, continue to cause contamination of water sources used by the birds. In domestic poultry, ducks shed virus for up to 17 days and pheasants shed virus for up to 40 days.

Concerning mechanisms of pathogenicity, experimental studies show that five amino acid changes transform low pathogenic viruses into highly pathogenic viruses; pathogenicity is associated with plaque size in MDCK cells. In ducks, mutations in internal polymerase genes rather than the HA appear to control high pathogenicity. It is not known, however, exactly which of the polymerase genes work to increase pathogenicity. Finally, it was pointed out that chickens are clearly not normal hosts for influenza A viruses; only viruses of the H5 and H7 subtypes replicate efficiently in chickens but this situation is changing: H9N2 viruses are endemic in chickens across Eurasia.

Discussion

Questions were raised about the extent to which H5N1 viruses spill over into the bloodstream of infected humans and whether this is necessarily associated with tissue damage. As very few autopsies have been performed, such questions remain difficult to answer. Reference was, however, made to evidence from two pregnant patients in China where virus was detected in the placenta.

Several participants sought explanations for the strong propensity of the H5N1 virus to infect children and young adults, causing severe disease with high case-fatality. In persons older than 50 years, infections were much less common Exposure history might be one explanation, as children tend to treat birds as pets or play in areas frequented by poultry. That hypothesis was not, however, considered adequate to explain all cases. Are immunological factors at work? Some evidence suggests a broader antibody response in the elderly. Could previous exposure to other influenza viruses help explain the distinctive age profile? Cytokine expression can also be related to age, and this might be another hypothesis to explore.

Concerning the potential high lethality of a wholly avian pandemic virus, some modelling studies have suggested that pandemic spread could not be fully sustained in the presence of very high mortality. All such matters remain difficult to predict.

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