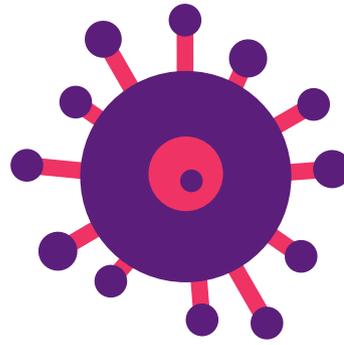


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QUESTIONS ON H5N1

BY ED YONG



Scientists now know that the deadly bird flu virus is capable of causing a human pandemic. That makes tackling the remaining unknowns all the more urgent.

The biology of the H5N1 avian influenza virus is rife with paradoxes. The virus is widespread, but hard to detect. It kills more than half of the people known to be infected, but thousands of those exposed have no apparent problems. It seems to be just a few mutations away from gaining the ability to spread from person to person, but despite more than 16 years of fast-paced evolution, it has failed to do so.

This week saw the publication of the second of two papers identifying mutations that give H5N1 the ability to spread through the air between ferrets. The papers, the latest¹ from a group led by Ron Fouchier at the Erasmus Medical Center in Rotterdam, the Netherlands, and the earlier one² by Yoshihiro Kawaoka at the University of Wisconsin-Madison and his colleagues, have been controversial because they offer what some see as a recipe for disaster — that they increase the risk of accidental or intentional release of a deadly human pathogen. But what is most unsettling about them, say many in the flu community, is the evidence they provide that the wild virus could spark a pandemic on its own. That threat makes the outstanding scientific mysteries about this tiny RNA virus — its genome just 14,000 letters long — even more pressing. Here are five of the biggest puzzles, and what researchers are doing to solve them.

1 WHY IS IT SO SUCCESSFUL?

H5N1 influenza gets its name from the combination of two proteins on its surface: haemagglutinin (HA) and neuraminidase. But there are many different strains of H5N1. The highly pathogenic strain that has grabbed headlines for more than a decade was first identified in 1996. Called Gs/Gd because it was found in domestic geese in China's Guangdong province, it is "totally different to any avian influenza virus in the past", says Robert Webster, a virologist at St Jude Children's Research Hospital in Memphis, Tennessee. Most avian influenza viruses ride harmlessly aboard wild fowl, occasionally flaring into lethal but short-lived outbreaks in domestic birds. The Gs/Gd lineage, however, has jumped back from poultry into wild fowl. It also infects mammals, including humans,

tigers, pikas, civets and more. It has spread to 63 countries, and is endemic in bird populations in six of them.

"What is so special about this virus that allows it to spread through the animal world so effectively?" asks Jeremy Farrar, a tropical-medicine specialist at the University of Oxford, UK. There are no firm answers.

China's crowded farms and markets, which offer a smorgasbord of potential hosts, might have selected for viruses that are adept at crossing the species barrier. The virus evolved quickly in 1999 and 2000, its family tree sprouting many new branches after efforts to stamp it out among domestic birds failed. During this time, one clade of the Gs/Gd lineage, known as 2.2, picked up a mutation in *PB2*, one of three polymerase genes that allow the virus to copy its genome. The mutation is widely considered to be an adaptation to mammalian hosts.

In 2002, for reasons that are still unclear, the viruses started hopping back into wild birds and killing them. At first, there were just a few isolated deaths, but in May 2005, clade 2.2 viruses killed more than 6,000 geese, gulls and ducks at Qinghai Lake — China's largest lake, and a major breeding spot for migratory birds. This outbreak heralded the start of a global tour in which the virus spread through bird trade and wild migrations to the rest of Asia, Europe and Africa. Vaccination controlled the virus in Hong Kong and Vietnam, but where applied haphazardly, it has helped to speed up the virus's evolution. In Egypt, it led to the birth of several new sub-clades³, and the country has had more new human cases than any other nation every year since 2009.

There is some good news: infections in wild birds have fallen sharply since 2006. But even as old lineages wane, new ones arise, such as clade 2.3.2.1, which has swept through poultry in Asia since early 2011. "That's the one that is of great concern to me," says Webster. "It seems to be becoming dominant and it goes into wild birds readily." H5N1 may be evolving faster than our ability to understand it.

2 **WHERE IS IT NOW?**
H5N1 seems to be both everywhere and nowhere at the same time, making it hard to predict when, where and whether it will bloom into a human pandemic.

Wild birds carry H5N1, but the virus can be hard to detect because very few become ill. No one knows how widespread it is in humans, either. As of 7 June, the World Health Organization had counted 606 H5N1 infections in humans, 357 of them fatal. Many think that the real number is much larger, which would mean that the death rate would be much lower than 60%. Peter Palese, a virologist at Mount Sinai School of Medicine in New York, looked at a number of studies that had found evidence of H5N1 infection in the blood of healthy people. He estimates that 1–2% of people in populations exposed to the virus become infected, but most have only mild or no symptoms⁴.

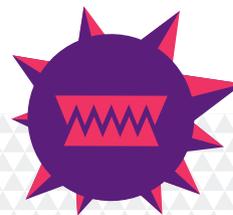
Palese's arguments are controversial. Farrar, who has treated patients in Asia, says that most of the cases he has seen have been severe. "If many mild infections were occurring, we'd expect to see some less severe patients in hospital, given the heightened awareness in the public and the medical profession," he says.

Underlying the debate about the infection rate is a poor understanding of how the immune system responds to the virus. People who become infected produce antibodies and T cells that recognize the virus, but no one knows how these responses rise

and fall over time, or how they manifest in people who show no symptoms. The signals could also be false alarms. "If you go into henhouses every day to clean up bird droppings that are loaded with virus antigen, you may get an antibody response without being infected," says Fouchier. More thorough surveillance of suspected cases will be needed to resolve the debate about how often people are infected, says Fouchier. Farrar adds that to understand how immune responses change over time, such studies will have to be done over several years. Many research-

The result, a cytokine 'storm' that floods the lungs with fluid and fatally damages surrounding tissues, is often what kills people. H5N1 triggers a more extensive storm than the human flu viruses H1N1 or H3N2 (ref. 5).

These factors may explain the severity of recorded cases, but not why infections are so rare. "Why is it that there are tens of thousands of kids running around playing with



"IN PRINCIPLE, H5N1 CAN BECOME AIRBORNE. THE CRITICAL QUESTION IS WHETHER IT WILL."

ers have called for better surveillance of domesticated and wild animals, too.

To Ilaria Capua, a veterinary virologist at the Experimental Animal Health Care Institute of Venice in Legnaro, Italy, the distribution of H5N1 is the most important issue, and the hardest to work out. "Any prediction about whether this virus will go pandemic is a function of where it is, how much of it there is and how possible the human–animal contacts are. But there are big black holes of information."

3 **HOW DOES IT KILL?**
Bit by bit, scientists are teasing out the genetic factors that make H5N1 so deadly. The virus has several mutations in its three polymerase genes that allow it to replicate aggressively, and patients who die carry the highest levels of viral RNA. Certain changes to *HA*, which codes for a protein that latches onto host cells, also allow the virus to infect tissues beyond the lungs and gut, including the brain. This all-access pass helps the virus to kill ferrets, mice and birds, but it is apparently less important in primates. "In humans, it still looks predominantly like a respiratory disease kills the patients," says Malik Peiris, a clinical virologist from the University of Hong Kong. Autopsies might paint a clearer picture, but Peiris says that these are rarely allowed in Asia because of cultural demands for whole-body burials.

H5N1 also drives the immune system berserk. Immune cells flock to sites of infection and produce inflammatory chemicals called cytokines, which attract more immune cells.

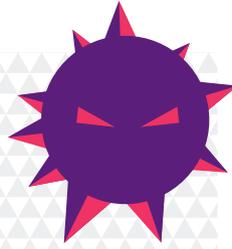
sick chickens, but we've only had 600 infections over nine years?" asks immunologist Anthony Fauci, who heads the National Institute of Allergy and Infectious Diseases in Bethesda, Maryland.

Palese suspects that the severe cases have simply inhaled high doses of the virus. But Peiris says that this cannot be the sole explanation. "Infection and disease are not directly proportional to exposure. Ninety-nine point nine per cent of the people who are massively exposed don't have disease, and don't have antibodies in their blood. But in people who get sick, the virus replicates like crazy."

Notably, cases are often clustered within families, specifically blood relatives. These people might be genetically susceptible to H5N1 infection, or others may have genetic variants that protect them. Identifying such variants will be hard because fewer than 300 people around the world have survived the infection, but studies are starting to reveal clues.

A few months ago, a group from the Wellcome Trust Sanger Institute in Hinxton, UK, found that the gene *IFITM3* plays a pivotal part in responses to some flu infections⁶. One variant of the gene, which encodes a stunted protein, was overrepresented in people who had been hospitalized with pandemic and seasonal flu strains, and even a mild H3N2 virus ran amok in the lungs of mice that lacked the gene⁶. Farrar has just completed a similar study, of 67 Asian patients who had been hospitalized with H5N1. The results, which have been submitted for publication, identify variants in two other genes that seem to confer susceptibility to the virus.

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4 WILL IT BECOME TRANSMISSIBLE IN HUMANS?

So far, people seem to catch H5N1 only through close contact with infected birds. To spread from person to person, the virus would have to become transmissible through airborne droplets. The two papers just published^{1,2} have shown that that is possible.

Fouchier's strategy was to tweak *HA* so that its protein recognized receptors in the upper airways of mammals rather than those on the surface of bird cells¹. He then allowed the virus to pass between ferrets until it evolved such that it started spreading through coughs and sneezes. Kawaoka took a similar approach², but he fused a mutated *HA* from H5N1 with other genes from a 2009 pandemic H1N1 strain. “In principle, H5N1 can become airborne,” says Fouchier. “The critical question is whether it will.”

One of the biggest questions about H5N1 is why it hasn't become transmissible after circulating for so many years — but no-one has a good answer. Many of the mutations that Fouchier and Kawaoka identified are already found in the wild. By searching surveillance databases, Derek Smith, a bioinformatician from the University of Cambridge, UK, found that many wild clades are already two to four mutations away from the sets that Fouchier and Kawaoka identified⁷.

Smith was unable to determine the actual risk because surveillance data masks the genetic diversity of the virus. H5N1 reproduces with errors, so any one patient carries a swarm of viruses with subtle genetic differences. The databases contain just the ‘consensus’ sequence, essentially a mash-up of the most common variant at every position in the genome. Only deeper sequencing, in which each position is read many times over, will reveal all the variants.

Even if the same combination of *HA* mutations that Fouchier and Kawaoka identified arises naturally, no one knows whether the resultant viruses would spread between humans as easily as they do between ferrets in the lab. It is also not clear how H5N1's other genes contribute to transmissibility, or whether different combinations of mutations would achieve the same effect. “These guys have only scratched the surface,” says Webster.

Fouchier and Kawaoka say that the value of their work lies in identifying the physical traits that make H5N1 transmissible. Some mutations allowed *HA* to recognize mammalian receptors, whereas others stabilized the protein. “If you take those traits, can you then make any flu virus go airborne?” asks Fouchier. The virulence of a transmissible strain is another unknown. One hypothesis suggests that as transmissibility goes up, virulence will become muted. An airborne H5N1 might recognize receptors in the upper airways, for example, but be less likely to descend into the lungs to cause the extensive damage inflicted by wild strains. “Theoretically, one could imagine such a scenario,” says Peiris. “But I wouldn't want to stake my life on it.”

Fouchier and Kawaoka's mutant viruses caused milder disease in ferrets than their wild counterparts do, but both men note that such comparisons are misleading because wild H5N1 has to be administered to the animals directly, which can introduce high doses deep within the lungs. And, Kawaoka notes, transmissible strains do not have to have a fatality rate of 60% to kill millions of people: the H1N1 pandemic of 1918 had a mortality rate of 2.5%, yet claimed around 50 million lives.

5 WHAT ELSE COULD CAUSE A PANDEMIC?

The Gs/Gd strain is what is known as a reassortant. It was born from a flu version of sex, in which different viruses infecting the same cell swap genes, and it probably includes genes from the H6N1 and H9N2 viruses⁸. Since then, H5N1's descendants have swapped genes mostly with each other. “H5N1 is not very sexually promiscuous,” says Capua. “It likes to reassort within its own lineage.” But the H1N1 strain responsible for the 2009 pandemic could shake H5N1 from its insularity. That strain is itself a cocktail of genes from swine H1N1, avian H1N1 and human H3N2 and includes a set of genes called the triple-reassortant internal gene (TRIG) cassette, which seems to make flu viruses more prone to reassortment. “That virus loves to mate,” says Webster.

Kawaoka's team has shown that the two viruses are compatible, and will reassort

spontaneously when they infect the same cells⁹. This is made more likely by their shared ability to infect pigs. Furthermore, Stacey Schultz-Cherry, a virologist at St Jude Children's Research Hospital, has found that reassortant viruses containing *HA* from H5N1 and other genes from pandemic H1N1 are better at replicating in human lung cells than either parent is, and that they become more virulent after a few rounds of replication¹⁰.

Wendy Barclay, a virologist at Imperial College London, cautions that although these experiments reveal that reassortment is possible, they do not quantify the odds that it will happen. “If you force the event, it'll happen, but I haven't seen anyone do the experiment in a more natural way,” she says. That would involve housing uninfected pigs with ones carrying pandemic H1N1, and poultry carrying H5N1. “Do they catch both viruses and do the viruses mix up?” asks Barclay. “It's an unknown and a pretty important one.”

The upside of an H5N1–H1N1 reassortant is that many people have already been infected with H1N1 and so might have some immunity. But few people have encountered any of the flu viruses that circulate in birds. “I think the great worry is that a purely avian virus somehow crosses over to us,” says Farrah. H5N1 tops the list of concerns because of the severe nature of the known infections, but other subtypes could escalate into pandemics first.

“H9N2 may be an equally plausible pandemic candidate,” says Peiris. It generally goes unnoticed, but has hunkered down among Asia's poultry, caused occasional outbreaks in humans and can reassort with seasonal flu. Some strains already have mutations that are associated with greater transmissibility in mammals. H7N7 is similarly widespread and under-reported. In 2003, it flared up in the Netherlands, infecting 89 people and killing a veterinarian. Virologists hope that by understanding the secrets that allow H5N1 to spread and kill, they are in a better position to assess the risk posed by other subtypes. “With flu, nothing is predictable,” says Capua. ■

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