

Transcript of the Question and Answer Sessions from the Fifth International Symposium on Avian Influenza

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Peter Woolcock. *Did the amino acid sequence change from emu to chicken passes as IVPI increased?* **Paul Selleck.** No changes were seen in a PCR product covering 200 bases around the HA cleavage site.

Daniel Perez. *During the serial passage of the H7 in chickens, were viruses passaged in eggs before going to chickens? (I mean ck to eggs to ck to eggs to ck or ck to ck to ck)?* **Paul Selleck.** The virus used for the first IVPI had been passed twice in eggs and between each pass in chickens the virus was reisolated in eggs from cloacal swabs (i.e., egg—egg—ck—egg—ck—egg—ck).

David Swayne. *Have surveys been done in wild birds to assess the influenza subtypes they are carrying? You mentioned the wild birds several times in the presentation associated within the farms and area.* **Paul Selleck.** Yes, immediately following the outbreak, a fairly stringent survey was done of the wild bird population in the area. Unfortunately, H7 viruses were not isolated from these birds. And in the past, surveys of other wild bird populations in Australia have also failed to yield any H7 viruses.

Anonymous. *Is there any new subtype of avian influenza now? And have any new methods been developed on evaluating pathogenicity of avian influenza viruses?* **Dennis Senne.** I guess I lose this one to Dr. Alexander by default. Currently there are still only 15 subtypes of influenza that are recognized. I am not aware of any new subtypes that have been described in the literature. As far as methods for pathogenicity testing, the standard tests are being used. The tests are to determine the amino acid sequence at the cleavage site of the hemagglutinin protein and the chicken pathogenicity test that Dr. Alexander referred to earlier and promised to tell us about on Wednesday. If there are any specific questions regarding the specifics of the pathotyping tests, we would be glad to discuss those, but I don't know that this is the appropriate time.

Hailu Kinde. *The transmurial oviductal edema and fibrinous yolk peritiruitis was also seen with H6N2 infection. However, we have not seen the severe tracheitis you saw with H7N2.* **Robert Eckroade.**

Okay, I was unaware that those lesions were occurring in your H6.

Anonymous. *In your opinion, what is the best sample to take for avian influenza directigen flu A test: cloacal or tracheal?* **Robert Eckroade.** If you really want to know, take both. Actually, our best results came from the lung or trachea. But sometimes when it is absent from there, you can find it in the cloaca, but we have had a few false positives in cloaca.

Patty Dunn. One characteristic about this H7N2 virus that we have been working with seems to be that it is more respiratory adapted. Of the samples that we are testing in the laboratory, we have far greater percentage of recovery from tracheal swabs than we do from cloacal swabs. So I think, like Bob says, if you want to really be sure you need to take both because you do not know the properties of the virus that you are working with are.

Anonymous. *How were you able to quarantine restricted flocks if the virus was considered to be low pathogenic?* **Robert Eckroade.** Well, let me just comment a minute; Patty Dunn is sitting here with me too. Keep in mind, low path avian influenza is not an OIE list A disease. The (federal) government is not the one that makes the decisions about what we are going to do about these low path outbreaks. This has to be an industry-driven but cooperative program with industry, academia, and state–federal government. The federal government I think up to now has played a more minor role. And so the decisions on how to quarantine, isolate, and depopulate all come from a committee action that decides on how to deal with these flocks until we find out better, we are going to do X, Y, and Z. And then, as I said, in this outbreak, the decision was made after depopulating many flocks, to not kill anymore. It was made by that same group that said look, this isn't working this time, so maybe we should just let some of these flocks live and try quarantine and market depopulation. The committee also thought that it was safe to allow marketing of those table eggs. The eggs moved on dedicated equipment, dedicated vehicles only on that day of the week, trying to remove any

possibility of transmission to the rest of the industry. And it worked. We think it worked fairly well. But again, that was an industry decision, driven by industry and then supported by the other parts of the group. **Patty Dunn.** Well, I just wanted to say that at any given time in Pennsylvania, we will have a flock that our department of agriculture has under quarantine because they found it antibody positive. They feel that they want to quarantine and send serum samples to Mr. Senne [at the National Veterinary Services Laboratories, Ames, IA] for analysis, and collect follow-up virus isolation samples. If they are negative by virus isolation, they are released to do business. But quarantine is the way of life with avian influenza in our state. It is usually a premise quarantine. In the cases that Dr. Eckroade talked about (from 1996–97), it was a regional quarantine. The 2001–02 cases that I talked about were premise quarantines, but if it had spread further, it would have been an area quarantine.

Anonymous. *What about paying the indemnity? Where does the indemnity money come from in Pennsylvania?* **Robert Eckroade.** Well, to start with, in the earlier days there never was any money available. The feds stood up and said absolutely no money for low path. The state stood up and said we don't have any money. Our state veterinarian in those days said I cannot sign on to that, and so the industry still made the decision to kill those first few flocks trying to control it. Gradually the industry built up some reserve money in a pool where they asked for donations and they built the pool up. That money is primarily used for very small flocks, backyard flocks, and gamebirds. Where they will go in and ask "Sir, we would like to buy your birds." "Would you sell them to us?" Then the industry pays for them themselves. Now more recently, the state government has made available a pool of money that will only pay, by state law, 66% of the cost of those birds and that's not much. That is not enough for some people. In Pennsylvania, they have come to believe that if we can get 66%, we're a lot better off than trying to fight this thing and not do it. So they take their licks and the 66%.

Anonymous. *With regard to bio-security, what specific practices are you employing? For example, what type of C&D is used in your situation?* **Kalid Naeem.** Basically, again, just different planning. There is no common policy for the control of H9 infection. So what some people are doing is that they slaughtered the flock and then the premises is disinfected and kept vacant for a couple of weeks.

And then they place birds after vaccinating against H9. Others disinfect the shed and place new flocks hoping that the disease will not strike again.

Anonymous. *You mentioned two treatments for broilers at risk or infected with H9: 1) treatment with amoxicillin. How was this administered? 2) Two, aqueous killed vaccine; how would it stimulate immunity?* **Kalid Naeem.** Okay, the amoxicillin is used orally in the drinking water. In a number of cases, we used it for 8–10 days, and some people have reported repeating it after a week's break. Many of them were able to curtail the infection. Second part was how does that aqueous-based killed vaccine work? Well, the aqueous-based killed vaccine is a normal vaccine which has aluminum hydroxide as an adjuvant, and I think its stimulation of immune response would be similar to the known mechanisms for how other vaccines work. The vaccine is injected subcutaneously.

Anonymous. *How long did it take for positive facilities to turn negative postdepopulation, and C&D—cleaning and disinfectant?* **Patty Dunn.** Basically, the houses were closed up, and the recommendation was to leave those houses closed and empty for 3–4 weeks before we did anything. So, we did not test immediately after depopulation. All along we did not do any extra testing, I should say. But when we went in, mostly between 3 and 4 weeks after depopulation, and did at least 10 environmental samples for virus isolation, they were all negative in all of the flocks. I cannot say when before that if there may have been remaining viable virus. The other thing is virus isolation on environmental samples could be hit-or-miss. I mean, we cannot absolutely say that there was no virus there, but based on some work Dr. Lu is going to present later about the persistence of the virus, we do not believe it is very great in those dirty environments. As for cleaning and disinfection, I do not know exactly what procedures were done, but I know the Pennsylvania Department of Agriculture did inspect what the owner did. And once the litter and the partially composted birds were both taken outside, they were not allowed to leave the premise, and they were piled and covered outside the chicken house. They were supposed to remain there for at least another 4 weeks before they did anything with that. I do not know if there are any plans to test that material at all.

Anonymous. *How do you test the environment for type A influenza?* **Patty Dunn.** Sampling varied depending on the set up of the house—the broiler-breeder house is a little bit more difficult. We took

15 to 17 samples from them. We took just gauze pads, and we did some drag swabs on the litter part. We did a lot of hand swabbing of the egg room, the slats. We got down through the slats, and dug into the manure, and took as many samples as we could that way. It was just spot sampling with gauze pads, and we put that directly into virus transport media and inoculated eggs. We do not tend to use an ELISA test on environment samples—not really a good idea for low virus titer samples and dirty samples, where you might have some false positives because of the interpretation of the color change involved, and the color involved in the dirt and the dust.

Anonymous. *With the H5 virus still present in Mexico, despite the vaccination program, is there still disease within vaccinated flocks?* **Dennis Senne.** I guess I cannot answer this question. Dr. Rivera (Mexico), would you care to comment on that question? **Eduardo Rivera.** The answer is no. And I should add that the virus that we are presently dealing with is, of course, the low path. We haven't seen any new break of highly pathogenic one since June 1995. That answers your question?

Anonymous. *You mentioned that the H9N2 in mid-east Asia is closely related to the avian influenza virus that caused human fatalities. Can an H5N1 and any H9N2 be closely related?* **Dennis Alexander.** No, I said that the H9N2 viruses that have been isolated and tested in some countries, particularly Pakistan, but also some from Iran and Saudi Arabia, I think, were closely related to the H9N2 viruses that have been isolated from humans in Hong Kong.

John Donahoe. *Is the H9 in the Middle East similar to the reference strain or is there some subtype variation?* **Dennis Alexander.** Yes, I cannot for the life of me remember which strain is the reference strain [Post meeting addition—A/turkey/Wisconsin/66 (H9N2)]. But, if you are talking genetically, you do get specific lineages of H9N2 viruses. Jill Banks has published a paper [Banks, J., E. C. Speidel, P. A. Harris, and D. J. Alexander. Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. *Avian Pathol.* 29:353–360. 2000.] looking at the HA1 gene. I think there were three genetic lineages. If you compare it with something like H7 viruses, the H7 genetic lineage just seems to be related to the geography of where the viruses were isolated. But this is much less marked with H9 viruses. We have always put that down to the primary host for H9 possibly being different to that of H7 and being the type of bird that moves more readily around the world than do

the flocks that may be the primary host of H7 viruses. So, yes, you do see genetic lineages. How they are divided does not appear too similar to how other viruses are divided. Does that answer the question?

Robert Webster. *Were the H6 vaccines in California efficacious and how long were they used?* **Dennis Senne.** Again, I might need to defer that question to Dr. Peter Woolcock, if he is present. We have not received a lot of feedback from the field in terms of the efficacy of the vaccine. I think the vaccine was an autogenous H6N2 vaccine. Typically, autogenous vaccines are usually restricted as to the farm/premises where the virus was isolated. Therefore, the vaccine probably has been used in a relatively low number of farms. Beyond that, unless Peter has something to add, I have no further comments. Okay, Peter said he would rather defer the discussion until tomorrow morning when he will present his talk on the subject. Thank you, Peter.

Antonio Zanella. *Are you sure that highly pathogenic avian influenza did not follow a moderate pathogenic avian influenza period in Pakistan? What is the situation of infectious bursal disease in your country and the relationship with avian influenza H9 infections?* **Kalid Naem.** If you are asking about the H7 highly pathogenic avian influenza outbreak of 1995, the low-path H7 outbreak was followed by HPAI, as we had isolated both the types. There is high incidence of IBD (infectious bursal disease) in Pakistan and that could be one of the factors which may be activating the pathogenicity of H9 avian influenza virus. But we have not tested it. And if you will allow me, I will suggest that we may have to redefine the definition of pathogenicity of AIV. Those influenza viruses that are capable to cause disease alone or in the presence of some other factors, they should be considered pathogenic under certain situations. Otherwise, you know, the question of vaccinating against H5, H9 vs. non-vaccination against others will continue. This will hinder the control of so-called low-pathogenicity H9N2 viruses. The work presented by our group here clearly indicates that induced immunosuppression results in enhanced virulence of H9N2. **Richard Slemons.** So the question for the records, experimentally have you looked at H9 influenza and birds with infectious bursal disease in combination and the answer for the tape—since I had to record these things last time, we are going to have this all on tape clearly—the answer was no.

Anonymous. *For Dr. Eckroade and Dr. Dunn, swine flu has killed people. Do you quarantine hogs for*

swine flu? Do you kill and landfill bogs for swine flu? If not, why do you for poultry? **Robert Eckroade.** I think for the audience we have to recognize there are two philosophies being used in this country dealing with avian influenza and infections. The philosophy in Minnesota, for years with turkeys, they control it very well using quarantine and market depopulation in an appropriate time. In Pennsylvania, probably because of the '83-'84 outbreak, where we had such dramatic losses, the industry is paranoid. The industry responds in a different way by attempting to immediately kill those first few flocks, hoping to control the outbreak. That works sometimes and sometimes it does not. We think it is a good start and if that does not work we can do some other things. **Patty Dunn.** I thought most of our swine were vaccinated for swine influenza. And I could say the same thing; I asked a horse person about equine influenza. I mean, I know there are not too many horse people that would let their horses go into the ground for equine influenza. **Robert Eckroade.** And we do not kill chickens with H1 either.

Greg Cutler. How do you handle quarantine of a large complex? What can you do about an uncooperative owner? We will take that part, what can you do about an uncooperative owner in your program? **Robert Eckroade.** Probably the answer is nothing. These are not regulated diseases, the state doesn't come in and say you have to do anything other than they can quarantine and restrict movement and require certain tests, but if that individual absolutely refused to be involved with a low-path H5, H7 I don't think anybody can do anything about it. The federal government is not going to do anything about it. The state is not going to do anything about it. What this really means is, is that if we really want to control avian influenza in this country, you have got to have the industry as part of the program. If they are not at the table, if they are not making a major portion of the decision of what to do about it, the program will probably fail. Unless some state or federal government is going to come in, require it, and pay for it. So, you have got to work with the industry to make this thing work.

Antonio Zanella. From 1998, have you found any seroconversion or virus isolation in the area? **Robert Eckroade.** Yes, Dr. Dunn just described the outbreak we had beginning in December 2001 that was just concluded. Four weeks after the first case, the last case was identified, and then several months later, we declared it over. So yes. The answer is yes. It is a separate introduction. Nobody thinks it is the same one.

Anonymous. If in the United States, we had H9 subtype avian influenza virus that affected humans, would the birds that tested positive for H9 be allowed to be vaccinated or sacrificed? It is open to the panel.

Khalid Naem. I think I would rather suggest vaccin[ating] human beings against H9, rather than sacrificing birds and going after all types of the susceptible birds. I would also go for vaccinating poultry against H9.

Anonymous. With the description of the disease you had, Dr. Eckroade, of the reproductive tract, and I realize these were in layers and not breeder birds, but this disease in breeder flocks, would we have to reevaluate vertical transmission of type A influenza viruses? **Robert Eckroade.** I think you would. Because with this disease there was a lot of virus present in the reproductive tract. I am not sure it's absent in other AI infections of birds, but there was a lot of virus in that tract of these birds. One could assume that there would be some transmission into that egg. Now how long it survives, whether it would ever survive incubation or what have you, of course, table eggs are not incubated, one would have to look at that. Now Patty Dunn, I remember that Dave Kradle did some work on those eggs, would you like to comment on that? **Patty Dunn.** I do not remember how many eggs, maybe Dr. Lu does, but we tested a lot of eggs from some of the these field infected flocks in '97 and '98 that were allowed to stand. We tested the shells and we tested the contents for virus by standard virus isolation. And we could never find any. And that's not to say that it's not there, but we sampled, I don't know, 6-8, 10 thousand eggs, maybe. And in this latest outbreak, we took 60 eggs from these broiler breeder flocks that were right in the heat of the clinical infection, and we tested again shells and egg contents and we couldn't find anything. Although, we also did egg yolk AGID to look for AI antibody, and we didn't find any in the eggs collected initially, but in eggs collected 8 days later we detected antibody in about 3 out of 60 of the eggs or something like that. But we could never find any virus. But maybe we just did not sample enough. We did not see that. We looked hard for that oviduct lesion in the breeders, and we did not see it.

Anonymous. The directigen flu A test has gone up from \$10 and test to \$20 a test now. Most people trying to buy that test right now say it's gone up drastically. So it's not quite as convenient for diagnostics. **Robert Eckroade.** Talk to your buyers. We went through the same thing where they canceled our contract, and we are still buying it for the same price through

the University of Pennsylvania hospital system, and they are still allowing us to get it. They took it away but they put it back. **Richard Slemons.** At the Ohio State University, we cannot. I have tried. **Robert Eckroade.** I heard from Virginia that there are very low supplies right now because human flu season is over and they only manufacture during human influenza season, so I think it is an availability problem sometimes as well. **Patty Dunn.** But the dot ELISA that Dr. Lu developed that was used in Pennsylvania is about \$.30 a test. We had the monoclonal antibody made on campus at our hybridoma laboratory. It is very similar in sensitivity and specificity to the Directigen, has some of the same problems, obviously, but I think there are some in-house tests that can be used very similarly because it is an ELISA test. We know how to make ELISA tests.

***Anonymous.** Is that published? That test?* **Patty Dunn.** It (information about the monoclonal-based DOT ELISA test for H7 AI virus) is in the manuscript. Has it been submitted yet? (asking Dr. LU in the audience) Yes, he (Dr. LU) wanted to wait. We obviously didn't have any field things to test them on and ... we didn't have any field samples to validate the test on until very recently. Unfortunately, we did not want the field outbreak to do that. But we used it experimentally on the '97-'98 outbreak alongside the Directigen using virus isolation as the comparison test. Hopefully, it will be submitted for publication.

***Dave Glauer.** How do you guard the security or do you even worry about guarding the security of your GIS data from freedom of information act?* **Robert Eckroade.** This is not a small problem; it is a very large problem in this country. Sherrill Davidson went to the university attorneys, went to the Pennsylvania Department of Agriculture attorneys, and determined that this was because of the manner in which it was collected, that is, under a guarantee of privacy, that it became university property not subject to freedom of information as it might be if it were in the offices of a government. And so she's gone that route, and the industry trusts that decision and has given us all this information. I can tell you that being in a meeting in December with several state veterinarians that would have liked to have had the same information when I presented a report on our GIS, said that their industry absolutely refused to give it to them. They were not going to just volunteer to give the information. If we ever have an outbreak of a foreign animal disease in this country, unlike the U.K., which was ready for it, we are in

trouble. But it is possible to work out these arrangements, I think, if the industry will agree under certain conditions that it could be used. The swine industry in Pennsylvania is now trying to work through the same thing with the privacy issue. But we feel comfortable because it belongs as proprietary information to the University of Pennsylvania. The poultry industry will fight to keep it from ever being given out. Well, we did do the work by collecting this data and putting it into a database where it otherwise wouldn't exist. So the university in fact treats it as intellectual property that is not subject to freedom of information. That is, I mean, we all know lawyers, and there are good ones when we need them and bad ones when we do not.

AVIAN INFLUENZA OUTBREAKS IN ITALY AND HONG KONG

***Wayne Collins.** Yesterday, the price of the Directigen test was discussed. What's the price in Hong Kong?* **Les Sims.** I do not know the exact price. I think \$20 USD is a bit higher than what we were paying, but I'll check that for you. (Postconference note: The price in Hong Kong is about \$13 per test).

***Anonymous.** In hindsight, is there anything about the low-pathogenicity avian influenza virus that could be seen as the predictor of mutation to high-pathogenicity avian influenza?* **Ilaria Capua.** Not really. But having seen what had happened in the past, we were very concerned about this. In fact, when we realized that the low-pathogenic virus was out of control, obviously this would have increased the possibilities of having a virus mutation, and we were expecting it. Although, I remember meeting with the farmers, and when I used to tell them, "Now look, this low-pathogenic virus that is causing so many serious problems in your turkeys is nothing compared to what could happen if the virus mutates," they said it's not possible not to have anything worse than this. Famous last words.

***Bublot Michel.** Do we know if H7N3 vaccine protects as well as H7N1 vaccines when challenged with H7N1 high-pathogenic virus?* **Ilaria Capua.** You mean, is there the same cross protection between H7N3 with the Italian highly pathogenic H7N1 and with the homologous H7N1? Is that the question? Well, my colleague will present the data of the cross-protection experiments that we did, because obviously, before we started vaccinating with the H7N3, we made sure that this would protect. And we did quite a lot of work. Anyway, we

investigated the clinical protection and that was of 93% with the H7N3 vaccine against the Italian H7N1. And I might just like to add a couple of things: firstly, from the vaccinated birds we did not find any virus in the muscle, which is very important from a commercial aspect, and that therefore viremia was obviously not established in the vaccinated birds. So, I think that 93% protection is good. It is good enough, considering the positive that comes from using a heterologous vaccine.

Richard Slemons. *Have any other HA and NA subtypes been found in chickens, quails, and pigeons in Hong Kong besides H5N1 and H9N2?* **Les Sims.** There have been some H6N1 viruses from quail and serological evidence of H6 exposure. But recently we've had a lot less H6 activity than we had about 12 months ago.

Eduardo Rivera. *Who pays for the depopulated commercial and retailed birds?* **Les Sims.** We have a law called the Public Health (Animal and Birds) Ordinance. Under this law there is a compensation provision that provides for compensation to be paid for any bird which is killed as a result of a disease control program. There is a ceiling on the amount that can be paid per bird. At the moment, that ceiling is about the current market value of live chickens.

Sue Trock. *Before the markets are reopened after the monthly depopulation with cleaning and disinfection, are they inspected by anyone to verify that they have been adequately cleaned and disinfected?* **Les Sims.** Could I go back to the previous question? For the monthly depopulation/rest day in retail markets there is no compensation paid to the retail traders. This is something the industry itself pays for. For any depopulation which is done because of a disease outbreak, the government will pay compensation for those birds. On market inspections, the Food and Environmental Hygiene Department has a team of health inspectors who inspect all these market stalls before they reopen to make sure they have been cleaned properly.

Anonymous. *But what if they don't properly depopulate, or they are not cleansed and disinfected adequately? Is there any penalty?* **Les Sims.** There is a penalty. They will find themselves without a market stall. They will lose their license.

Rob Webster. *Did the Muscovy ducks show disease signs?* **Franco Mutinelli.** In 1999–2000, 25 backyard flocks composed of mixed avian species, ducks and geese included, were affected by HPAI. In one outbreak mortality was also observed in two domestic geese and two Muscovy ducks. One

Muscovy duck exhibited incoordination and tremors.

Anonymous. *The outbreak subsided during the summer months, July and August, and again came back in October to December. Was this due to enhanced biosecurity because of the hot season (i.e., susceptibility of the virus to increased environmental temperature)?* **Ilaria Capua.** I suspect it was due to both. Obviously, the virus is more susceptible to the high temperatures that are present in Italy in the summer, and the farmers were obviously more aware of the situation. And mind you, putting up biosecurity measures is not something you do overnight, especially if you don't have any money and you have to implement some behavior (changes) and some physical equipment like arches and pumps for washing trucks and so on. So, it did take quite a while to have them going, but then I think both the oncoming warm and the implementation of biosecurity contributed to a reduction in outbreaks.

David Suarez. *What have been the control measures taken in Mainland China as to control H5N1? Do they publicly recognize the problem?* **Les Sims.** I am not in a position to comment on the situation in the Mainland, which is not under my jurisdiction. This question would best be directed to Mainland authorities, who have worked very closely with us to deliver birds that meet the testing requirements we set.

Robert Webster. *Will vaccine now be used on a regular basis for H5N1 control in poultry in Hong Kong?* **Les Sims.** I'll talk some more about this on Wednesday in the late breaker session, but we have made a decision to introduce vaccination for one year as a control measure in one small zone of approximately 20 farms, where we have continued to find virus. The last infected farm in this zone was detected around 20th March. We just depopulated that farm and another farm that was also virus positive in mid-March. We have introduced vaccination into the farms around these two infected farms.

Bublot Michel. *What is anti-N1 response (kinetics of appearance, percent of positivity) in birds vaccinated with H7N3 vaccine and challenge with H7N1?* **Ilaria Capua.** Well, someone will address this issue on tomorrow, I think, or on Wednesday. But in fact, the antibodies to the neuraminidase are more precocious than the antibodies to the hemagglutinin. They appear a couple of days earlier.

Bublot Michel. *Were there sentinel birds in the vaccinated flocks?* **Ilaria Capua.** Yes. We started out

with sentinels; Stefano Marangon will address this issue. At the beginning we obviously didn't have the discriminatory test when they decided to vaccinate. So, the control of the field situation was done through sentinel birds. Unvaccinated sentinels. It is very, very difficult to keep a sentinel as such and identify a sentinel; therefore, we had sentinels mingling with vaccinated birds. That is why we found the discriminatory test so useful. Because, when we found seropositive birds among the sentinels, they were tested with the discriminatory test, and, therefore, we were able to say these are vaccinated birds not infected sentinels. So, yes.

Andrea Miles. *I believe you mentioned that the use of sentinel birds was more sensitive than environmental samples. Please describe how you use sentinel birds.* **Les Sims.** I'm not really talking here about sentinel birds. I'm talking about "routine" dead birds within a flock of essentially healthy birds. There is a distinction there. We don't have sentinel birds within our normal flocks. Basically the whole flock acts as a sentinel. When we move to vaccination, we will be using unvaccinated sentinel birds. But for our normal flocks, when I talk dead bird monitoring, I am just talking normal dead birds you find on a daily basis in a poultry flock. And, we use these as a focused or selected sample that is more likely to give us a positive test result.

David Halvorson. *Can you expand on your statement that there was very, very limited spread from a vaccinated flock?* **Ilaria Capua.** Well, Stefano Marangon will expand on that statement tomorrow. He'll show you how the infection spread. But correct me if I'm wrong, Stefano, but I think there was only one vaccinated farm that got infected. And there was no spread from that farm to neighboring farms, vaccinated or unvaccinated. Am I right? Yes.

Ian Brown. *Have studies been done in humans with occupational exposure to H7N1? Any evidence for zoonotic transmission?* **Ilaria Capua.** Yes, studies have been performed. Dr. Donatelli, she works in Rome. I can't remember exactly the figures, but I would say that approximately 700 sera collected from exposed people like farmers, veterinarians, technicians, were tested with two different serological tests by the serum neutralization and the single radial hemolysis test and they all resulted negative. And also, some tests were performed for virus isolation on pharyngeal swabs and they were also all negative. So, we have absolutely no evidence of transmission to humans. In fact, I inoculated myself

with low-pathogenic virus attempting to inoculate a chicken, and I'm still here.

Carol Cardona. *Did you see a difference in susceptibility between broiler chicks and layers? Alternatively, how do you explain the high number of outbreaks in layers and the low number in broilers?*

Ilaria Capua. We had a lot of outbreaks in layers because the layer industry is a much dirtier industry than the broiler industry. Obviously broiler breeders have much better biosecurity levels than layers, because we have a lot of layer farms in Italy, and probably worldwide, do not have the all-in-all-out system. So they are constantly restocking. It is also very difficult to keep a layer farm clean. There is a lot of movement, the egg trays, and the eggs—they all go to the egg-collecting plants and so on. In our opinion, when the infection gets into a circuit, it will spread within that circuit. It finds its way to spread. With reference to the first question, the different susceptibility of layers than broiler?, what I can say is that, in fact, I agree with what Les said. In the layers, the spread of infection inside the farm was much, much slower than in the broiler or in the broiler breeders. Probably because of the amount of feces that got into contact with each bird is lower than if they're reared on the ground. I wouldn't say there are some clinical signs that can be different. You can all see it in the book. But I wouldn't say that there is a different species susceptibility.

Anonymous. *Did you say infections of layers, which weren't kept in cages, or were all those kept in cages?*

Ilaria Capua. No. We also saw some floor-raised layers. In fact, in that case, it spread more rapidly.

Anonymous. *Do you collect tracheal swabs from birds in live markets while monitoring for avian influenza virus?*

Les Sims. Our live bird market samples are either dead birds from which we will normally collect cloacal samples or fecal swabs collected from trays under cages. If the whole dead bird is submitted to the laboratory, we will take cloacal, tracheal, and/or lung samples for culture. The majority of our samples from the live bird markets have been collected from under the cages, so they are fecal samples.

Anonymous. *To reach eradication of avian influenza virus through vaccination, do you consider that a program of 1.5 years was enough? And what's your suggestion to, in using the long-term vaccination program?*

Ilaria Capua. In our experience, the 1.5-year vaccination program worked. But, I think a lot of it depends on the situation you have when you begin vaccinating. Because when we began vaccinating, there was not any virus around. Well, it

was around, but we did not have any outbreaks. I mean, it was probably somewhere because then it reemerged. So, I think it is very, very important, in fact, I would not want this Italian experience misinterpreted, i.e., “we have the key to solving all the problems and vaccination is the key is what you need.” I think that we were successful because we have a very good territorial system in place with just all the flocks are georeferenced. Does that make sense? I mean, you know exactly where they are located, and you know exactly the relationships between all the flocks. We had a very, very intense monitoring system. I mean, we performed about 160,000 HIs just for the first year of vaccination. We are Italy; we are not the United States, so we did do a lot of work and a lot of monitoring. And, we knew exactly where the virus was, and that’s how you control it. We were very, very prompt at identifying the reemergence of the virus. With reference to a longer vaccination program, I still think you need to have a system in place. It is not the vaccination by itself that will solve the problem. You need to have a system in place, and that’s why in my last slide I said you need to have a flexible and efficient data-processing system, because the information that comes from the field must be analyzed and must be immediately available for decision makers. Because if you stamp out a flock 10 days after or 15 days after it was infected, you are too late. Although with the vaccine, the amount of virus that is shed in the environment is less. We have seen this, and we have also seen this experimentally. So, I think from one infected farm, if it’s not vaccinated, let’s say, hypothetically, you could infect 10 other farms. If it is vaccinated, maybe, hypothetically, you could infect four or five. But you need to know where it is or else vaccination can be dangerous, in my opinion.

David Halvorson. *Given what we know about the roles of hemagglutinin and neuraminidase in reducing virus entry and exit from cells, wouldn’t autogenous vaccine be expected to reduce virus shedding more efficiently than a DIVA vaccine?* **Ilaria Capua.** It probably would. But I think that you have to make a cost-benefit evaluation of what you are doing. Is it more important to reduce the virus shedding of, say, one log, or is more important to know exactly where your infection is? In our experience, it was very useful to know exactly where the infection was, because then you can do something about it. I mean, homologous vaccine is not going to reduce your shedding level to zero—it is not. It’s probably going to reduce it, I don’t know how much, but it’s

probably going to reduce it a bit more than a heterologous vaccine. I think it’s more important to know where you are with field infection and what you’re doing.

Ron Fouchier. *Are differences between low-pathogenic avian influenza and high-pathogenic avian influenza restricted only to the HA gene and basic cleavage site only?* **Ilaria Capua.** I have to say that we haven’t done a lot of the work that we would’ve liked to do. We have a collection of about over 600 isolates, but I think we are around 700 or 750 isolates, from so many different species. And obviously, we have just recovered from the epidemic and we haven’t had time to look at the rest. Some work has been done by Jill Banks and mainly with the HA gene. But these viruses are available for any virologist who is willing to enjoy himself with them.

David Henzler. *What numbers of samples (i.e., cloacal swabs, feces samples, and blood samples) are taken from each of the local farm sources, especially in Hong Kong and Mainland China?* **Les Sims.** Birds coming into Hong Kong, are tested before they leave the farms in the Mainland, and the samples are tested in the Mainland. I understand they are collecting 30 blood samples per batch of poultry within 3 days of export. The birds are then retested at the border; another 14 blood samples from the consignment of birds are collected as randomly as possible. If any dead birds are found, then they also tested. For our waterfowl, we routinely take blood and cloacal samples. So again, we collect 14 samples from every consignment of birds. We have a similar arrangement for our local farms in that we go to those farms within 5 days of marketing to take blood samples and check for any unusual mortality. If we find dead birds, then we test those. We are not doing routine cloacal swabs or tracheal swabs on chickens. If we find dead birds then we will test these for virus.

Jeanet van der Goot. *Can you explain why mainly turkey farms and layer farms were affected during the highly pathogenic avian influenza outbreak?* **Ilaria Capua.** Layer farms, as I said, because they are dirtier farms. Turkeys, we showed, are more susceptible from a pathological point of view to the mildly pathogenic avian influenza virus. But we suspect, and what we’ve seen from the field, is that turkeys are more susceptible to the virus, and I mean they need less virus to get infected. We have some evidence, particularly with the low-pathogenic virus, that when there were chicken farms and turkey farms next to each, the turkey farms got infected and

the chicken farms did not. So, we think turkeys need less virus to get infected, and they probably also shed more than chickens.

Robert Webster. How is the H7 vaccine standardized? **Ilaria Capua.** This was a commercial vaccine made by Merial for Pakistan. So, in fact, it was the only vaccine available, and it is a licensed product.

P. J. G. Kuhne. And what was the vaccine schedule in turkeys and chickens? What was the background of the schedule? **Ilaria Capua.** Stefano Marangon, I think, will go into more detail of this. In fact, we only vaccinated the turkeys, the meat turkeys, and some layer farms that did the all-in-all-out system. The broilers or the broiler breeders weren't vaccinated. And the turkeys were vaccinated three to four times; the males were vaccinated four times. And the layers three times, I think, three or four. I would need to go back through the records. But we evaluated the antibody response in the lab before we decided to use the vaccine. Obviously, there was no experience, particularly in turkeys that are not very good reactors, and we set up a vaccination schedule and program in the lab, evaluated it, and then used it in the field.

Dennis Alexander. How many doses of vaccine were administered? **Ilaria Capua.** For 15 million birds, we administered approximately 45 million doses of vaccine.

Dennis Alexander. So each bird had three doses? **Ilaria Capua.** Yes, more or less.

Anonymous. How would you explain the absence of disease in the mountains of this same region? **Ilaria Capua.** I don't know. There [are] a lot of things we don't know. We have not had time to look at them. Perhaps, Stefano, who is our epidemiologist, will be able to give us some light on this. I know they are working on this. But I don't know.

Anonymous. Is the chicken-human transfer truly unique or has current diagnostic technology demonstrated an event that has occurred previously? For example, what work has been carried out without previous human pandemic strains in interspecies transfer? **Les Sims.** I think I would rather leave that one to the public health experts who will present papers in a couple of days' time; Nancy Cox's team would be in a better position to answer that. All I can say, on interspecies transfer, is that prior to 1997, there had not been any clinical disease in man apart from the odd case of conjunctivitis in people exposed to other avian influenza viruses. The work done in the Pennsylva-

nia outbreak by Rob Webster's group demonstrated that people carried virus for short periods of time but did not become systemically infected. However, this is a difficult area to work in, because the diagnostic tests are very difficult to do. There have been a lot of problems doing the serology in man. You cannot just run an HI test on human serum and hope to get meaningful results.

Can I just make one other point on the previous question? We do not do agar gel diffusion tests because we have both H9 and H5 influenza viruses. Therefore, we can't use this test to pick birds that are H5-positive birds, so we have to use hemagglutination inhibition as our primary serologic test.

Veronique Jestin. Since H9N2 infection has been recorded in Hong Kong as giving cross protection to infections with H5N1, could the H6 vaccination practiced in Italy mask signs of H7 infection? **Ilaria Capua.** I don't know. Though I can say the H6 vaccination in Italy is not taking place any longer in most of the turkeys, because one of the problems we had was the reactivity of the turkeys, that they are, as I said, not very good reactors. And so we asked the companies to stop vaccinating against H6 and H9 in order to give a bit less antigenic stimulation to these birds. So in fact, presently only very, very little number of turkeys are being vaccinated for H6 and H9. But we asked them to get rid of it. I don't know, I really wouldn't know.

Dennis Alexander. It was a H6N1 vaccine that they used? **Ilaria Capua.** It was a H6N2 vaccine and the H9N2. That was the first thing we asked them when we decided we would use a heterologous vaccination strategy.

Dan Karunakaran. How did you depopulate the farms? By what route was the vaccine administered? **Ilaria Capua.** The farms were depopulated mainly with CO₂. In some cases, however, with HPAI, most birds in the house were already dead by the time the culling was organized. The vaccine was administered subcutaneously.

Veronique Jestin. Is the intravenous pathogenicity index (IVPI) test performed in chickens a good test? **Franco Mutinelli.** Yes.

Veronique Jestin. Has the IVPI test been performed in turkeys? **Franco Mutinelli.** Yes; The result of the IVPI in turkeys was 1.0; still a low-pathogenicity AI virus.

Jeanet van der Goot. If performed in turkeys, were the birds SPF? **Franco Mutinelli.** No, they were conventional.

AVIAN INFLUENZA ECOLOGY AND EPIDEMIOLOGY

Dennis Alexander. If quail are necessarily intermediary for viruses to adapt to chickens, does this mean that such an adaptation would not occur in countries where there are no quail, and what does the role of turkeys in the adaptation of viruses to chickens?

Rob Webster. Yes, I think it is an oversimplification to say that it's only quail. I think that there are probably many other species involved. We just lack the knowledge. Turkeys certainly. Ratites certainly. It's very likely that pheasants and quail and many other species are likely to be involved in this adaptation of viruses to chickens. It is oversimplification to say just quail.

Dennis Alexander. Since influenza A viruses are known to survive exceptionally well in feces, why are viral transport media considered necessary?

Britta Hanson. We try to avoid taking feces samples just off the ground so that when we take them directly from an animal, we know which species and what age it was. I do not know if that is answering this question. Then we use the transport media because it helps depending on what type you use. As you can see we had better success with the brain-heart infusion broth, as it seems to protect the virus long-term so that we can store it in our lab. It takes us months to get the test done, and we just could not leave the feces samples unprotected or out at room temperature. We need to freeze the samples.

Dorothy Geale. All your cases are related to clinical disease reporting (passive surveillance). Is there any active surveillance in California? And are there any plans?

Peter Woolcock. Active surveillance is done predominantly by the individual private companies. Surveillance is also performed on a proportion of serum samples submitted to CAHFS.

Dorothy Geale. Are there any plans to routinely go in and check flocks for avian influenza?

Peter Woolcock. Again, this is done by the private companies, as well as by routine submissions to CAHFS.

Dorothy Geale. Can you speculate on the lack of avian influenza isolations in shore birds in your study compared with previous North American studies?

Ron Fouchier. I can always speculate. One option could be that our timing was wrong. From what I have heard about Dr. Webster's studies is that they sample American shore birds during spring migration season. We sampled European shore birds in the summer and early fall. However, we have also done some serological screens and found no hard

evidence for influenza infection in European shore birds.

Ian Brown. What was the level of genetic diversity in the HA genes of the H1N1 virus isolates in your studies? And I'll add on to that, did you see evidence of swine lineage or of avian lineage and so on?

Laura Campitelli. We have not been able to analyze the H1 strains at genomic level, yet, although we plan to do it in the near future. We have analyzed only the H5N2 virus isolated in 1993 to see the correlation with the highly pathogenic strains that circulated in 1997.

Ian Brown. What level nucleotide identity defines unique genotypes in the Nanchang markets?

Rob Webster. Between 600 to 1000 nucleotides of each gene was sequenced, and they were analyzed phylogenetically. Separation into different groups is based on differences in phylogenetic relationships.

Dorothy Geale. In the Netherlands 1999–2000 study, they examined 9000 birds; previous North American studies examined approximately 3100 isolations. How do you feel sample size may have influenced the hemagglutinin and type percentages you reported?

Britta Hanson. Well, we tried to sample at least 300 birds every year. They only catch so many at this location. You know there's a lab time constraint we have. If we could collect 9000 samples that would be nice, but realistically, that is years of lab work for the type of testing that we do. So I think that we feel in our lab that we have a pretty good representative sample just based on roughly 300 birds a year from that location.

Anonymous. Do you think there was any bias because it was a relatively smaller number than was in the larger Dutch studies? Could there be any bias in your sampling?

Britta Hanson. No. Certainly I guess there could always be bias in any study. I do not know what we could do to increase that given the amount of time and money that we have.

Anonymous. You mentioned a progression in receptor binding preference in the quail H9 from the alpha 2,3 to the alpha 2,6. What selective pressure would be driving this evolution in quail?

Robert Webster. Probably on the distribution of alpha 2-3 and alpha 2-6 receptors in the quail, although this has not yet been determined. We are inclined to think that we have only alpha 2-3 in avian and alpha 2-6 in humans; this is probably an oversimplification.

Paul Selleck. Has the sensitivity and specificity of your PCR test relative to virus isolation in eggs been determined, and how do you know that PCR-negative swabs did not contain virus?

Ron Fouchier. We

have determined sensitivity and specificity, and this information can be found in our article in the *Journal of Clinical Microbiology*. The sensitivity is about 100-fold higher than virus isolation in eggs or isolation of human viruses in cell cultures. The portions of the matrix gene that we used to design the primers are highly conserved. We have shown that using these primers, we can detect virus representing all influenza virus subtypes, isolated from all geographical areas and from all different hosts identified to date.

Anonymous. Could you please explain, what are the control measures, if any, to control the outbreaks of avian influenza in California? **Peter Woolcock.** Basically, it is not regulated, so we are looking for voluntary cooperation between industry and the states' agencies. And that is basically what is in operation at the present.

Anonymous. You mentioned a reoccurrence of disease in one flock; [Were] the premises depopulated between occurrences or were these in the same birds? **Peter Woolcock.** They certainly were not in the same birds because they were 2 years apart. I do not think they were depopulated, because the previous birds would have been moved on.

Anonymous. Was there ever a period where all the birds were out of that particular farm or was there basically a continual introduction of new birds into that farm? **Peter Woolcock.** New birds were certainly introduced, but I do not know what the time interval was between introductions.

Anonymous. Was that farm ever completely depopulated? **Peter Woolcock.** I don't know.

Michiel van Boven. Are the flocks being depopulated, or are they voluntarily being depopulated or obviously the state has not forced them to depopulate. **Anonymous.** For the record, the state has no regulatory authority because it's a H6N2 virus, and they are relying upon cooperation with the industry to try to control this program—a statewide program to control it.

Anonymous. In your opinion, the ability of the H9/H5 strains to spread to humans is mainly due to their internal genes or to their binding preferences of the H9/H5 hemagglutinin? **Robert Webster.** This was answered years ago by Rudy Rott; the ability to transfer between species is polygenic.

Richard Slemons. Are type A influenza viruses maintained in wild birds other than waterfowl and shore birds? Are gulls a natural reservoir? **Robert Webster.** The distribution of influenza A viruses in different aquatic birds is still poorly resolved. Ducks and gulls are certainly involved, and reports from

this conference show that many other species of birds are potential reservoirs.

Anonymous. Swabs for the Directigen test require a transport media. What is your recommendation for that type of media and the dilution? **Britta Hanson.** I'm sorry, I really would not feel qualified to answer that question. I am not familiar with that system. I do not know if anyone else is familiar with that could answer.

Carlos Romero. Anyone on the panel, is there a good cell culture system to routinely isolate avian influenza viruses of similar comparable sensitivity as that of embryonated chicken eggs? **Peter Woolcock.** We have compared a couple of cell lines, and chicken embryo fibroblasts were not as sensitive as eggs. **Robert Webster.** Eggs first, MDCK second, and there is a pig lung cell line that one of the current postdoctoral fellows (Sang Seo) developed in my lab. This pig lung cell line is available from the America Type Culture Collection. **Peter Woolcock.** I would like to add that for primary virus isolation, embryonating chicken eggs are the most sensitive.

Patty Dunn. Bobwhite quail in the Americas are a different genus and species from Old World quail. So what species of quail are you referring to in Nanchang and China, and has there been any work on experimental transmission, specifically on Bobwhite quail? **Robert Webster.** The answer is no. To my knowledge, no specific transmission studies have been done on Bobwhite quail, but serological studies in Bobwhites in the United States show 100% positivity to influenza! The bottom line is that the breeding and release of Bobwhite quail is a potentially dangerous practice from the perspective of spreading influenza!

Anonymous. You did not sample any quail. Do they not exist in your region, and given what Dr. Webster has said regarding quail, would such a test be considered essential? **Ron Fouchier.** We have sampled a few quails, but as in the U.S. they may not really be wild quail. Quail and pheasants were bred and put out in the wild again for hunting purposes. There are not many wild quails left in The Netherlands, but we have sampled a few, which were all negative.

Giovanni Cattoli. What is known about the influenza receptor pattern in quail regarding receptor types and receptor distribution? **Robert Webster.** This is currently under study, and that will be known in the near future.

Anonymous. Getting more viruses from chickens—could it be because you are looking or testing more?

Could it be because there is more chicken production than ducks? **Robert Webster.** The available information is based on studies of Ken Shortridge, who did poultry market surveillance in Hong Kong through the 1970s and 1980s. In those studies, he rarely isolated influenza viruses from chickens, and the majority of isolates were from ducks. In the present study, slightly more influenza isolates were obtained from chickens than from ducks; thus, the available information indicates that there is an increasing prevalence of influenza viruses in chickens as compared with the 1970s and 1980s.

Anonymous. *How do the new H5, H6, H9 isolates replicate in mammalian species (mice/ferrets)? What is the role of the new receptor specificities? Can an isolate have dual specificity, and is that a bigger threat to humans?* **Robert Webster.** In mice, the H5N1 influenza viruses replicate in the lungs, and a subset of isolates spread to the brain. In contrast, the H5N1 influenza viruses in ferrets replicate predominantly in the lungs. The H6N1 and H9N2 influenza viruses in mice and ferrets replicate predominantly in the lungs, but in mice these viruses can be rapidly adapted to become pathogenic strains, with spread to the brain. Regarding receptor specificity, the H5N2 and H6N1 influenza viruses contain variants that combine with α 2-6 terminal sialic acid, which is typical of human influenza viruses. Thus, there is concern that these variants have a human-like receptor specificity and have increased potential to spread to humans.

Karen Burns. *Why do quail preferentially shed in trachea and not feces?* **Robert Webster.** The molecular basis for this tissue specificity is unresolved and is under study.

Robert O'Conner. *How can you say that chicken isolations were equal to duck isolations? You sampled more ducks, but can you extrapolate that chickens equal ducks? Your bar graphs show chickens are less than ducks, especially for H9.* **Robert Webster.** Yes, indeed, we sampled more ducks but took this into account when expressing our results. After correcting for the fact that we sampled twice as many ducks as chicken, we find that 1.3% of chickens and 1.2% of ducks were shedding influenza viruses; thus, in the present studies, chickens and ducks were about equally infected with influenza viruses.

Ron Fouchier. *I am surprised about experimental infection of A/New Caledonia in quails. Is there other data available on infection of quail with human flu A & B?* **Robert Webster.** We were also surprised about the ability of A/New Caledonia/20/99 (H1N1) to infect a proportion of the quail but

would stress that only two of the nine birds showed low levels of virus for 4 days. Studies are ongoing to determine if other influenza A or B viruses will replicate in quail.

Robert Webster. *What changes in chicken raising would explain the upturn in H6N2 prevalence? Are all houses screened for small birds?* **Peter Woolcock.** I do not have a definitive answer to either question.

Anonymous. *Did you take measurements to stop the H6N2 outbreaks?* **Peter Woolcock.** I am not sure I understand your question, but any measures taken would be accomplished by the individual companies. Remember, low-pathogenicity AI is not a regulated disease.

David Halvorson. *How is H6 being controlled in California?* **Peter Woolcock.** Again, this is under the control of the individual companies.

ADVANCES IN MOLECULAR BIOLOGY AND MOLECULAR EPIDEMIOLOGY

Yoshi Kawaoka. *Can you make GuineaFowl/02 virus with the HA cleavage site PEKPKKR/G highly virulent by passaging it in 14-day-old eggs using the Max Brugh method?* **David Suarez.** We have looked at that, and so far we have not made that virus become highly pathogenic, and we are also looking at it with another method using a day-old chick model. We have not been able to make it go highly pathogenic with either system right now, but we have been hindered in part because of the lack of animal space to continue to do these studies.

Anonymous. *You mentioned that H7N2 was introduced into the live bird markets in 1994. Where did it come from?* **Dennis Senne.** We really don't know where it came from. As Dr. Suarez commented earlier, it likely was introduced by bringing waterfowl into the live bird markets. A lot of the live bird markets in the United States have waterfowl, chickens, turkeys, quails, and pheasants, all mixed together. We heard this morning that Hong Kong has now stopped the practice of allowing waterfowl and quail into the markets there. We have not restricted this practice in the United States. We do not have the legal authority to do so and so it is difficult. Likely, it came in as a waterfowl isolate, became adapted to poultry, and has persisted in the live bird markets since 1994.

Daniel Perez. *Are avian influenza viruses isolated from New York markets routinely tested for replication in mammalian species, such as mice or pigs?* **Dennis Senne.** We have not done such studies in our

laboratory. We are primarily a diagnostic laboratory. We rely on our research counterparts to take it to the next level. Dr. Suarez, do you have any knowledge to whether such studies have been done on the live-bird isolates? No, it's not been done, sorry.

Daniel Perez. *Are there other viruses in Hong Kong poultry markets that carry a PB2 gene with a lysine at position 627?* **Malik Peiris.** As far as I know, none of the other H5 viruses we now have in the poultry retail markets have this mutation.

Robert Eckroade. *Does anyone know the identity of the individual who made the connection between the live bird market and outbreaks of avian influenza in commercial poultry?* **Dennis Senne.** I think that it might have been Dr. Dave Kradel that made that first observation. I know it was someone from the Pennsylvania diagnostic lab or a veterinarian who made that observation. Okay, Dave Kradel was the one.

David Halvorson. *Are there live bird markets in California that could be a reservoir of H6?* **Richard Webby.** I am not that sure; I believe there are some markets, but not many. Is that correct, Peter? (Yes.) As I said before, we would like to extend our study to look at more H6 viruses from that region of the U.S. If anyone does have any of those viruses, we would be very interested to have a look.

Anonymous. *Was the PB2 E to K change found consistently in the other human H5N1 strains originally identified as highly pathogenic in mice?* **Yoshi Kawaoka.** No. Not all of the H5N1 human isolates that are pathogenic in mice have that mutation. However, we recently did an experiment in which we changed the PB2 of the nonpathogenic 486 virus with that of another highly pathogenic H5N1 virus that does not have the E to K mutation. This PB2 gene made the nonpathogenic virus highly virulent. In other words, there are other mutations that convert a PB2 that does not support high virulence in mice to one that does support high virulence.

David Halvorson. *In the week of April 8th, were all the live bird markets depopulated or only the positive ones?* **Dennis Senne.** They were all depopulated; all 122 markets were empty and down for 3 days.

Susan Trock. *Are there not other large U.S. cities (besides) than those in the eastern U.S. that have live bird markets? Do they also circulate AI viruses?* **Dennis Senne.** There was a National survey done (in the late 1980s, I believe) to find out where live bird markets existed in the U.S. Many of the larger cities did have live bird markets, but they were not

being routinely monitored for presence of avian influenza virus.

Ian Brown. *Do you have any information on the antigenic diversity of H6N2 viruses given the genetic heterogeneity in the HA?* **Richard Webby.** We have made chicken antisera to the initial isolates and used this in HI assays against some of the more recent ones. They are antigenically similar. The HA gene does appear to be a single introduction and of the one lineage. The H6N2 heterogeneity is not occurring in the HA gene.

Kanta Subbarao. *What is the genotype avian human swine of the H1N2 turkey isolate from Missouri—Internal gene segments?* **David Suarez.** I have to go back and look at that, since I do not remember offhand. I think the PB2 and the PA were avian influenza, the N2 and PB1 were human influenza, and the rest were classical swine.

Richard Slemons. *What environmental or host factors could be driving reassortant among avian influenza viruses in Hong Kong?* **Malik Peiris.** I think one of the points is that with the great expansion, particularly large kill chicken, and poultry industry in that region over the last decade, or so I suppose. That possibly is one of the things that may be bringing a difference of the evolution of these viruses in that region in particular.

Gus Koch. *Did you look for the scale of replication of the swan virus isolate after air-sac inoculation of chickens? And, what is the selective pressure upon the virus population?* **Yoshi Kawaoka.** Initially it replicated only in the air sac. What is the selective pressure? We don't know.

Chang Won Lee. *Is there any difference in rescue system or strategy between human and avian influenza virus? If no, how about the efficiency?* **Yoshi Kawaoka.** There is no difference in terms of the rescue system. We made three human viruses, four or five avian viruses, and one swine virus. There is no difference in how you make viruses from plasmids. There are differences in terms of efficiency. And this depends on the replication efficiency of the original virus. Of course, virus that grows well is easy to rescue.

Jonathan McCullers. *You explained how a virus might make its way from a large farm to a live bird market, but how does a virus such as these H7N2s make its way from a small urban live bird market to large farms, as you suggest is happening?* **David Suarez.** The traffic is obviously going both ways. So the trucks are coming from the farms to deliver birds to the market, picking up dirty crates. I do not think it is inconceivable to think that the crates would not

be properly washed or the truck disinfected before it goes back out to the farm. I think that is the most likely scenario, although the direct epidemiological ties often are missing. We do not have the smoking gun to show that somebody did come from the markets to the index farms to start these outbreaks.

Robert Webster. *What is the extent of homology between the HA1 of 2000 versus 2001–02?* **Malik Peiris.** The HA gene of the H5 viruses of 1999, 2000, and 2001 has been well conserved. The HA cleavage site is also largely been conserved, although a few viruses have the loss of one basic amino acid at the HA cleavage site.

Stephen Lindstrom. *Because NS2 knockout express viral proteins without virus replication, how does vaccination with NS2 knockout compare with vaccination with DNA vaccines?* **Yoshi Kawaoka.** We never compared the efficiency of the DNA vaccine and the NS2 knockout, so it is difficult to answer this question.

Stephen Lindstrom. *How were NS2 knockouts quantitated and genome confirmed? Similarly, how were infection and gene expression by NS2 knockout viruses confirmed?* **Yoshi Kawaoka.** You take the virus from cells transfected with plasmids and infect new cells with these virus particles. After immunostaining, you see protein expression. By counting the number of cells that express viral protein, you can calculate the number of infectious particles.

Anonymous. *Have you tried any double gene combinations in H5N1 systems to look at combined effect on pathogenesis?* **Yoshi Kawaoka.** In one of the initial experiments, we made all of the single gene reassortants and tested their virulence, but we have not made any double gene combinations.

Anonymous. *Is the connection known between the live bird markets and the current outbreaks in Virginia and North Carolina?* **David Suarez.** Yes. Looking at the sequence analysis of the viruses from the index cases in North Carolina and Virginia, the viruses are related to the live bird market lineages with the three basic amino acids and the 24-nucleotide deletion. It has gotten all the characteristics of a live bird market isolate.

Guus Koch. *Do mice that are immunized with the replication-incompetent mutant develop an antibody response? And, what was the dose and route of immunization?* **Yoshi Kawaoka.** The amount of virus intranasally inoculated into mice was about 10^5 infectious particles. There was an antibody response determined by HI.

Anonymous. *Your comparison of CA isolate's sequence to waterfowl or live bird markets did not*

include flyways or markets in California. Could this influence your results? **Richard Webby.** This is certainly a valid comment. Different flyways of waterfowl do have different reservoirs of virus, as, one would suspect, would live bird markets. Logically one would assume that the most likely source of the Californian H6 viruses would be West Coast bird reservoirs. The paucity of data in our study from flyways and markets in this region simply reflects the fact we do not have such samples. The only conclusion we can draw from our study is that the donor reservoir remains unknown.

David Halvorson. *What unique control methods are being used in Hong Kong now?* **Malik Peiris.** 1) The rest day, with the aim of reducing virus "amplification in the retail markets," 2) Targeted vaccination as an adjunct to control of a HPAI virus, 3) Regular surveillance (serological) of imported poultry and local poultry farms (serological and virus isolation), 4) Regular surveillance (fecal swabs for culture) of apparently healthy poultry in retail and wholesale markets, 5) Virological surveillance of dead poultry in retail/wholesale markets.

Guus Koch. *Do mice that are immunized with the replication-incompetent mutant develop an antibody response? What was the dose and route of immunization?* **Yoshi Kawaoka.** Yes, each mouse was intranasally inoculated with 10^4 infectious particles.

Kanta Subbarao. *Does the E627K mutation in PB₂ account for the pathogenicity of all of the HK H5N1 viruses that were highly pathogenic for mice? Do you speculate that the mutation occurred in humans or chickens?* **Yoshi Kawaoka.** No. There are some mouse-virulent viruses without the E627K mutation. Considering the mutation rate of influenza viruses, it was present in chickens, but viruses with the mutation were selected in humans.

Daniel R. Perez. *Did all Group 1 H5N1 viruses contain lysine in PB₂ e position 627 (and vice versa for Group 2 viruses, glutamic acid)?* **Yoshi Kawaoka.** No. There are some mouse-virulent viruses without the E627K mutation.

Robert Webster. *What was the subtype of the swan virus?* **Yoshi Kawaoka.** H5N3.

Anonymous. *What ensures continued safety of vaccines attenuated viruses if coinfecting = natural virus and reassortant = new NS₂ or matrix gene?* **Yoshi Kawaoka.** Genes used to make replication-incompetent viruses do not enhance the virulence of typical viruses. Thus, the reassortants are not more virulent than the currently circulating viruses in the field.

Anonymous. Does the PB₂ 627 mutation hold true for all high- and low- path H5N1 viruses? If not, how can you explain the path differences in absence of the PB₂ 627 mutation? **Yoshi Kawaoka.** No. There are some mouse-virulent viruses without the E627K mutation. There are other PB2 mutations that achieve the same effect.

Susan Trock. In your work with mice, can repeated passage through mice increase virulence? Have you had any experience with length of carriage or number of virions/volume of feces? **Yoshi Kawaoka.** We did not passage the virus in mice. We did not isolate viruses from feces.

Anonymous. Ten of 16 H5N1 viruses isolated from Hong Kong in 1997 were high virulence to mice. However, only three of them have amino acid K in PB₂ 627. Do you have any idea about the molecular basis of high virulence of those viruses that do not have amino acid K in the PB₂ 627? **Yoshi Kawaoka.** There are other PB2 mutations that achieve the same effect as the E627K mutation.

Michel Bublot. How is replication-incompetent vaccine produced (since it does not replicate in cell culture)? The replication-incompetent virus contains only few point mutations in NS₂. What is its stability? Does it reverse to virulence or replication competent? **Yoshi Kawaoka.** By transfecting cells with plasmids. Since this virus does not replicate in cells, the likelihood of the emergence of revertants is limited.

Ron Fouchier. You seem to be sure that H7 in industry originates from LBM. What's the evidence that it could not be the other way around? Phylogeny alone is not sufficient evidence. **Dennis Senne.** The issue of reintroduction *vs.* maintenance of AIV in the LBM system has been debated for several years. There is precedence for the maintenance hypothesis dating back to 1986, when the LBMs were first recognized as a significant man-made reservoir of AIV (Senne *et al.*, Proceedings of the 3rd International Symposium on Avian Influenza, 1992). In 1986 it was determined that the source of low-pathogenic H5N2 virus that reappeared in commercial poultry in Pennsylvania following the successful eradication of the HPAI H5N2 virus in 1984 was most likely the LBMs. Following the outbreak of HPAI, surveillance for AIV was at a very high level in northeastern states, and there was no evidence to show that the H5N2 virus was present in poultry outside the LBMs. As for the current situation in the LBMs involving the H7N2 AIV, the phylogeny data does provide support for the hypothesis that H7N2 AIV is being maintained

within the LBM system and is evolving. If the virus was evolving independently from several sources outside the LBMs, I think we would see more genetic diversity in the isolates. Additionally, in 2001, a descriptive and surveillance study of suppliers to New York and New Jersey live bird markets was conducted (see poster abstract by Bulaga *et al.*, this Proceedings) in over 190 known suppliers from nine northeastern states. The survey was conducted by testing for presence of virus and antibodies to avian influenza. No virus or antibodies to the H7 AIV [were] detected. Given a 40%–60% prevalence rate within the LBM system, it is hard to believe that the H7N2 virus could be introduced into that many markets only from outside sources. However, it should be pointed out that H7N2 AIV has been isolated from chicken samples directly off supply trucks on a few occasions, so we do know that it is also coming from outside sources, but I think it is primarily being maintained within the market system.

David Halvorson. What exactly do you mean we have tried over and over to stamp out H7 in live bird markets since 1994? **Dennis Senne.** In the United States, control of low-pathogenic avian influenza virus is the responsibility of the individual states. Since 1994, between 1500 and 8000 samples per year have been collected from the LBMs by the states and tested for presence of AIV by virus isolation. When the H7N2 virus is detected in the LBMs, the states have been requiring the markets to sell off the birds and clean/disinfect the premises. Historically, this approach has been successful in eliminating H5 and H7 infections on several occasions, but it has not worked with the recent H7N2 AIV.

Bob O'Conner. AI-monitored flock for LBM—What specific test will you use as your method for declaring a flock negative: AGID, Directigen, V.I.? **Dennis Senne.** I do not know what tests will be used in the future; however, historically most flocks have been monitored serologically, but there are some flocks (mostly ducks) that have been monitored for presence of virus as well. I am not aware of any flocks being monitored by using the Directigen test (antigen capture immunoassay).

Dorothy Geale. What was the regulatory rationale for the synchronous closure of all live bird markets in northeast U.S.A.? Why not regional closures (*i.e.*, all New York one week, all New Jersey another week, etc.)? Is there documented cross contamination? **Dennis Senne.** We consider the live bird markets in the northeast U.S.A. to be a live bird market system and

we wanted to shut down the entire system at the same time. This would eliminate any transfer of birds from one market to another and stop all movement of vehicles, crates, and birds, thus giving us the best chance to clean up the positive markets. I am not sure what you mean by cross contamination, but there are occasions where birds are exchanged between markets to meet customer demands.

Robert Webster. *Are we waiting for mutations or reassortants to make H7N1 become lethal? Are the other genes of the viruses homogeneous? Are reassortants with a HP HA lethal?* **David Suarez.** Considerable effort, by both state and federal officials, is being expended to try and control the H7N2 virus in the live bird markets. However, the longer it is allowed to remain in the live bird markets or other commercial poultry, the risk of it mutating to the highly pathogenic form of the virus exists. The H7N2 viruses from the first 3 or 4 years of surveillance showed considerable sequence variation in the internal genes, suggesting that reassortment with different viral gene segments were occurring commonly. However, recent viruses all seem to have the same internal gene constellation with much less sequence variation, suggesting that the virus has found a gene constellation that is well adapted for chickens. We have not performed this (reassortants with a HP HA) type of experiment.

IMPACT ON PUBLIC HEALTH

Daniel Perez. *Vaccination is currently used to prevent disease of highly pathogenic avian influenza in avian species. Which measures does CDC have in place to monitor the spread of highly pathogenic avian influenza from vaccinated birds to humans?* **Jackie Katz.** I would have to say that there is nothing that CDC would have in place, and I would think that it would be something that the agencies that initiated the vaccination would be monitoring. And I guess if there was evidence of infection still in those vaccinated bird populations, then CDC would be interested in looking at individuals, humans that were occupationally exposed to those birds. But probably that is all we could do at that point.

Anonymous. *What method of phylogenetic analysis did you use?* **Ann Reid.** It is the PAUP analysis. Does that answer the question for whoever is out there?

Anonymous. *Can you please justify the use and sacrifice of non-human primates for this research when no new data is available from previous mouse and ferret studies?* **Ron Fouchier.** It is my feeling that

either humans or primates are going to give us a clear answer on the pathogenesis of this virus and not mice and ferrets. At this point in time, macaques represent the only animal model that will show a similar pathogenicity as humans. Of course, we tried to limit the number of monkeys; as you saw we only used four and no more than four.

Carolyn Bridge. *What additional research steps would be made based on the results that you've found so far? And how have these studies really added anything to what was already known about the pathogenesis of these viruses in humans and other animal models?*

Ron Fouchier. At present, we have no other plans to use these monkey models, beyond the detailed analysis of samples collected from the experiments shown. What it has added, I think, is the understanding that systemic replication is not required to explain the pathogenesis in humans, or at least in primates; the multiple organ dysfunction syndrome may not be due to systemic replication but the result of an acute respiratory distress syndrome. That is, in short, what is new that came from this study and has not been found in other animal models.

Anonymous. *How were the influenza network members selected, since you have only a few veterinarians involved?* **Klaus Stohr.** Actually, I think there are two questions. The first one is how were the participants in the AIN selected? The second one could be: Why haven't been more veterinarians involved?

The members of the AIN were selected based on their expertise and their activities in influenza surveillance and control at the human–animal interface of the disease. One of them is Dr. Rob Webster, Head of a WHO Collaborating Centre. He has been working on influenza at the human–animal interface for the last 25 years. In addition to Rob Webster, we requested participation of two more Directors of WHO Collaborating Centres; colleagues who have been intimately involved in human global influenza surveillance for decades and who play a leading role in making annual recommendations on the human influenza vaccine composition. They represent the human and public health side of influenza surveillance and control. At the same time, the latter two colleagues have close interaction with scientists involved in animal influenza research. Furthermore, we have invited the Director of the OIE and Food and Agriculture Organization Influenza Reference Centre, located in Weybridge, U.K., who have collaboration on animal influenza worldwide. In addition, we wanted to include scientists with current studies in animal and

bird influenza surveillance and ongoing research on the epidemiology of the disease in Asia and thus asked Dr. Hitoshi Kida from Japan, Dr. Kanzhen Yu, the Director of the Chinese Veterinarian Research Institute, who is also Director of the National Avian Influenza, and the Influenza Research Team from the Hong Kong University to join the AIN. All of the members in the AIN are renowned experts in the area of animal influenza surveillance and research at this human animal interface, and most of them are veterinarians.

Yoshi Kawaoka. *Did you try to isolate virus from a chicken that died upon IV inoculation with PR8 virus? If so, where did the virus grow, and what was the virus titer?* **David Swayne.** As luck and fate would have it, that chicken died on a weekend when I was out of town. So, we do not have any tissues for virus isolation, and we have repeated several of these experiments. Occasionally, you get a non-highly pathogenic influenza virus that kills a chicken, and with one of the H9 reassortants, we had a bird die. We repeated the experiment, and we did not get any more deaths. So one bird death with a low virulent or non-pathogenic or non-highly pathogenic virus is not unusual. It is just a random occurrence, which in our experience is mainly from infection in the kidneys following intravenous challenge.

Malik Peiris. *What criteria would you use to decide if and when a virus isolated in live poultry markets is a public health threat to humans?* **Jackie Katz.** I guess since 1997, we have to consider any avian virus could be a threat, but I believe it would not just be a single isolate. It would be a fairly widespread outbreak, and hopefully we would be able to get some serologic evidence in humans that there was some transmission, either serologic evidence or evidence as we had in the H5 and H9 cases of human infection. And once that evidence was obtained, we would consider it a substantial threat.

Kanta Subbarao. *How did you determine the dose to use to infect the macaques?* **Ron Fouchier.** We did not really. One cannot do this in monkeys. The only dose we tried was this dose. We had previously done experiments in chickens with the same dose. That is all the information we had.

David Halvorson. *Regarding the need for “enhanced exchange and communication,” how do trade embargoes in response to low-pathogenicity avian influenza affect open exchange of information? (Moderator comment: There is a need to exchange information, but because of trade embargoes, there may be a tendency not to share the information as widely. So how can we*

work around this?) **Klaus Stohr.** This is actually the crux. How can we encourage countries to report on the isolation of animal viruses of known animal pathogenicity but of unknown human pathogenicity? It is recognized that not all outbreaks of highly pathogenic avian influenza viruses in OIE member countries are actually notified. The reasons could be that notification would entail that the country would be subject to an export ban and other embargoes. However, this issue might change, hopefully in the nearer future, in the context of the emergence of *in vivo* low-pathogenic avian influenza strains, which cause economically relevant disease. The recent findings in Italy and now in the U.S., that low-pathogenic avian influenza strains can become an important economical fact for agriculture, might change reasons and interest in notifying isolation of any avian influenza virus irrespective of its pathogenicity at the time of isolation. I think that as long as these animal viruses are not an economical factor for agriculture, it is going to be very difficult to encourage the reporting of low-pathogenic or medium-pathogenic avian influenza strain emergence in birds. The proposal by one of the scientific communities of the European Commission to make low-pathogenicity avian influenza subtypes H5 and H7 notifiable in the future might be a possible solution to the obvious conflict.

Robert Webster. *What were the cytokine levels in the infected macaques? And for how long was virus shed?*

Ron Fouchier. The cytokine experiments are ongoing; we are studying the cytokine levels and cytokine mRNA levels in different tissues at the moment. I cannot give details about that yet. The virus shedding went on until the animals were euthanized. We did not perform a clear time-course analysis experiment. Since virus shedding was seen already at day 1, we can conclude that virus shedding occurred from day 1 to day 7, at least.

Anonymous. *Did you completely section the organs, specifically the brain, or only sample specific regions? And a follow-up question: did you look at other H5N1 isolates?*

Ron Fouchier. We did not look at other H5N1 isolates, since we only had one. The brain was sectioned quite detailed; different regions such as cerebrum and brain stem were sectioned and multiple sections were checked.

Anonymous. *And other organs as well?* **Ron Fouchier.** From all other organs, multiple sections were taken. For the organs where necrosis was observed, immunohistochemistry was performed in more detail.

Anonymous. *Does CDC have plans to study*

serological responses among live bird market workers in the U.S., and why hasn't this been done previously?

Jackie Katz. CDC would love to study poultry workers in live bird markets, and we have explored the possibility with various organizations and groups, and we keep coming up against various issues that include the types of ethnic populations and the emigration status of the individuals that are working in these live bird markets and just the willingness to work with the government agency. And that is the overriding problem we are facing. But if anybody has any ideas how we could work either with occupationally exposed poultry workers or individuals who work on farms, my group and I would love to talk to you.

Anonymous. *In your chicken trial, how old were the chickens that you used for virus inoculation, and then how frequently postinoculation did you collect samples?*

Yumi Matsuoka. The sample was collected day 3. The virus isolation you are talking about? Tissue samples were also collected on day 3.

Anonymous. *And the age of the chickens?* **Yumi Matsuoka.** Four weeks.

Dorothy Geale. *Did the 18 human cases of H5 (H5N1 from Hong Kong) show pathological lesions in any other tissue than the respiratory tract and lymph nodes?* **Ron Fouchier.** First of all, not all 18 cases were examined. Six persons died, but even not all six were studied in detail. There is very limited information available on those individuals. There was, if I am not mistaken, multiple organ dysfunction syndrome in these people. I would also like to make one additional remark; these people generally died much longer after infection as compared to the monkeys we studied. Because of ethical reasons, we had to terminate the experiment at day 7, and most humans died later than day 7 postinfection. Some of the differences, like in our case, lack of evidence for multiple organ dysfunction syndrome and lymphopenia, may be related to the date relative to the moment of infection.

Anonymous. *Does primate susceptibility to the H5N1 viruses give some indication that this species may be a reservoir or a source of H5N1?* **Ron Fouchier.** We have serum samples from many different animal species in our freezer. We have screened these for influenza in the past and found no evidence for H5N1 influenza, including in the samples collected from monkeys.

Veronique Jestin. *Are the molecular factors that enable avian H7N7 to cross the species barrier known?* **Jackie Katz.** I would say no. And I guess, since these viruses did not really cause a true respiratory

infection, I think they still might be considered not to have crossed the species barrier, although there [has] been transmission to humans, they have not caused influenza like illness. Unless anybody else knows the information, I do not believe it has been studied on a molecular basis.

Thomas Rowe. *Could you speculate why there are no clinical signs or no fever in the H3N2-infected monkeys?* **Ron Fouchier.** My speculation is going to be restricted to the fact that we find poor virus replication in monkeys infected with H3N2. We inoculated, in that case, with 5×10^6 TCID₅₀ of influenza virus H3N2 at the same sites, but we isolated only 10^4 virus back in the subsequent days after infection. So probably it has something to do with the virus titers produced in the lungs.

Anonymous. *If H9 and H5 avian influenza viruses can infect humans after exposure to chickens, why can't these viruses pass horizontally from human-to-human, since infection in humans assumes the proper receptors?*

Jackie Katz. I guess we really do not understand very much about the human-to-human aerosol transmission. And one thing, at least in animal models, these avian viruses do not appear to replicate to nearly the same extent as, say, a human H3N2 virus in the upper respiratory tract of a ferret, which is the experience I have. That would be one reason perhaps that these avian viruses are not transmitting, just because they are not producing as much virus into the bronchial airways that would be there to be expelled and transmitted. But it probably goes beyond that, and, as I say, the molecular basis is not really understood. It was, I think, assumed, or one possibility was because the receptor specificity was different, at least for the H5N1 viruses, that still bearing the avian-like receptor specificity was a limitation in human-to-human transmission. But we see the H9N2 viruses, at least many of them currently circulating, have acquired the human specificity. But in our limited studies, they still do not appear to be readily transmitting from human to human. So there is just a molecular basis there that we do not understand, and that we actively need to do a lot of research on.

PATHOBIOLOGY AND PATHOGENESIS

David Henzler. *Based on your report, or others, is there any evidence that meat from birds infected with high-path or low-path avian influenza could be infectious to persons consuming the meat?* **Terry Tumpey.** I think that is a difficult question to answer because we just do not know whether or not

exposure to poultry meat could contribute to the transmission of virus to humans. From Dr. Jackie Katz's talk yesterday, the evidence indicates that exposure to live poultry, and not poultry meat, is a potential risk factor for virus transmission to humans. **David Swayne.** Yes, to follow up on what Dr. Jackie Katz stated yesterday, the risk assessment on the epidemiology was that preparing or consuming poultry was not a risk factor. It was actual contact with live poultry that was a risk factor.

Huaguang Lu. *What species of the fowl or duck I guess of the 14-day embryos fowl eggs, chicken eggs is that correct?* **Jill Banks.** Yes, we were using chicken eggs.

Huaguang Lu. *Did you have any specific reason to use 14-day-old embryos?* **Jill Banks.** Well, we were using 14-day-old embryos because this model has been shown to increase the pathogenicity of some avian influenza viruses. And we were hoping to change the phenotype of an abnormal virus, which has a multibasic cleavage sign but zero IVPI, into one that has the predicted high pathogenicity for chickens. So that is why we went that route.

Huaguang Lu. *What has happened to the duck meat after the H5N1 virus was isolated?* **Terry Tumpey.** It is my understanding that any shipment container that comes up positive for H5N1 influenza virus is discarded during the quarantine period. The quarantine period can be as long as 30 days.

Huaguang Lu. *Did you know of any epidemiological investigation back to the meat processing plant or the original duck farms?* **Terry Tumpey.** No, I have not heard of anything.

Giovanni Catollo. *Are there any explanations about the fact you did not see additional glycosylation sites in the viruses passed in the 11-day-old embryos compared to the 14-day embryos?* **Jill Banks.** No, I do not think I have an explanation for that, but it is clear that there is something different in the cellular makeup of 14-day-old embryos *vs.* younger ones. And as you know, there has been a publication suggesting that the proteases are very different in 14-day-old embryos and that this selectively hampers the replication of avirulent viruses, leading to a replicative advantage for virulent mutants. I do not know whether this has an effect on the selection of viruses with additional glycosylation sites and changes the viruses in this case. But clearly there is something different in these embryos.

Stefano Marangon. *Is there any possible explanation of the observed higher RO value of the high-path AI virus?* **Jeanet van der Goot.** Well, we did not

look at the virus titers in this model, but this could be one of the reasons for the higher Ro value, because there is more virus excretion of the high-path AI virus. Of course, there are always two things: one is the susceptibility of the susceptible animal; the other is the amount of virus excreted by the infected animal. A higher virus titer could be one of the explanations.

Anonymous. *Have you looked at tissues from avian influenza-infected birds for apoptosis?* **Stacey Schultz-Cherry.** Yes, we have, and you do see apoptosis as well as necrotic cell death. It is not one or the other. And there is evidence of apoptotic cell death in areas where there is virus as well as areas where there is no virus, such as in the spleen. And we think that may be due to cytokines at the local site.

Robert Webster. *Have you done studies on the H5N1 virus in geese, and how long was the virus shed in mice?* **Terry Tumpey.** Well, unfortunately, we just looked at day 4 postinfection in mice, and we detected high titers of infectious virus in lung tissue. Virus shedding for other related H5N1 viruses, such as the Hong Kong/97 viruses, has been more closely looked at. For the H5N1 viruses that are not lethal in mice, the virus is cleared anywhere from 7–9 days after infection. We have not looked at geese at all with this particular isolate.

David Halvorson. *How do you know the wild birds in your studies had not been previously exposed to avian influenza?* **Laura Perkins.** We did do serologic testing on the birds prior to inoculation, and all the birds that were inoculated were negative. That was through AGP.

L. Shirley. *Were the rats used in your study white lab rats or Norwegian, (wild- or common-type rats)?* **Laura Perkins.** They were basic lab rats, Simonsen albino strain.

David Swayne. *I think you said they were SPF; is that correct?* **Laura Perkins.** Yes.

Ian Brown. *How do you account for the wide variation in transmission between experiments with the low-path AI?* **Jeanet van der Goot.** This is one of the things that strikes us, too. We found much variety in the experiments. One of the things I did not show in this presentation was that we only found the trachea swabs to be positive in the low-pathogenicity strain. This is not what we expected. This could be one of the reasons: there was only excretion by the trachea. On the other hand, in this kind of experiment there is always a chance process, so variable outcomes can happen. One should, when you find this variety of outcomes, do more experi-

ments. But it is something that is inherent to this model because of the small number of animals.

Richard Slemons. Will freeze-thawing three times decrease the infectivity of type A influenza viruses?

Terry Tumpey. Yes, we know from laboratory studies that freeze-thawing virus samples will reduce the infectivity of the virus. But in order to get the virus fully out of muscle tissue, the scientists in South Korea found that the best approach is the freeze-thaw samples multiple times.

Richard Slemons. Why were duck carcass samples freeze-thawed three times before virus isolation, I assume vs. when they take the meat and grind it up?

Terry Tumpey. From what I understand, our collaborators in South Korea have tried different approaches to isolate virus from animal carcass. However, they indicated that their best approach is to freeze-thaw the samples in order to obtain the greatest virus yield.

Anonymous. Did you use SPF ducks, and where did you get them?

Laura Perkins. Cornell University actually has SPF ducks for whoever is interested. The ducks used in Terry's experiments were not SPF, but they were obtained from Privett Hatchery. These ducks are high-quality birds from a commercial producer located in New Mexico.

David Swayne. The ones in Dr. Perkin's study were SPF ducks?

Laura Perkins. Yes.

David Halvorson. Did anyone look in chicken meat in Hong Kong for high-path AI virus?

Terry Tumpey. I am not aware of any studies that specifically looked for highly pathogenic avian influenza in chicken meat in Hong Kong. Under experimental conditions, we can detect infectious virus in chicken meat following infection with many of the Hong Kong/97 H5N1 influenza viruses, including the recent duck meat (Duck/Anyang/AVL-1/01) virus. So, high-pathogenic avian influenza goes systemic in the chicken and can be detected in multiple sites, including muscle tissues.

David Swayne. And, Laura, you want to add any addition comments? Okay, I will make a comment as the moderator's prerogative. Yes, we have done studies with several of the '97 Hong Kong H5N1 viruses, and you can identify such viruses in muscle. In fact, it is more frequent to find the virus in muscle of chickens, for example, than with 2001 Duck/Anyang H5N1 experiment that Dr. Tumpey reported. Furthermore, we found it more frequently in chicken samples than we did in the duck samples.

David Halvorson. What is the significance of the loss of amino acids in the stalk region of the neuraminidase?

Jill Banks. The significance is that glycosyl-

ation of receptor binding sites moderates the effectiveness of hemagglutinin binding to host cells. So reduced NA activity due to a stalk deletion can be balanced by additional glycosylation of the HA near the receptor binding site. This may be an important determinant for cell tropism and host range.

Anonymous. Is there any difference between different strains of influenza virus and their ability to produce apoptosis in vitro and in vivo?

Stacey Schultz-Cherry. Oh, good question. Not that we have found to date. There may be different kinetics, but we have not yet found a virus that does not induce apoptosis, and hopefully through the use of virus rescue systems, we can start to identify which viral proteins are involved, and we can start to modify those and find the virus that doesn't induce cell death.

Anonymous. Do you know of the proteins that are implicated in apoptosis?

Stacey Schultz-Cherry. Well, that was the first thing that we went about studying. We found that numerous proteins when expressed in cells (NS, NP, NA) could all induce apoptosis. So, we took the opposite approach to try and find the cellular pathway, and then we will identify which viral protein maybe the signals.

David Halvorson. In your experiments, what is the significance of the failure of low-path AI-infected birds to seroconvert?

Jeanet van der Goot. The only explanation so far that we have come up with is that it could be a superficial infection. **David Swayne.** I will make a comment. Part of this may be a sensitivity of the AGP test to detect seroconversion and that you may have low-level virus replication in some studies, but inadequate seroconversion. So it depends on the sensitivity of those two assays, the virus isolation detection and the AGP serology.

Charles Beard. What is the relative importance of glycosylation sites and basic amino acids and the HA cleavage site in determining the pathogenicity of H5 or H7 avian influenza viruses?

Jill Banks. The major contribution is the number of basic amino acids at the hemagglutinin cleavage site. If we do not have these, we do not have a highly pathogenic virus. But there are some viruses that have multiple basic amino acids at the cleavage site and do not necessarily show the high pathogenicity that you would expect them too. And that's one reason why we're looking at the impact of additional glycosylation sites.

Susan Trock. On your slide, you expressed concern for human health associated with recovery of virus from

duck meat. Would you expand on this? **Terry Tumpey.** Well, I think we should be aware that poultry meat can potentially harbor infectious virus in skeletal muscle. We have to be careful not to raise too many red flags in terms of public health implications right now. These are just initial studies taken from two to three experimentally infected ducks, so more work needs to be completed with this virus and other highly pathogenic avian influenza viruses.

Sue Trock. Currently Virginia is sending some infected flocks to processing. How do you address the public's concern regarding eating sick chicken/turkey?

Terry Tumpey. I would refer to my previous answer for this question. **David Swayne.** I may add a little comment to that. In the United States, for slaughter for human consumption, there has to be no clinical illness. So the bird would have to be showing no clinical signs to be slaughtered and used for human food processing.

Rick Bright. In your studies, this is comparing the mouse and the chicken studies and the infective doses, is there a difference in the infective dose given in the mouse studies vs. the chicken studies? **Terry Tumpey.** No, we tried to keep everything consistent, and we used the dose of 10^6 EID₅₀ given intranasally. The intravenous test for chickens is a 1:10 dilution of allantoic fluid, so the dose for that particular route is different. For the chicken pathotyping experiments we performed both intranasal and intravenous tests, but for all intranasal tests, a dose of 10^6 EID₅₀ was used.

David Swayne. Maybe I'll focus this a little more. Do you know what the mouse infective dose or the chicken infective dose would be for these viruses?

Terry Tumpey. No, at this point we have not conducted experiments to determine the mouse infective dose or chicken infective dose for these viruses. We just used our standard dose of 10^6 EID₅₀ for each of the species tested.

Susan Trock. Have you identified low-path AI from duck, turkey, or chicken meat? **David Swayne.** I would say that I have not seen low-pathogenicity avian influenza viruses in skeletal-muscle fibers of experimental studies. However, a study conducted in the early '90s with a visiting scientist from Egypt, Dr. Adel Shalaby, we found AI viral antigen in the epithelium of the abdominal air sacs in some of the challenge groups. Therefore, if you remove tissues like the kidneys, you will sometimes get positives on virus isolation because of the overlying/attached air sac membranes. We also have air sacs that go up into the keel bone and humerus on the wing, so there

would be obviously a potential for air sac contamination of the associated meat products if they contained bone.

RISK ASSESSMENT, REGULATIONS, AND TRADE ISSUES

Dorothy Geale. What is the definition of commercial poultry in the U.S.A.? **T. J. Myers.** There is not a statutory definition of it. So it is whatever you want it to be. I am sure that question is being raised because there is some overlap between the commercial poultry system and the live bird marketing system. As Lindsay mentioned, we do see spent laying hens in the live bird marketing system. So there is some overlap. But, for the purposes of this discussion, it is open to interpretation; whoever is looking at it.

Ken Rudd. The EU regulation shown yesterday banned the use of so-called GMO vaccines for the control of AI. What does the USDA view on the use of such products in the U.S.? **T. J. Myers.** We have licensed a (fowl) poxvirus-vectored avian influenza recombinant vaccine for H5. If the USDA felt a need to use that vaccine in the U.S., it is licensed, it is available, and we would use it.

Anonymous. Have studies been done showing that meat infected with LPAI will infect poultry by the oral route? **Howard Pharo.** Not that I know of. **Dennis Alexander.** Well, I know some studies were done some time ago. In fact, they did fail to establish infection by that route. And I think that was because the dose was too low, but I do wonder if birds would necessarily get infected directly by the oral route. If you see what I mean, because the virus could get into water or something else in the environment and infect them by the conjunctival route or even the respiratory route.

Carol Cardona. What percent of total suppliers were sampled? **Lindsey Garber.** That would probably be hard to say because our list of suppliers is incomplete. In the first phase of the study, where they were visiting markets, they discovered several suppliers that were previously unknown by going through some of those records. So I could not even venture to guess what percent of suppliers were actually participating in the study. **T. J. Myers.** Let me see if I can add to that. We included in the study every supplier that we knew of, so we looked for anyone we could identify.

Carol Cardona. In the live bird markets, how would AI-positive flocks be detected? And as a follow-up, time delays in diagnosis or seroconversion may alter the risk

of spread. How would this problem be addressed? **T. J. Myers.** Currently, each state has their own surveillance program. New York has been doing routine surveillance in the live bird markets on a quarterly basis. In the past, New Jersey has done it on an annual basis. Are they currently increasing that surveillance? I am not quite sure how frequently they are visiting the markets right now. As to the length of time between testing a market and getting results back, that has been a big concern of ours for quite a while. The surveillance that has been done in the past has been based on virus isolation. So when virus isolation results are received 2 weeks later, all the birds that were tested have long since left the markets. That was why, with the epidemiology study, it was important for us to use that opportunity to validate the PCR test that David Suarez's lab developed. We found in that study that the PCR test has good sensitivity and specificity for determining whether a particular market is positive or negative. It is not quite as useful on a bird-by-bird or sample-by-sample basis. But on a market-by-market basis, it is a very good test for determining a positive market. That is now available for use to get results within 24 hours to tell us whether or not a market is positive.

Anonymous. How does OIE consider the level playing field when the U.S. surveillance and diagnosis of AI far exceeds that of other OIE countries? **James Pearson.** The OIE *International Animal Health Code* and the SPS Agreement both state that, and import sanitary measures can only be put in place if a similar level of protection is applied internally by the importing country. Consequently, a country should not put restrictions on U.S. imports because of the surveillance program if they don't have a similar one. Unfortunately, such restrictions are put on, but if the importing country does not have a similar surveillance program, the restrictions can be challenged and hopefully removed. The *Code* also specifies that a country should have a monitoring and surveillance program if they want to be considered as free of disease.

Anonymous. Should the U.S. stop looking less vigorously? **James Pearson.** No, you should not stop looking, as HPAI can be a devastating disease and early detection is important also, as stated previously, monitoring and surveillance is necessary to justify the U.S.'s free status. As far as trade is considered, aggressive surveillance can have ramifications, but they can be dealt with, as discussed in the previous question.

Zheko Kounev. Under what circumstances would

USDA consider use of vaccine against H5, H7, or H9?

T. J. Myers. That is something that is going to need to be discussed on a case-by-case basis, I think. In the past we have received requests for the use of the vaccine. And in one case in Utah—in 1995 or 1996, not quite sure which year it was, 1997 maybe—we used an H7 vaccine. It was a highly concentrated poultry area with very rapid spread similar to the recent Italian situation, when they decided to use vaccine. If the U.S. has a situation like that again, we would consider use of vaccines in that case. Other requests for vaccine use have come in where we only saw sporadic cases over a longer period of time. We have turned down those requests, such as in Pennsylvania in 1997 to 1998. It really has to be decided on a case-by-case basis.

David Halvorson. Do you think changing the 92/40EEC directive is justified when it's based on an assumption that all H5s and H7s have HPAI potential? **Dennis Alexander.** I am not too sure exactly what angle that question is coming from. I think that there is. The assumption is that all of them can mutate to virulence—at least all H5s and H7s. There is no good evidence to suggest that there are some that cannot. So although it is an assumption, it is the assumption that needs to be disproved, not the other way around—that you could never prove that all of them can mutate to virulence. So if somebody was to come up tomorrow with cast-iron evidence that there were certain types of H5 or H7 viruses that it was impossible for them to mutate to virulence, then that would confound the assumption. But until then, I think the assumption would be overridden. It is a very good hypothesis, but I do not think that you could ever say that it was fact that they could all mutate to virulence.

Britta Hanson. If all H5 and H7 viruses were to fall under stricter control measures, do you think there will be any pressure to implement regulations or population reductions of wild birds that are known reservoirs of the H5 and H7 subtypes?

Dennis Alexander. That is one of the big considerations that you have to take into account, and obviously I do not think that would be an appropriate control measure at any time. Unless perhaps around individual farms or something like that. Where the OIE was coming from when it redefined Newcastle disease was the concept that although you made Newcastle disease in all birds notifiable disease, the way you actually handled them and the way you allowed trade with countries that had Newcastle disease in different categories of

bird would be quite different. So if it was in commercial poultry, we would do one thing, in wild birds another. But, in fact, they never actually got around to defining that compartmentalization. The logic would be that if this was the case for one disease, we could also apply similar compartmentalization to other diseases. The way you would handle the disease in different types of birds if you were going to have slaughter policies for all H5s and H7s could be quite different and would not necessarily result in slaughter regardless of the type of bird. **James Pearson.** Dennis was involved in this (compartmentalization concept). There was a proposal for Newcastle disease, as Dennis said, that wild birds and poultry were separate into distinct compartments. The commercial compartment would include live poultry markets, but it would not include wild birds. It was drafted and submitted to the general session of OIE twice with changes. I think that at least many of the countries believed it in principal, but they had trouble with the way that it had been drafted. Maybe it is not possible to draft it. It is not going to be submitted this May. There were some definitions that will be background for the next year will be submitted, so we are still a year away from compartmentalization. The Newcastle and the AI chapters I mentioned refer to each other. The AI chapter refers to the Newcastle. So when the Newcastle is adopted, the AI chapter will, unless somebody objects, be the same.

Anonymous. *What trade or what sanctions can OIE put on countries that do not report diseases?* **Jim Pearson.** I think we are doing about as much as we can. The Director General has announced that the OIE will pursue all suspected disease reports from any reputable source. If there is evidence of disease outbreaks, that haven't been reported, we will contact the Chief Veterinary Officer (CVO) and ask for an explanation. This usually results in an immediate response with a report or an explanation of why it hasn't been reported. We also have asked the OIE Reference Laboratories to report any isolations of highly pathogenic avian influenza that they make. If there has been no report to the OIE, the CVO will be contacted for an explanation.

Maria Pittman. *I don't know if you can answer this, but is USDA going to allow vaccination of turkeys in Virginia, and what will be the trade restrictions for live poultry and products if that happens?* **T. J. Myers.** You are correct; I cannot answer that. As I mentioned, there is an assessment team currently in Virginia, and obviously vaccination is one thing that

is being discussed. But I do not know what that decision will be.

Anonymous. *Do live birds from the Northeast move south to live bird markets in South Florida?* **T. J. Myers.** I think Dennis Senne already mentioned that the other day. We did see an H7N2 virus with the same genetic characteristics as that in the live bird markets in the Northeast in one of the live bird markets in Florida. What year was that, David? (**David Suarez.** 2001.) So there are some links there. Again, as Lindsay Garber has mentioned, we can only deal with the suppliers and the trade that we know about. There is a lot of movement of birds that we do not know about, so that does complicate the situation.

Eric Gonder. *Under option 3, why was vaccination not included?* **Dennis Alexander.** Actually, the EU SCAHAW did consider vaccination as a separate issue within the debated deliberations. If whoever asked that question wants to read what they have to say about vaccination, they can go to the website I put on the last slide, or I can give it to you if you did not get time to write it down [http://europa.eu.int/comm/food/fs/sc/scah/out45_en.pdf]. Vaccination was considered, as I said, as a separate issue, but, in fact, the committee concluded that you should not vaccinate against H5 or H7 viruses.

Robert Webster. *Under that proposed definition, how will the definition distinguish between low-pathogenicity AI and non-pathogenicity AI?* **Dennis Alexander.** I do not believe it is actually seen as distinguishing between low-pathogenicity and non-pathogenicity H5 and H7 viruses; all these are included. For other subtypes will depend on the intravenous pathogenicity index (IVPI), as it is at present in the EU.

Richard Slemons. *Is there a difference between the scoring methods between the IVPI for avian influenza or Newcastle disease?* **Dennis Alexander.** Not if you use the IVPI test for Newcastle disease. But within definitions that refer to using pathogenicity index tests for Newcastle disease (i.e., the OIE and the EU definitions), which are more or less the same now, it is recommended that the ICPI test is used for Newcastle disease and the IVPI for AI. The scoring for ICPIs is zero if the bird is normal, one if it is sick, and two if it is dead. So the maximum score is two rather than three in the IVPI.

Gloria Tam. *How are daily cleaning and disinfection actually done, leaving in mind that there are birds around?* **Lindsey Garber.** The markets were either emptied of birds or the birds were moved to a different area within the market. The

response was a general policy of cleaning and disinfecting daily, but it would not necessarily occur every single day.

Anonymous. *Is that consideration of new edition on HPAI for OIE? If yes, when the new edition will be published as the research is in procession?* **Dennis Alexander.** The next edition of the OIE Manual should appear in 2004; by that time the OIE may have readdressed the definition of HPAI and taken into account the deliberations of the EU SCAHAW; if changes are made these will presumably be incorporated.

David Suarez. *You suggest the GF/MA/02 isolate should be considered highly pathogenic, but no highly pathogenic H7 without an insert has less than five basic amino acids. Explain further why you think it should be considered highly pathogenic.* **Dennis Alexander.** What I actually said was I thought GF/MA/02 [cleavage site motif -KPKKR/GLF-] would probably fall within the EU definition since this states: "... viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin" compared to the OIE definition, "If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be highly pathogenic." I thought all HPAI H7 viruses had an insert of at least one amino acid, although not all HPAI H5 viruses do. It looks like a basic amino acid at position -4 may be all important.

Peter Woolcock. *If the new EU definition is adopted, what about H5/H7 in ducks and other non-chicken/turkey species, including wild birds/ornamental collections?* **Dennis Alexander.** The argument against stamping out LPAI H5/H7 infections in commercial ducks is that there is no evidence that H5 or H7 viruses have mutated to virulence in them in the past. However, the converse argument is that if allowed to spread among commercial ducks, this would represent a reservoir for spread to other poultry, where mutation may take place. The recommendation to change the definition must be seen in the context of Directive 92/40/EEC, which states in Article 1: "This Directive defines the Community control measures to be applied in the event of an outbreak of avian influenza in poultry..... This Directive shall not apply where avian influenza is detected in other birds....."

Antonio Zanella. *Because turkey is the most susceptible domestic bird, why is the ICPI test not also done using this bird?* **Dennis Alexander.** It is true

turkeys appear to be more badly affected than chickens by LPAI viruses in the field, but presumably this is because they are more susceptible to respiratory infections. What purpose would be achieved if IVPI tests were done in turkeys instead of chickens? I don't believe there is any dispute over viruses designated HPAI under current definitions, and the aim of pathogenicity tests is to detect such viruses.

Dorothy W. Geale. *You introduced three options for the EU: a) status quo, b) statutory reporting AI, H5, H7 (any), and c) different control. 1) Did the third option include statutory reporting? 2) Could multiple basic a.a. be considered in the definition? (i.e., 2a.a vs. 1 a.a.-3 is regarded as HPAI.) 3) Will this EU decision come to OIE for consideration?* **Dennis Alexander.** I pointed out the options the EU SCAHAW had considered. Option 3 was an intermediary position, where all H5 and H7 infections would be notifiable, but statutory methods of control would be different for HPAI under current definitions [i.e., stamping out] than for H5 and H7 viruses that are LPAI viruses under current definitions. I think there are plans for the OIE to review the definition of HPAI.

Antonio Zanella. *Is it possible for the presence of HPAI viruses in a population of LPAI virus?* **Dennis Alexander.** It is possible for mixtures of viruses to exist when cultured in eggs. This can cause problems in diagnostic laboratories when different H subtypes coexist. I can see no reason why HPAI and LPAI viruses of the same subtype should not be present in the same egg-grown population. If this did occur, it would be expected that the mixture would still be HPAI in *in vivo* tests. There could be more of a problem with nucleotide sequencing, but it may be possible to discern the presence of a mixed population.

David Halvorson. *Why do all your options for commercial poultry include stamping out LP H5 and H7? What is the scientific basis?* **T. J. Myers.** USDA is concerned about the potential for any H5 or H7 AI virus to mutate to a highly pathogenic virus. This potential provides the scientific justification for a regulatory response to these infections. However, the response necessary to control low-pathogenic infections could conceivably be less aggressive than a response to a highly pathogenic outbreak. These are the issues that will need to be discussed with the states and the industries before a policy or regulations can be established.

Robert Webster. *Are the steps in control of AI in Hong Kong being adopted in the U.S.? Is any consideration being given to separating aquatic birds*

from gallinaceous birds? **T. J. Myers.** Any suggestion to improve the control measures used in the U.S. live bird marketing system, including separating aquatic and gallinaceous birds, will be considered.

Patricia Dunn. Does “stamping out” mean the same thing as depopulation, as you used the former term in your talk? Is the USDA close to allowing use of vaccine in Virginia? **T. J. Myers.** Depopulation specifically refers to the act of killing and disposing of infected animals, while stamping out usually refers to the entire range of activities (quarantine, depopulation, cleaning/disinfection, restocking, surveillance, etc.) that are part of eliminating a disease outbreak. At the time of this meeting, discussions are ongoing with the State of Virginia and the Virginia poultry industry regarding vaccine use options.

Dan Karunakaran. On controlling H7 in commercial poultry in the U.S., would you differentiate “turkeys” from “chickens,” as you do LBM, and allow vaccination and control slaughter in turkeys? **T. J. Myers.** At this point in time, no generalizations are made by USDA regarding H5 and H7 vaccine use in either chickens or turkeys; each request is considered on a case-by-case basis.

Dorothy Geale. Is the USDA committed to controlling H5 and H7 LPAI infections to the extent of legislation of reporting all H7 and H5 (+/- voluntary or legislated control)? **T. J. Myers.** As discussed in my presentation, the USDA has not made any decisions on what regulatory changes should be made regarding H5 or H7 (LP) infections, including whether or not such infections should be reportable at the Federal or state level. Public input on this issue will be critical in the USDA’s decision-making process.

Calvin Anthony. You mentioned indemnity for HPAI. Do funds exist for this? If so, where? Also indemnification of live birds? Where? **T. J. Myers.** Commodity Credit Corporation (CCC) emergency funds can be accessed to pay indemnity for HPAI outbreaks, and were used in 1983–84. If a program is developed that includes indemnity for LPAI infections, the USDA would need to request funds in its annual budget for this expense, rather than asking for emergency funds each time a flock needed to be indemnified. This would be analogous to the funding approach taken over the years in the bovine brucellosis and tuberculosis control programs.

Stefano Marangon. The success of stamping out measures is linked to prompt identification of any LPAI outbreaks (epidemics). Do you think that it will be a feasible option for an infection which can spread

asymptotically? **T. J. Myers.** The USDA will need to work with the States and the poultry industries to clearly define its surveillance goals and methods before defining what stamping out measures should be taken in response to the surveillance results. We will need to take into account the shed and spread characteristics of the virus as we develop a feasible approach to controlling LPAI infections.

Nancy Cox. Why is it not possible to close live bird markets since they pose a threat to animal health and a potential threat to human health?

T. J. Myers. Outlawing the live bird markets would not eliminate customer demand for this product and would simply drive this marketing system underground. The challenge we face is to manage or mitigate the risk rather than to outlaw it.

Jeanet van der Groot. You mentioned the MAF website. What is the address of the website? **Howard Pharo.** <http://www.maf.govt.nz/mafnet/>.

Anonymous. Why were the spent layers the most prevalent type of fowl possessing H7N2? **Lindsey Garber.** The reason for the high prevalence in spent white fowl is not completely understood. Positive white spent layers were found in several different markets and had been supplied to the markets by several different suppliers, so it cannot be explained by clustering (either in markets or suppliers). One possible explanation may be related to length of time the birds spent in the market. The white spent hens we tested were in the market a median of 4 days (also mode of 4 days), whereas overall, the median was 2 days (mode = 1).

Eduardo Rivera. When you talk about live bird markets opening 7 days a week, does that mean the day off they had no birds on the premises? **Lindsey Garber.** We asked two separate questions: 1) number of days per week the market was open for business, and 2) does the market have one or more days per week that the market is empty of birds? Surprisingly, these two variables were not significantly associated with each other. The number of days per week the market was open (7 vs. <7) was significantly associated with presence of H7 in the market, regardless of whether or not the market had days empty of birds. When looking at these two variables together, only 19% of markets that had a combination of being closed at least one day per week and also having at least one day per week empty of birds were positive for H7, whereas 70% of markets with the combination of being open 7 days per week and no days empty of birds were positive. In general, birds were moved elsewhere within the market when cleaning and disinfecting

occurred, especially markets that cleaned and disinfected daily.

Anonymous. *How do new test methods become adopted by OIE and recommended for the diagnostics and standards manual?* **James Pearson.** The *OIE Manual of Standards for Diagnostic Tests and Vaccines* is revised every 4 years, and the authors are encouraged to add new techniques that have been validated. The *Manual* is then sent to the OIE Reference Laboratories and to the Member Countries for review and to add new procedures if appropriate. The Standards Commission of the OIE coordinates this process. The final version must be approved by the OIE International Committee.

David Suarez. *Reports to OIE within 24 hours—Are these for suspicion of disease or confirmation of disease?* **James Pearson.** The *International Animal Health Code* states that confirmed cases of List A diseases must be reported. The *Code* states the following as far as requiring the reporting suspicion of a *List A* diseases: A provisional diagnosis of a disease should be reported if this represents important new information of epidemiological significance to other countries; The Chief Veterinary Officer is responsible for making the decision as to what should be reported but the decision will be reviewed by trading partners.”

Nancy Cox. *What proportion of HPAI outbreaks do you think are actually reported to OIE by member countries?* **James Pearson.** I believe that almost all the outbreaks that are confirmed by the OIE Reference Laboratories are reported. However, there are probably cases that are not confirmed that are not reported.

Baltus Erasmus. *Hypothetical situation: Would there be legitimate reasons for trade sanctions against a country using strategic prophylactic vaccinations against H5 or H7 AI only in valuable breeding stock under state control coupled with necessary surveillance, sentinels, etc.?* **James Pearson.** The vaccination of breeding stock should not result in trade sanctions as the *OIE International Animal Code* does not forbid the use of vaccine. It does require surveillance, which will be complicated by vaccine. However, as stated in the question, it is possible to establish a surveillance system that would detect highly pathogenic avian influenza in vaccinated birds.

FIELD EXPERIENCES IN CONTROL AND ERADICATION

Dorothy Geale. *What surveillance was done in nonvaccinated area? How was it conducted—at*

slaughter/placement? **Stefano Marangon.** A monitoring program approved by the EU Commission was implemented in nonvaccinated areas of the region. Meat-type farms were serologically monitored at slaughter on a sample base (10 blood samples per farm). Furthermore, all breeder and layer flocks were inspected and serologically tested at least every 2 months.

Susan Trock. *How did you handle hatching eggs from infected breeder flocks? Please comment regarding your concern for the hatcheries and hatching eggs.* **Stefano Marangon.** AI virus is not transmitted vertically. Nevertheless, there could be a risk of cross contamination at the hatchery. In case of HPAI infection, all the hatching eggs produced on the infected premise were traced back and destroyed. In breeder farms affected by LPAI virus, hatching eggs were not traced back and destroyed, but strict biosecurity measures and disinfection procedures were defined and enforced both on the farm and at the hatchery.

Stephen Lindstrom. *If flocks are vaccinated with H7N3 to discriminate between vaccinated birds and those infected with H7N1, how would a potential outbreak of H7N3 be dealt with or surveyed for?* **Stefano Marangon.** Taking into account that AI epidemics are rare events, the probability that such a situation occurring is low. Nevertheless, in the only one vaccinated flock which became infected, overt clinical symptoms and a sharp rise of the weekly mortality rate (7% in one week) were observed in sentinel (nonvaccinated) birds, while no clinical symptoms and no increased mortality was seen in affected vaccinated animals. Furthermore, the LPAI virus was isolated from sentinel birds and typed. This means that a potential outbreak of the H7N3 virus strain could have been easily suspected and diagnosed.

Anonymous. *What would you have done if instead of turkeys, you had broilers in regard of use of vaccination to control LPAI in Italy?* **Stefano Marangon.** We took into account the possibility to vaccinate broilers only once at the hatchery and to monitor the efficacy of this vaccination scheme in the field, in order to evaluate if it was possible not to revaccinate the birds on the farm.

Gloria Tam. *What was the rationale for choosing to vaccinate for one and a half years?* **Stefano Marangon.** It was based on a qualitative risk analysis on the possible persistence of the LPAI virus in the vaccination area. In fact, at the beginning of the vaccination program, the LPAI virus was still circulating among poultry farms in the region, and

the last LPAI outbreak was stamped out at the end of April 2001. It was agreed with the European Commission that, taking into account the epidemiological situation, Italy could have brought to an end the vaccination program at an early date. In fact, due to the favorable results of the monitoring activities carried out both inside and outside the vaccination area, vaccination was stopped starting from the 1st of March 2002.

Jeanet van der Groot. *Did you find a significant difference in the height of the HI titer between the groups inoculated with 50,000 EID₅₀ and 500,000 EID₅₀?* **Huaguang Lu.** Yes, very significant. The HI titers (120–140) of birds inoculated with 500,000 ELD₅₀ were more than two times higher than those (50–60) inoculated with 50,000 ELD₅₀ during the peak period of 4, 5, and 6 weeks pi.

Guus Koch. *How do you envisage the inactivation of AIV on a larger scale in manure in the field?* **Huaguang Lu.** Just leave the manure in the field house and completely close the house without doing anything. AIV contained in the manure will be inactivated within 1 to 3 weeks depending on temperature; increasing the house temperature will speed up the inactivation process. Our experimental studies indicated AIV was inactivated within 6 days with field chicken manure at an ambient temperature (~60 F) condition. Our field experience: during the AI outbreak in PA last December, two broiler breeder flocks affected with AIV were all depopulated 14 days after the outbreak; the two houses were closed with manure inside. AIV was not detected after 3 weeks (<3 weeks, not tested) of closing houses without doing anything; the house temperature was between 35–40 F at that period.

Michel Bublot. *Was the infection in the vaccinated flocks detected on vaccinated birds (DIVA) or in sentinel birds?* **Stefano Marangon.** The presence of the infection was detected on sentinel birds (virus isolation and seroconversion). At that time, January 2001, the N1-N3 discriminatory test was not available yet. Sera collected on vaccinated birds were frozen and used to validate the N1-N3 discriminatory test: 8 out of a total of 10 sera gave positive results to this test.

Khalid Naeem. *Have you found H9N2 and NDV from the same flock? If yes, do you see any interaction between the two viruses?* **Malik Peiris.** Yes, H9N2 and NDV are isolated from the same flock and in fact, sometimes from the same specimen. We are unable to comment on whether there is an

interaction or disease synergism between these two viruses.

Khalid Naeem. *Did you find H9N2 and H5N1 from the same flocks?* **Malik Peiris.** Yes, we have isolated both H5N1 and H9N2 viruses from the same farm. It has been shown that H9N2 viruses may cross protect against H5N1 viruses isolated in 1999 (i.e., containing Goose/Guangdong/1/96-like internal genes) (Seo *et al.* J. Virol. 76:4886–4890. 2002.). There is no data on cross-protection *vs.* more recent H5N1 isolates.

David Suarez. *Where [are] the H9N2 viruses coming from that reinfect the markets?* **Malik Peiris.** H9N2 viruses are endemic in poultry in these regions, the Dk/HK/Y280/97-lineage viruses in chicken and the Qa/HK/G1/97-lineage in quail. These are not HPAI viruses. In general terms, they appear to have relatively little impact on production.

Anonymous. *Do the songbird markets in Hong Kong play any role in the epidemiology of AI?* **Malik Peiris.** We have done limited studies on isolating influenza viruses from songbirds. Though influenza viruses can be isolated, they are not the subtypes we routinely isolate from the poultry markets or farms. Thus, the viruses from songbirds do not seem play a major role in the ecology of viruses in the poultry industry.

Michel Bublot. *Do you observe any difference in the efficacy of different vaccines (inactivated *vs.* fowlpox recombinant) to eradicate MPAI?* **Eduardo Rivera-Cruz.** No, if the recommended vaccination calendars are followed. Fowlpox influenza vaccine is used mainly in broilers.

Anonymous. *Are there any data to indicate that the eradication would not have happened without vaccinations?* **Eduardo Rivera-Cruz.** We have no data in that respect, but the field experience when we faced the HPAI was that poultry around an infected farm was vaccinated with the inactivated-emulsified vaccine, and most of the time, the HPAI virus did not spread around.

Antonio Zanella. *What are the most concentrated state areas of poultry in Mexico?* **Eduardo Rivera-Cruz.** Jalisco, Puebla, and Comarca Lagunera.

Guus Koch. *Use of the recombinant vaccines allows one to monitor for infection by serology. Was such testing performed? If so, what was the result?* **Eduardo Rivera-Cruz.** The recombinant fowlpox influenza vaccine elicits very low titers of antibodies, if any, for a very short period of time. However, when a vaccinated flock is infected with a LPAI virus, the titers increase dramatically.

Anonymous. *How do you explain this rapid decline*

in antibody titer (of field exposure) compared to vaccination titer? **David Henzler.** These were mildly pathogenic H7N2 avian influenza infections; hence, the poultry infected (primarily laying hens) were not stimulated immunologically as strongly as if it were a highly pathogenic influenza virus. With vaccination (at least with killed vaccines), each bird is individually injected. The dose (antigen) is predetermined to be sufficient for the bird to develop a protective titer. When each bird is injected, there is no question as to whether each received an infectious challenge equivalent. In our 1996–98 H7N2 avian influenza outbreak, field reports from farmers noted the infection would start at one area of the house and take some time before mortality spread from the localized area of introduction to other areas in the house (several days to 2 weeks). The antibody response was determined by the agar gel immunodiffusion test. Hence, declines in antibody were assessed relatively from multiple temporal random samples taken from individual birds or eggs (egg-yolk antibody). This slower pattern of spread is expected with more mildly pathogenic viruses. Additionally, when large populations of poultry are exposed to an infectious organism, a factor known as flock immunity is important. This is where the flock response to a challenge agent is dependent not only on the infectious dose itself but the individual immunities of each bird, which are not exactly alike. Other components, such as bird-to-bird transmission (horizontal spread) through respiratory or gastrointestinal excretions, also play a role.

Peter Holt. What was the reason for the poor and/or rapid decreasing antibody titers? **David Henzler.** This is somewhat explained in the previous question. Primarily this H7N2 influenza virus was a slower moving agent, less immunologically stimulating than a highly pathogenic virus, and bird-to-bird transmission within the flock was necessary for continued spread. In this outbreak the virulence of the agent did not increase in time, as demonstrated in the field by similar flock morbidity and mortality patterns.

Dorothy Geale. Will your experience with depopulation on moderately pathogenic AI impact Pennsylvania's current policy of depopulation? **David Henzler.** Yes. Each influenza virus subtype appears in the field to have its own unique characteristics in terms of likely poultry species most infected and the within-flock pattern of infectious response. In the 1996–98 Pennsylvania H7N2 avian influenza outbreak, most of the birds infected were laying hens.

In the outbreak center there were numerous meat birds—primarily commercial broilers—however, none were known to become infected. As we began to depopulate large flocks of commercial birds (80,000 to 123,000 bird flocks), an apparent pattern of infectious spread to neighboring flocks within a 1 to 1½ mile (1 to 2 km) area was evident. The methods employed for depopulating these birds spread the infection to nearby flocks. In time, protocols used for depopulation improved to the point where this was not as large a factor in flock-to-flock spread, but the risk was still sufficient to look at alternative methods to depopulation and subsequent on-farm burial or transport to community landfills. The flocks remaining in production were closely monitored for any evidence that the agent would remain in the flock or continue to pose a risk for area spread of influenza virus. With strict premises/flock quarantine and biosecurity and a closed-loop system of egg transport vehicles and egg packing supplies, it was clear we were able to allow the flocks to immunologically recover from the initial introduction of this influenza virus. These methods were successful in containing the outbreak and preventing widespread occurrences of additional cases in an area of Pennsylvania that contains the largest density of poultry. Further large economic losses to the producer (flock and eggs) were prevented and adverse environmental consequences avoided (disposal of very large numbers of poultry carcasses).

Anonymous. Are eggs that are positive for antibody to H7 AI still susceptible to infection by H7N2 virus?

David Henzler. Avian influenza is not a known vertically transmitted disease. I do not know if chicks hatched from eggs which had antibody to H7 avian influenza are susceptible to subsequent influenza infection.

Anonymous. Define the strict biosecurity measures used in control. What specific components of depopulation furthered the spread of the AIV to nearby flocks?

David Henzler. The biosecurity measures early on included an area quarantine. The zone contained all of the known infected flocks, with additional area surrounding those to likely include any new exposed flocks based on the particular pattern of spread with this mildly pathogenic avian influenza virus. This quarantine remained in effect for many months. To move poultry in or out of the defined zone, a permit had to be granted from the Pennsylvania Department of Agriculture. When flocks were diagnosed as infected or suspected to be infected, a portable high-pressure spray disinfectant unit was

transported to the premise. A Pennsylvania Department of Agriculture official determined the best location for placement (most often at the end of the farm lane), and mandatory disinfection of personal vehicles and all other vehicles was required for a period while the flock was most likely to spread virus. Service calls by industry technicians were halted and restricted to phone reports. One large commercial feed mill within the zone developed a routing plan for clean side of exiting feed trucks which were disinfected with a stationed employee all hours of the day and night with a separate generator hook-up for night lighting at the disinfectant station. The plan implementation [was] approved and monitored by officials of the Pennsylvania Department of Agriculture. To reduce this risk of eggs or dust, feathers, and organic matter on the outer surface of eggs or transport materials, we maintained eggs cooled on farm for additional days and had a “closed-loop” pattern of egg packing materials (plastic or fiber flats), egg carts, pallets, and other transport equipment along with a dedicated truck to further processing (egg breaker plant). All materials were then returned to the same farm after additional sanitation of materials and the truck at the breaker plant.

Early depopulation methods were done as soon as possible following diagnosis of avian influenza infection. These methods employed industry and government depopulation crews including either on-farm burial equipment or off-sight landfill disposal equipment and personnel. In large commercial cage layer facilities, this involves relatively large numbers of people (often a dozen or more) removing hens from cages and throwing them into covered or partially vinyl tarped 20- or 30-yard lined industrial waste disposal units. Many feathers and dust are either exhausted out the ventilation system or become airborne with all the activity of the depopulation crews. Attempts to use landscape cloth fixed on wooden frames to surround pit exhaust fan housings were unsuccessful in furthering the spread to nearby flocks.

Dorothy W. Geale. *What is the nature of the surveillance program in Mexico—active or passive?* **Eduardo Rivera.** Active.

Anonymous. *Could you please give an update on the recent outbreak of LPAI H5N2 in the state of Nuevo Leon?* **Eduardo Rivera.** On February 11, 2002, an avian influenza virus (LP) H5N2 was isolated and identified from broilers originated in Pesqueria, Nuevo León. An epidemiological survey, done for three consecutive times, for all poultry farms in

Nuevo León was initiated, resulting in 94 farms with positive avian influenza serological results. The total number of poultry farms in the state of Nuevo León was 284. Quarantine was established in the positive farms, and poultry were allowed to be vaccinated against avian influenza. So far, no more serologically positive avian influenza poultry [have] been found in the state.

Gloria Tam. *When you depopulated, did you just do it on a farm basis, or did you draw up a circumscribed area? And, if so, what was the radius and the rationale for such a distance?* **Eduardo Rivera.** In general, we depopulate on a farm basis.

Nancy Cox. *How are cloacal swabs or environmental samples containing feces treated before testing with Directigen or Dot-ELISA for positivity. Any special diluents, treatment, etc.?* **Huaguang Lu.** We place cloacal swabs or environmental samples in virus transfer medium (VTM, without serum) or PBS, centrifuge at ~1500 rpm for 10 minutes, filter through a 0.45 filter, and then follow the procedures of Directigen or Dot-ELISA tests.

Anonymous. *What is the sensitivity of dot blot test vs. Directigen and virus isolation?* **Huaguang Lu.** We conducted two comparison studies as follows: 1) H7N2-infected SPF experiment chickens: Dot-ELISA: Se = 42.86%; Sp = 87.50%; Directigen: Se = 38.1%; Sp = 87.5%; (2) H7N2 outbreak of broiler breeder flocks: Dot-ELISA: Se = 62.32%; Sp = 90.91%; Directigen: Se = 57.97%; Sp = 95.45%. Note: Se = sensitivity; Sp = specificity.

There was no statistical difference between the Dot-ELISA and Directigen test for their Se and Sp in the detection of AIV from clinical specimens. The test specimens include tracheal swabs, cloacal swabs, and environmental swabs. The Se and Sp were calculated based on virus isolation results in embryonating chicken eggs as a standard reference test. The Se of the Dot-ELISA or Directigen in the detection of AIV *vs.* virus isolation varies depending on the virus concentration in a test specimen or infection status of an AIV-positive flock. Results from our studies indicated that the Se of Dot-ELISA ranged from 25% to approximately 90% in testing various specimens collected from H7N2 experimentally infected SPF birds and H7N2 field outbreak flocks. The sample collection was done two to three times per week.

Anonymous. *Have you evaluated the infectivity of H7N2 in composted or buried carcasses from depopulated flocks? What method of carcass disposal would you recommend?* **Huaguang Lu.** Not really done. But one time we collected body fluids from

carcasses of AI-positive birds piled in a flock house for less than a week, and all specimens were negative for AIV. Also, based on our studies of chicken manure inactivation for AIV, within field chicken manure, AIV lost infectivity in 24 hours under 30–37 C and less than a week under 15–20 C temperatures. So to leave the carcasses in the field house and increase the house temperature (30–37 C) would be an option to destroy the AIV infectivity and avoid spreading.

Anonymous. *Was the disinfectant efficacy ever evaluated in the presence of organic matter? If not, would you expect the efficacy of the disinfectant to be lower under field conditions?* **Huaguang Lu.** Not done. In field condition, it might be lower efficacy in comparison with a laboratory condition, but would not be much.

Gloria Tam. *How far can your virus survival and inactivation data for H7N2 be extrapolated to other subtypes of AI? What would be your recommendations for disinfecting H5- or H7-soiled farms and LBMs?*

Huaguang Lu. Although all the virus survival and inactivation data were done for H7N2 virus, those data should be extrapolative for other AIV strains, since they are the same enveloped viruses and they are sensitive to heat, lower pH, and disinfectants. For disinfecting H5- or H7-soiled farms and LBMs, any effective disinfectants (e.g., DC & R, Virkon) would work well. Heat and disinfectant combination for a chicken house will enhance the inactivation process.

Dorothy Geale. *You stated that cage or manure samples should be components of any surveillance program in your conclusions. Could you comment on the reliability of serological sampling only?*

Huaguang Lu. In our experimental bird trials for H7N2 virus, birds showed good serological immune response to H7N2 virus. Antibody titers (HI test) were detectable from the second week of infection to >20 weeks. Serological sampling only should be reliable as virus isolation, but there is a time delay (~1 week) for serological result in comparison to virus detection or isolation. On the other hand, AIV is usually detectable during the first 2 to 3 weeks of infection and not detectable after 4 weeks. Thus, serum samples should also be an important component of AI surveillance.

Anonymous. *One of the problems with GIS databases is maintaining the confidentiality of the information. In your opinion, who should maintain the database? (Note: In the U.S., most poultry companies are reluctant to assign responsibility for the GIS database to government or a university because of the*

Freedom of Information Act.). **Manfred Ehlers.** There is no equivalent to the Freedom of Information Act in Europe. In Germany, we have a strict Privacy Law that also restricts access to this type of GIS/database data. The database is maintained by a University technology transfer center that acts as a private company. With this, we do not follow University regulations. I do not know, however, if that is valid for the U.S. as well. What I would propose is a certified company which acts as an in-between for universities and private companies so that private data are protected.

Anonymous. *How can we prevent GIS data from falling into the hands of bioterrorists?* **Manfred Ehlers.** The database must not be maintained in an open network environment. Actually, a stand-alone set-up seems the most secure solution to me. Data exchange can be done by CDs or password protection. However, once you produce a CD, you can probably find everything on it. But is it a real threat?

Anonymous. *The GIS system seems like a wonderful epidemiology tool for retrospective analysis. But given the extensive, detailed background information required to be entered, how useful is it likely to be for predictive purposes or real-time outbreak monitoring and control (i.e., without the supporting information databases)?* **Manfred Ehlers.** The currency and the accuracy of the database is the most important feature. You can make use of the power of GIS only if and when you have the proper data to go with it. Otherwise, it is just a 'dumb' map (or even worse: garbage in => garbage out).

Dorothy Geale. *Has analysis been done to identify trigger points related to NNI, GINI, or kernel estimation (which are measures of density) (i.e., could any of these indices be a predictive indicator of outbreak potential)?* **Manfred Ehlers.** This is actually part of the current research. We hope to find a suitable set of parameters (besides density, distribution pattern, etc.) that would indicate the risk for each potential location.

VACCINES AND ANTIBODY-BASED DIAGNOSTICS

Jeanet van der Goot. *How do you explain the cross-reactivity after two or three vaccinations with the DNA vaccines in the HI test?* **Chang Won Lee.** I don't know the immunologic basis for that, but there are several observations with influenza virus or with other avian viruses that repeated vaccination will elicit some nonspecific reaction. And I also want to

say that cross reactivities were inconsistent with heterologous antigen. We got different results, either no or higher cross reactivity, with different heterologous antigens.

Kanta Subbarao. *What dilution of sera did you use in the N1N3 IFA test?* **Giovanni Cattoli.** 1:250. That was the working dilution.

Guss Koch. *Is the influenza HA incorporated in the Newcastle disease (ND) virus particles? And could this explain the lower replication rate?* **David Swayne.** The sequence data shows that there is obviously insertion of the cDNA of the avian influenza (AI) H7 gene in the right location, between the P and the M genes of the Newcastle disease virus. The AI H7 gene is incorporated into the recombinant ND virus and expressed as evident by Northern blot analysis and indirect fluorescent antibody tests. Now the questions are how well does the virus replicate and how well is it expressed quantitatively? The data suggest the recombinant replicates to 3 logs lower titer in respiratory tract of chickens compared to a standard B1 ND vaccine. The latter question is an important issue to further explore.

Guss Koch. *Would insertion upstream of the NP or between NP and P gene give a better vaccine in the view of the expression gradient of Newcastle disease virus genes?* **David Swayne.** That is a good question, and I do not know the answer. Your suggestion is worthy of exploration as to the best site for gene insertion.

Anonymous. *Is it possible to have cross protection against other H types of avian influenza viruses?* **Giovanni Cattoli.** We have shown that the vaccine strain provides good protection against a challenge by HP H7N1 virus. Based on that data, I assume there would be a good protection against other H7N1 subtypes. Of course, you have to test it in advance, but I expect that there would be good protection against other H7 avian influenza subtype. **M. Tollis.** Well, possibly you can have some cross protection, cross-protective activity between H7N3 vaccine and other H7 strains. But I could not assure you. I mean, possibly. It depends also on the homology between the two viruses. You know you have always to consider how distant they are.

Amer Silim. *Do you plan to avail, that is, to sell or give your antibodies for research or diagnostic purposes? And also, do you hope to do similar work on neuraminidase antigens?* **Chang Won Lee.** We are also planning to produce antibody against neuraminidase and nonstructural proteins. We are willing to provide the antibody or the plasmid with a material transfer agreement. Talk with Dr.

David Suarez about getting the antibodies or the plasmid.

Anonymous. *What type of correlation did you find between your serum and egg-yolk antibody ELISA?*

Maria Tollis. A very good—99%—concordance (as I showed in my presentation, under only one circumstance we found a substantial difference) was demonstrated. Such a good correlation was also demonstrated for the antibody titers in serum and egg yolk derived from the same chicken.

Anonymous. *What is the protection level (%) of existing inactivated vaccine compared to that of the rNDV vaccine?* **David Swayne.** In these particular studies, we did include an inactivated AI vaccine to compare to the rNDV. We are going to do some additional studies, and at that time we will compare both of them. Also, we will probably include a live AI virus vaccine group along with the inactivated AI vaccine and rNDV.

Robert Eckroade. *Considering that you would have applied this as spray or water delivery, possibly resulting in further reduction of efficacy, do you think this approach is still feasible?* **David Swayne.** If there was preexisting immunity to Newcastle disease virus, it could reduce efficacy of the vaccine by interfering with vaccine replication and produce insufficient antibody titers for protection against AI challenge. Similarly, preexisting immunity against fowlpox reduces efficacy of the fowlpox recombinant AI vaccine. If the birds in the field have been either vaccinated with fowlpox or have been exposed to field strains of fowlpox virus, efficacy of the fowlpox recombinant is greatly reduced.

Veronique Jestin. *What is the protection afforded to turkeys with the inactivated vaccines? Why the choice of the HPAI instead of the LPAI vaccine strain?* **Maria Tollis.** It is important to consider the different species used to standardize the vaccine. We just standardized our H7N1 vaccine in chicken, so I cannot tell you about the protection in turkeys, because we did not look at turkeys. Regarding the choice of the HPAI instead of LPAI for the vaccine strain, we used a low-pathogenic influenza virus for the vaccine. We used a highly pathogenic avian influenza virus for challenge. And for international rules, OIE, European Community, we have to use low-pathogenic avian influenza viruses to make vaccines out of them. So, I mean, in any circumstances, we could have used highly pathogenic avian influenza viruses. Maybe this was, in some instances, not very clear in the presentation. Only low-pathogenic avian influenza viruses can be used for the vaccine. We used a low one.

Michel Bublot. *What was the stability of the rNDV AIV H7 for HA expression after in vitro passage and in vivo in chicken passage?* **David Swayne.** We have propagated and passaged the rNDV virus several times through 9-to-10-day-old chicken embryonating eggs. After that process there was still expression. Now the question may be, how much expression is there of the AI H7 hemagglutinin protein by the rNDV. We have not quantified the protein in any of the systems, other than, obviously, in the live bird experiments; we know the AI is expressed, especially because we detected some antibodies, and we know that we can detect the H7 hemagglutinin protein in tissue cultures.

Anonymous. *When testing egg yolk by the ELISA tests, do you have any problem with nonspecific reaction due to the presence of excess lipids in the yolk material?* **Maria Tollis.** Not at all. That was a very good result.

Robert Webster. *For how long after infection was your test positive for N3, and how many months lapsed?* **Giovanni Cattoli.** Actually, we have no data on that, only about the appearance of the antibody. So far, we tested the appearance of the N1 antibody response: we wanted to know if it was earlier or if it appeared later compared to the HI to be sure that we had no window in between.

Rafael Fernandez. *How is the serological response to Newcastle disease virus proteins when using this recombinant NDV vaccine?* **David Swayne.** With the current rNDV construct, the NDV HI antibody response after one or two immunizations is lower than with a single immunization using the field strain of NDV B1 vaccine. So we are talking about titers that generally range from zero in some birds to probably 1:20, maybe up to 1:30 or 1:40. I did show the data on the rNDV vs. field B1 vaccine replication in inoculated chickens. Part of the low serologic response is the rNDV did not replicate as good as the field B1 NDV virus. As a result, we are not getting as high NDV HI or AI H7 HI titers as we need. Therefore, we are not getting as good of protection against both Newcastle disease and avian influenza as we need.

Calvin Anthony. *Instead of using B₁, why not consider using a cloned LaSota virus? Better spread, less reactivity.* **David Swayne.** The only infectious clone to NDV we have available from our collaborators, Adolfo Garcia and Peter Palase at Mt. Sinai Hospital, is the B1 clone. We agree that looking at a LaSota clone may improve replication and spread and should be pursued.

Guus Koch. *Why use H7N1 and not the available*

H7N3 commercial vaccine for this study? The HI titer induced is rather low, which may be caused by the inadequate formulation of the H7N1 vaccine. **Maria Tollis.** The H7N1 vaccine was selected and prepared "ad hoc" and used for standardization of the minimum requirements of a potential candidate for vaccination of chickens because the vaccine virus strain was homologous to the field virus strain causing LPAI in Italy (it represented the first Italian field isolate of LPAI) and shared 98.1% homology with the amino acid sequences of H7N1 causing HPAI in Italy. In the light of these considerations, it is the choice to use the H7N3 vaccine (made by an HPAI virus strain isolated from outbreaks of HPAI in Pakistan) that appears questionable!

Immunization with the standardized HA contents in the inactivated H7N1 vaccine induced GMTs ranging from $10^{3.19}$ /ml (with 5, 2.5, 0.5:g HA), to $10^{2.74}$ /ml (with 0.25:g HA) and $10^{1.88}$ /ml (with 0.1: g HA) (which means approximately GMTs ranging from a maximum of 1:1550 to an intermediate of 1:550, and a minimum of 1:76) on day 21 after the administration of one dose of the different vaccine preparations. These titers (or at least those induced by 0.5 and 0.25:g HA, these latter ones included in the vaccine preparations chosen for establishing the vaccination schedules) appear to be extremely high, and such a magnitude of the immune response has been considered as the main indicator of a predictive protection in vaccinated birds.

MOLECULAR DIAGNOSTICS

Eric Gonder. *What are the specific problems with using real time RT-PCR at pen-side?* **Erica Spackman.** Probably the primary problem with that is nucleic acid extraction, and, as Dr. Perdue mentioned earlier, for things like bacteria, mostly for human pathogens, cartridge-type methods for extracting DNA for specific pathogens are being developed. But for AI, nothing has really been developed. And in order to get a clean preparation and because of the equipment involved for the extraction, it would be really difficult to do at pen-side.

John Pasick. *What test samples have been used with this technology, and what time is required from sample receiving to test result?* **Richard Collins.** The testing time, from taking the clinical swab to isolating RNA to detection, can be all achieved in one working day—typically, 4–6 hours.

Dennis Senne. *What types of samples?* **Richard**

Collins. Cloacal swabs, pharyngeal swabs, anal swabs, blood, and internal organs have all best tested specifically with this system.

Anonymous. *What is the cost of these portable PCR devices?* **Michael Perdue.** The Idaho Technology machine is around \$50,000. Right now the Cepheid is \$28,000. They are getting cheaper. Like anything else, as they make more of these things and they are used more widely, the price will come down, and the reagent costs likewise will come down. But they are not inexpensive at the moment.

Anonymous. *Have you compared the sensitivity of your NP-based PCR results with M-based PCR tests?*

Karen Dybkaer. No.

Anonymous. *Can you detect virus from chicken nests?* **Richard Collins.** Yes. We have tested cage sweepings and market swabs with this system.

Veronique Jestin. *Since the cleavage motif of each five strains can be very variable, how do you design a universal primer for this region?* **Richard Collins.** I am not sure you can design a universal primer for H5 pathogenicity. That is a highly variable region that spontaneously arises during the evolution of the virus. This system was specifically tailored for the viruses circulating in Southern China and Hong Kong and is ultimately based on the goose/Guangdong/96 cleavage site, which has remained unchanged from 96, through to chicken/Hong Kong/97, A/Environment/Hong Kong/99, chicken/Hong Kong/2001, chicken/Hong Kong/2002—all the hemagglutinin cleavage site sequences are essentially unchanged. The primers can tolerate a couple of nucleotide mismatches, provided they are in an internal region and not at either end. Having said that, I accept that the pathogenic cleavage site is highly variable, so you would have to tailor each system specifically for each region. I think I am right in saying that the highly pathogenic Italian sequence for the cleavage site is the same as Hong Kong, but evolved independently. So the system does have applicability to other areas as it is.

Giovanni Catolli. *What are costs of the different tests?* **Erica Spackman.** Well, the costs for the RRT-PCR test, whether it is the subtyping test for the hemagglutinin or the matrix test, it is about the same. The materials cost is about \$8 per test. **Dennis Senne.** They are getting cheaper. It is half the price of the Directigen test.

Stephen Lindstrom. *What are the advantages of NASBA over other detection methods such as RT-PCR or real-time RT-PCR.* **Richard Collins.** The principal advantage is the huge degree of amplifica-

tion. Because you get 100 to 1000 copies of your target sequence with T7 RNA polymerase, you can get 10^9 – 10^{12} amplification in 2 hours. In contrast, most PCR systems give you 10^6 in the same period. It is very robust. Because of the huge degree of amplification, you can pool 20 samples together. So only one of those 20 has to be positive; you can still get a positive result.

Jill Banks. *Can multiple/degenerate probes be used for the cleavage site, to allow detection of multiple cleavage site motifs that often occur with HPAI outbreaks?* **Richard Collins.** We have not specifically tested it, but it is certainly an option.

Anonymous. *Did you do any titration comparisons between RRT-PCR and virus isolation? I think you presented some data on that, relative sensitivity.* **Erica Spackman.** Yes, we have done that. We have looked at the EID₅₀. We did get better sensitivity with the matrix test than virus isolation. But it was a little bit less sensitive for subtyping. This was done by looking at RNA directly extracted from dilutions inoculated into eggs, which were tested by RRT-PCR.

Anonymous. *Is there a difference in sensitivity between cloacal and tracheal swabs in the RRT-PCR?* **Erica Spackman.** We have not actually looked directly at a difference in sensitivity between the two. With the live bird market samples we will detect more positive tracheal samples, but that is probably just due to the replication of the virus in the trachea. As far as any inherent sensitivity differences between the two types of swabs, I am not really aware that there is any. We have not looked at that directly though.

Anonymous. *How many samples do you need to run over time to be sure that the results will detect the RNA if it is there?* **Erica Spackman.** I guess the question is addressing reproducibility. Theoretically, if you are trying to detect the virus, hopefully you would be able to detect it in one reaction per sample. Of course, with any assay, with a low level of virus present, you are going to have less reproducibility. So running multiple samples will give you better sensitivity, probably. But usually, we just use one reaction for one sample format.

Anonymous. *In your RRT-PCR do you amplify a reporter gene at the same time to validate your test?* **Erica Spackman.** I believe this is asking about whether we use a positive control template for the RRT-PCR test. Yes, we run a reaction with *in vitro*-transcribed RNA from the assay target gene with every set of test reactions to ensure that the test is working properly.

LATE-BREAKING ISSUES

Eduardo Rivera. *How many micrograms of HA antigen does the vaccine contain?* **Dr. Sims**. I'm going to take a rain check on that question and come back with a written answer. (Postconference note. The company supplying the vaccine chose not to provide this information.)

Charles Beard. *Is vaccine being used oil emulsion or alum? Do you have required interval between the use of oil emulsion and processing?* **Dr. Sims**. The vaccine is a water in oil emulsion. We are basically following the standard set by USDA on the withholding period for other oil-based avian influenza vaccines.

Michel Bublot. *Inactivated vaccines will be used in Hong Kong. What about the fowlpox vector H5 vaccine? Will it be allowed? (and similar question: Veronique Jestin. Since the fowlpox recombinant H5 vaccine exhibited good protection against H5 Hong Kong 1997 strains, why were inactivated vaccines chosen?)* **Dr. Sims**. We have a problem with the fowlpox vector H5 vaccine in that all of Hong Kong's day-old chicks are hatched outside of Hong Kong, so we don't have direct access to them and can't, at this stage, arrange vaccination in the hatcheries. We also have multiple suppliers of day-old chicks, and the logistics of getting those birds vaccinated once they arrive in Hong Kong precludes the use of this vaccine at this time.

Dorothy Geale. *Is there a discriminating test for the killed H5 vaccine you are using vs. wild H5 infections? Or are you developing one?* **Dr. Sims**. Because we'll be using a H5N2 vaccine, we should be able to use a discriminatory test such as the DIVA test system used in Italy, measuring anti-N1 antibody. The only complication we may face is that we have had H6N1 influenza virus in Hong Kong previously. At the moment, we don't believe this virus is circulating widely, so I still believe that we will be able to use this test. The other thing we'll be doing in vaccinated flocks is to monitor unvaccinated sentinel birds as well as individually labeled vaccinated birds. We will be able to monitor these birds for antibody responses and disease as well.

Anonymous. *In order to decrease the spread of LPAI in LBM, would it be possible to consider the distribution of antiviral substances such as Zanamivir?* **Dr. Mullaney**. I don't think that I am the best person to answer that question.

Eduardo Rivera. *After cleaning did you use any disinfectants in the live bird market?* **Dr. Mullaney**. Yes, the markets were required to be without birds,

cleaned of all organic debris and obstructing materials, and then disinfected. Phenolics were used in most cases for residual action. In the markets in New England, the teams additionally used chlorine disinfectants after the initial inspections of the market's efforts.

Anonymous. *Are there plans for more market closures, as done in Hong Kong?* **Dr. Mullaney**. Not at this time. This was an initial effort to coordinate a closure of the retail markets in the Northeast. At this point closures are not required routinely by all states. Markets are monitored periodically but at different intervals, depending on the manpower of each state. Markets that test positive may be required to be without birds and have cleaning and disinfection. The markets may reopen the same day in some instances.

Dorothy Geale. *What is the scientific rationale for simultaneous closure of widely separated markets (i.e., regulatory action requires scientific documentation). Was closure on a sequential state-by-state basis considered? Why just retail, not wholesalers simultaneously (on a state-by-state basis)? (and similar question: Anonymous. Under what authority were LBM closed)?* **Dr. Mullaney**. This retail marketing system has consistently tested positive for H7N2. These markets, while in six states, are supplied basically by the same supply system. In New York City alone there are almost 80 of these markets, so in this sense, they are not widely separated. Indeed, some of these markets have the same owner or family connection contributing to movement among the markets themselves. To address the question of this particular virus circulating within the markets themselves, a simultaneous closure was a must to prevent the establishment of the markets themselves as a reservoir. It is important to point out that this closure was not a federally mandated program but was carried out under the authority of the individual state's Department of Agriculture. The USDA coordinated the effort for uniformity of time and conditions.

As to the wholesalers and dealers, there was much consideration to that, and ideally they need to be directly involved in future closings. The addition of other states into this closure late in the program's development also extended the network of suppliers, dealers, and wholesalers involved. To include the wholesalers directly added to predicted legal challenges of not including all under the same conditions. It is important to point out, however, that while these wholesalers and dealers were not formally a part of this closure, indirectly they were

closed, as many were unable to move their product into the retail level for the 3-day period.

Veronique Jestin. For environmental samples, have you determined the minimal number to collect on a statistical basis in order to declare that the environment is now not infected? And if so, what's that number? **David Suarez.** No, we haven't looked at that. I mean, the statisticians will tell you, at least for animals, how many clinical samples that you have to collect to determine if a flock is infected. So you might estimate the number of environmental samples to collect based on the numbers of animals present. However, I don't know of any way of showing statistically how many samples need to be taken to ensure that an environment is free of viral contamination. I don't think it's quite the same as looking at the individual animal. And so, no, we really haven't done that.

We certainly sampled areas that we thought were likely to be positive. For example, we've done studies in the past looking at different areas in the market, which was covered by an earlier paper. For example, we looked at the office in the live bird markets because they weren't disinfecting the office. We suggested that this might be a place where the virus could hide out. But when we tested the offices, we had very few or no positive samples. So we concluded that the offices were not a major risk factor. So we are taking samples based on previous research and using an educated guess for the correct number of samples to take.

Anonymous. Are the poultry companies that ship hatching eggs to Delmarva fumigating them? Do they send baby chicks too? **Bruce Akey.** No baby chicks, just hatching eggs are going as far as I know. I don't know if there is additional fumigation done before they are shipped.

Eric Gonder. Are eggs from infected broiler breeders moving out-of-state? **Bruce Akey.** No.

Anonymous. Has vaccination been considered to stop the spread? **Bruce Akey.** The USDA did grant approval for the use of an existing H7N2 vaccine in turkey breeder replacements only. However, segments of the poultry industry, concerned over trade implications of the use of vaccine, have made it clear to the state that they do not in fact want vaccine used at this time.

Conley Byrd. What size quarantine zone are you using? Are breaks within company farms? Or are adjacent farms involved? Have any backyard birds been infected? **Bruce Akey.** We've quarantined the infected premises. We draw a 1-mile circle around those and test in that area looking for additional

infected farms. As far as adjacent farms coming positive, there have been some of those. But in reviewing the outbreaks so far, we're not seeing a pattern that suggests that airborne spread is a big contributor at this point. The way we're seeing it pop up here and there so far looks like it's probably people and equipment that are moving this thing around.

And the backyard flock issue: we have tested a few that were immediately adjacent to some of the earlier houses that broke, and they were negative. We have not found any infected backyard flocks yet.

Carlos Romero. What was the time elapsed between the day that the problem was identified and the day of diagnosis of AI? **Bruce Akey.** March 7th was the day that the company vet went to the farm where clinical symptoms were reported. He took samples that day and we tested them here and they were AGID negative. The samples were sent into NVSL for virus isolation. On the 11th, additional blood was pulled and sent into our labs and it was AGID positive by the following morning, the 12th. So we had our first indication on AGID on the 12th of March. And on the following day, the 13th, NVSL reported the results of virus isolation as being positive and was able to type it as H7N2. So approximately 5 days between the first clinical symptoms and the first lab confirmation of AI.

Josh Hatkins. Given the density and proximity of the farms in Hong Kong, why did you just depopulate on a farm-by-farm basis—particularly since your second cluster was only 5 km from the index case? **Dr. Sims.** Initially, we had a quarantine zone which we did depopulate eventually, because most farms in that zone became infected. When infection occurred at a second site some 5 km from the first site, we decided to monitor developments closely and, in the end, the result has been successful in that only four farms were found to be infected in the second site. One of the problems we face in Hong Kong is deciding where to draw boundaries for quarantine zones because all chicken farms are close together. It comes down to a decision as to whether you quarantine and/or depopulate virtually all the farms or you concentrate on a smaller number of farms. In this case we chose the latter approach.

Dorothy Geale. One of the control methods was to retest the market. What was the sensitivity for this test for early infections? Or with flock negative serology is this concern negated? **Les Sims.** Whenever we are doing testing, we're doing blood testing. But we recognize that serology is not the most sensitive test for this in terms of picking up early infections. But

we're also looking for dead birds. If we find dead birds then we'll do Directigen tests on those birds. If we find anything in those, then we would stop that consignment, hold it back, and retest it to find out what was going on.

Kelly Preston. How are birds identified for the health certificate requirement? **Les Sims.** These birds are all coming from one farm in one truck, so we know how many birds have left the farm. The farmer has to call up to say how many birds he's actually going to be delivering to the wholesale market on that day. And that determines the number of birds that could arrive in the market from that farm. Because the poultry are in cages coming on one single vehicle, and the vehicle can only pick up birds from one farm, we therefore know where the birds have come from.

Nancy Cox. Has the inactivated H5 vaccine in use in Hong Kong been shown to provide protection against viruses bearing the A/Goose/Guangdong/96 hemagglutinin? **Dr Sims.** Not yet. However, we anticipate that this vaccine will provide cross protection, based on work done in Dr. Swayne's laboratory using other AI vaccines against Hong Kong H5N1 viruses.

Conley Byrd. How were birds disposed of that were destroyed? How long after destruction before a farm may repopulate? Have you established "triggers" for pre-emptive slaughter? If so, could you share them? **Dr Sims.** Birds were disposed of by burial at landfill in purposely dug trenches. Birds were placed in plastic bags for transportation. Farms could reopen once they have thoroughly cleaned and disinfected their farms and met new biosecurity requirements. This will require at least 1 month. The trigger for slaughter in this outbreak was the presence of HP H5N1 virus.

Andrea Miles. For your dead birds monitoring, how did you coordinate bird pick-up so that you did not create a biosecurity risk? **Dr Sims.** We mobilized a very large team of people and vehicles to minimize the number of farms that each person visited. We instructed those entering farms how to avoid spreading infection and also provided them with appropriate protective clothing and footwear. Personnel visiting farms avoided contact with live poultry and did not enter sheds where birds were raised on litter. The possible risk of cross contamination had to be balanced against the need to determine the exact extent of infection. This could only be done by doing tests on dead birds from all farms.

Paul Selleck. Could you please elaborate on the different genotypes described in your presentation? **Les Sims.** This information will be published at a later stage by Dr. Guan Yi and the team from the

University of Hong Kong. Briefly, there were seven distinct "genotypes" detected in Hong Kong during the 2002 outbreak. Six of these were detected in birds in retail markets. Two of these six were also found in local farms, and one was only detected in local farms. The differences in gene sequences indicated that we were dealing with multiple incursions of virus into markets and farms.

Anonymous. Will disinfectant label claims now have to have new directions for AI or whatever in light of the new discoveries of PCR? **Dr. Suarez.** No, I don't think so. I mean, the label claims will remain the same, since they refer to inactivation of the virus. The RRT-PCR test will be particularly good for testing animals during outbreaks like in the LBMs, but it isn't really applicable for testing environmental samples, because it can't distinguish live and dead virus. So no, I don't think it would have any impact on label claims.

Howard Pharo. Have there been definite links shown to exist between live bird markets and the commercial poultry farms by which infections have shown to pass back through the farms? **Dr. Suarez.** Yes, as we had mentioned yesterday, the molecular epidemiology looking at the sequence of the hemagglutinin gene has shown that the same viruses that we've been seeing in the live bird market in 2001–02 are related to the outbreaks in Virginia, North Carolina, and the outbreak in Pennsylvania at the end of 2001. So the molecular epidemiology demonstrates that a link exists. However, it is my understanding that a good direct classical epidemiological link between the Virginia outbreak and the live bird market systems has not been documented. So we're not sure where the original virus introduction occurred from, but we certainly suspect, based on the sequence information, that there must have been some sort of contact involved. **Bruce Akey.** No, we don't have a definite link at this point in time. We know that there are people in Virginia that do ship birds to the live bird market. Not from the commercial poultry companies themselves, but we do have people that raise ducks and geese and some chickens and so on that do ship to the live bird market. But we don't have a definite epidemiological link for this particular outbreak at this point in time.

Dorothy Geale. Is Virginia ordering destruction without compensation/indemnity from USDA? Or is there state- or industry-based indemnity? **Bruce Akey.** At this point there is no indemnity from USDA or from the state or from the industry. I was shocked as anybody in the room when our Attorney General said that we had the authority to do that.

There is no indemnity. There is no promise of indemnity. However, there are possibilities for indemnity both from USDA and the state. We have had situations in the past, on much smaller scales, with cattle and tuberculosis, where the farmers involved got some indemnity from the USDA or the state and then were able to go the state legislature and get additional monetary relief. But at this point in time there is no indemnity being paid on these birds. And nobody is being promised any.

Trisha Marsh-Johnson. *How many integrators are currently known to have positive flocks? Do the 79 flocks include any in North Carolina?* **Bruce Akey.** The 79, the number 79 is just Virginia premises. As to how many integrators are involved, there are five companies that operate up here in the valley. Four of the five have had at least one positive farm so far.

Zheko Kounev. *Do you have data or an idea of how the virus has been spread?* **Bruce Akey.** Looking at the spread on GIS maps, it looks like there is the possibility of airborne spread, but it looks more likely like it's being spread by the movement of people and equipment. I'm sure anybody that's from a heavily poultry populated area knows that in general the poultry growers, they or some other member of their family, may not only be involved in the poultry houses but may also be involved elsewhere in the poultry industry; at the plant or driving a feed truck or helping their brother-in-law or whatever. So there are plenty of opportunities for people to have moved this thing around on us.

Guus Koch. *How is manure disinfected and disposed?* **Bruce Akey.** Currently we're using the same protocol that North Carolina is using. After depopulation, the houses are closed up for 2 weeks and not touched. At the end of 2 weeks, the litter is either composted in the house or outside on at the farm before it can be spread. The house is completely cleaned and disinfected and inspected by one of our state inspectors before the house is released. In broiler farms that have the capacity to heat up the house, the houses are also being heated up to about 100 degrees for 2 days.

Klaus Stöhr. *Could blood samples be taken from depopulation teams to look for avian influenza antibodies?* **Bruce Akey.** We've talked with our Department of Public Health about this issue and I know that they have heightened their surveillance for any flulike illnesses. The Virginia Department of Health is proposing to do such a study in collaboration with the CDC.

Guus Koch. *Your internal [positive] control is probably a plasmid. What measures did you take to prevent false positives because of [potential cross] contamination of the live bird market samples.* **Erica Spackman.** First, our positive control is actually *in vitro*-transcribed RNA. And we don't go to any special measures with the PCR for the prevention of contamination in the way we handle the samples other than that we keep everything separate (samples, controls, and reagents). So it's basically a physical or logistical separation.

Guus Koch. *Do you test the samples from the live bird markets in duplicate or did you retest some? Can you elaborate on the reproducibility of the tests?* **Erica Spackman.** For the general testing, no. We only test each sample one time. As for looking at the reproducibility, most of that's been done with *in vitro*-transcribed RNA, and some with the live bird market samples, but it's been done separately from actually producing the results for the test a (clinical samples have been used for reproducibility testing). But when implementing it (the test) diagnostically, we just test everything once. **David Suarez.** I have one additional comment to that. Some of the samples are being tested multiple times because the positive samples are being retested with the H7 and H5 test. Almost all the samples from the live bird market samples also test H7 positive. So we have an independent test that correlates the results from the original test sample. Of course, if it's negative on the first test, then we aren't going to retest it with the H7 test. We wouldn't expect any additional positives from the subtype-specific test, since the sensitivity of the matrix test (the type A test) appears to be better than the subtype-specific test. However, one concern is the possibility of a false-negative test because of PCR inhibitors in the sample. That was part of the justification for a multiplex test using an internal control.

David Swayne. *In the 2002 outbreak, you indicated no human infections, presumably hospitalized cases. How about serologic evidence of human infection without disease?* **Les Sims.** Hong Kong has a very intensive human influenza surveillance program, based on throat samples from people with influenza-like illnesses seen at outpatient clinics and private medical clinics. No H5 viruses have been detected in this program since 1997. The table of results for year 2002 can be found at the following web site: <http://www.info.gov.hk/dh/diseases/index.htm>. Serology was not done.