FTS-CDC-PHPPO

March 9, 2004
12:00 p.m. CST

Coordinator — Good afternoon and thank you all for holding. I would like to remind parties that all lines have been placed on a listen-only mode until the question and answer session of today's conference. Also, today's call is being recorded. If you have any objections, you may disconnect at this time.

I would now like to turn the call over to Ms. Shelly Langford. Thank you, ma'am. You may begin.

S. Langford — Good day and welcome to the 2004 Public Health Teleconference Series on Infectious Disease. This is Shelly Langford, State Training Coordinator at the Washington State Department of Health Public Health Laboratories in Shoreline, Washington.

Today's teleconference is being hosted by the Washington State Department of Health and sponsored by the National Laboratory Training Network, in cooperation with state public health laboratories.

Welcome to our teleconference, "Serologic and Molecular Amplification Assays for Arthropod-Borne Viruses". A few notes before we begin: After the program, each participant needs to register and complete an evaluation form documenting your participation. It helps us to continue to bring high-quality, cost-effective training programs in a variety of formats. To do this, go to www.phppo.cdc.gov\phtnonline. The verification code is Arbovirus. Again, the Internet address is www.phppo.cdc.gov\phtnonline and the verification code is Arbovirus.

When you've completed the registration and evaluation form, you will be able to print your certificate of attendance. You have until April 9th to complete this process. These instructions are in your original confirmation letter and the general handout. They were also e-mailed to each site representatives this morning.

At the end of this program, if time permits, we will open a brief question and answer period. Again, we're on a listen-only line. We cannot hear you. You can only hear us.

Again, welcome and thank you for joining us. We have 50 sites from across the United States listening to this teleconference. Today's speaker is Dr. Robert Lanciotti, Section Chief of the Diagnostic and Reference section, Arbovirus Diseases branch of Centers for Disease Control and Prevention, who speaks to us today from Fort Collins, Colorado.

Dr. Robert Lanciotti received his bachelor's degree in chemistry from Loyola College in 1982. In 1983 he was employed as a research chemist within the virology division of the United States Army Medical Research Institute for infectious diseases in Frederick, Maryland. His research involved the antigenic and molecular characterizations of arthropod-borne viruses, with emphasis on novel approaches to vaccine development.

In 1985, Dr. Lanciotti began working at Maryland Medical Laboratories in Baltimore, now known as Quest Diagnostics. Between 1985 and 1989 he designed immunologic and molecular bases assays for human

immunodeficiency virus, Human Cytomegalovirus, Hepatitis B virus, and human Papilloma viruses.

During this time, Dr. Lanciotti also completed his Master's of Science degree in biomedical sciences at Hood College in Frederick, Maryland. In 1989, Dr. Lanciotti began working at the division of vector-borne disease infectious diseases of the Centers for Disease Control and Prevention in Fort Collins, Colorado. His research involves studying the biology, evolution, and phylogeny of arthropod-borne viruses.

He also developed molecular amplification-based diagnostic assays for the rapid detection of the arthropod-borne viruses for use in CDC's diagnostic laboratory. Concurrent with his work at the CDC, Dr. Lanciotti also completed his PhD in microbiology at Colorado State University in 1994. In January 2000, Dr. Lanciotti was appointed chief of the Diagnostic and Reference Laboratory at the CDC, where he is currently employed.

It is my pleasure to introduce to you and to welcome our speaker, Mr. Lanciotti.

Dr. Lanciotti Thank you for that very kind and lengthy introduction. I had forgotten half of those things that you said I did, but it's a pleasure to be here. Once again, thanks. What I'm going to try to do is remember to tell you which slide I'm on, so that we can all stay together. So right now I'm looking at the first slide, the title slide, and just to point out that what I'm going to attempt to do in this presentation is to talk about the neurologic and molecular assays that we use for arboviruses.

Most of the time I'll talk about West Nile virus, because that's obviously the virus we've spent a great deal of time working on. However, the types of tests that are used are really identical. For serological, we may switch a monoclonal antibody here or there, and for molecular we'll switch primers. But really the template for the kind of test that we do really remains the same, so I'll do that.

I'm going to talk, in the beginning of this talk, a little bit about arboviruses in the United States, just to sort of set the table for what's out there, what patterns do we see, and so forth.

Let me go to the second slide. This is just a summary of the medically important arboviruses that are found in the United States. It's certainly

not a comprehensive list. There are many other arthropod-borne viruses that are out there, but I just want to list the important ones here, the ones that we are occupied with testing most often. The first two there are Eastern and Western Equine encephalitis, or alphaviruses; they are in the genus alphavirus, single-stranded RNA viruses.

Flaviviruses, just below that, are the two that we're most often concerned with is St. Louis encephalitis and West Nile virus, although there is occasionally some dengue that comes across the border that we are involved in testing, as well as testing for dengue and travelers. Powassan is a tick-borne flavivirus. It's very rare, in terms of finding it in humans, so it's listed there, but, again, not of great concern to us.

Finally, in the bunyavirus genus, the most often encountered virus that we deal with LaCrosse encephalitis, primarily disease of children and young adults, but as you can see there are several other bunyaviruses, Jamestown Canyon and so forth. Those, to a much lesser extent, are associated with human illness.

So the next slide, just to expand a little bit, will talk about the distribution of these viruses. As you can see, eastern equine encephalitis, we consider

it primarily a disease in the eastern part of the United States, east of the Mississippi River in general. You can see I just want to point out that not a huge number of cases when you look at this 38-year period from ’64 to 2002. We haven’t updated these slides yet, but essentially the pattern really hasn’t changed, about five cases a year of eastern equine encephalitis. We really don’t see large epidemics of eastern. We just see this kind of steady state of less than ten cases per year, most of the cases coming from the southeastern part of the U.S., from Florida and Georgia.

Western equine encephalitis has a slightly different pattern. You can see that it’s similar to eastern in the sense that there is this steady state of about eight cases per year. However, there are epidemics of western. We haven’t had one in quite a while, but as you can see 1964 to ’66 epidemic and the ’75 epidemic account for 65% of all the cases that we have of western. We actually have not seen any cases since 1997, I believe, and there have only been four since 1990, so we really don’t see a lot of western equine encephalitis human activity.

The next slide is LaCrosse encephalitis. Prior to West Nile, this was actually the most often-encountered arbovirus infection of humans in the U.S. You can see also 3,000 cases, a much greater number of cases per

year, 76 cases per year than compared to the alphaviruses. Again, primarily young children, young adults disease can cause encephalitis. There is a fatality associated with it, so this is a virus of concern to us, primarily in the eastern part of the United States.

Next slide is St. Louis encephalitis, which is a flavivirus very closely related to West Nile. You can see here a much greater number of cases than, again, compared to some of the other arboviruses. St. Louis really does have epidemic cycles. There are really years and years of virtually almost no activity. We'll detect a handful of human cases in these off years, but it has the potential for very large epidemics.

The last one, the largest one on record is the '75, '76 SLE epidemic, which took place kind of in the Mississippi Ohio Valley, in that region of the country. You can see that about 50% of all those cases of St. Louis can be accounted for during those epidemic years. If you were to look at the history of St. Louis, you'd see, again, very little activity for a number of years and then a spike of an epidemic that occurs, and then it disappears again. So, as we stand today, we're in a time - we have not seen a real large SLE epidemic since the mid-'70s.

Now let's talk about West Nile. I'm going to give a little more detail, since this is of great interest to everyone in the current state of affairs in the U.S. Just to review, as you can see, in '99, we have five seasons of West Nile that we've dealt with. In '99 we really only caught part of that epidemic, but, as you can see, 62 cases really confined to the New York metropolitan area.

The virus then spread, the following year, to a few more states, but really kind of a quiet year compared to '99. Two thousand and one was not really a huge difference in terms of the number of human cases, although virus activity was detected in a lot greater number of states. Two thousand and two was when things really took off. As you can see, we jumped to over 4,000 cases, 39 states involved in reporting human cases.

In 2003, up almost to 9,000 cases and almost year-round activity from the first case in the end of the March, all the way through the end of November. So, essentially year-round activity of West Nile, although most cases obviously occurring in the summer, late summer months. We're now at a point where, in terms of human cases, every state in the continental U.S. has reported human cases except for two.

The next slide is a theoretical image that we put together here, based on a lot of work we've done, both with looking at human activity, looking at data that's coming to us from the blood banks, where we're seeing a large number of infections, and we can follow some of these people. This is going to get refined as time goes on, this human infection iceberg.

Essentially things are holding up according to this model, as we're coming to learn. About 80% of people that are infected with West Nile remain asymptomatic. About 20% develop what is referred to West Nile fever, which is a syndrome of a fever, myalgias, and, in a good percentage of cases, a rash, but it's self-limiting and not really of great concern.

Then we move on to central nervous system disease, which is about less than one percent of the time. In that category there are case fatalities. This will be refined as we get more and more data from blood donors. Again, there were a lot of people that donated and were infected. Blood was screened. We can follow these people and find out what occurs following to their infection.

So the next several slides are just to give an image of the spread of West Nile virus in the United States. Again, the first one here, 1999 virus. Now

this encompasses all activity, not just human cases. So this would be human cases and horse cases, bird surveillance, mosquito surveillance. So basically the next few maps are showing anywhere that West Nile has been detected throughout the country.

So, in 2000, on the next slide you can see the virus spread to New York, New Jersey, and to Maryland and to North Carolina. In 2001 some activity really all throughout the eastern part of the United States. Two thousand and two, again, is corresponding with the other table that I showed where there was a great epidemic of human activity. See here now we've got virus activity of some kind throughout most of the United States. In the next slide, in 2003, continued spread of the virus and, again, a large number of human cases.

So the next slide talks about some of the surprises that we encountered in 2002 that coincided with this very large epidemic. We discovered that West Nile could be transmitted through organ transplantation. This was documented and reported in the literature, but essentially there was a donor, a West Nile-positive donor that, subsequent to dying, donated two kidneys, a heart, and liver and all four recipients were infected, with a fatal outcome in one case.

We also discovered that West Nile can be transmitted through blood transfusions. I'll have a slide to show a little bit more detail about this, but basically, in 2002, we followed a large number of suspected transfusion-related cases. We confirmed 23 of those. We believe there were probably many more out there that we never heard about, but obviously of great concern to public health. The FDA became involved, and private industry, and a lot of things developed subsequent to this discovery to begin a process that we now have of screening all blood products.

We also discovered that West Nile could be transmitted through breast milk. There was one case that we confirmed. We've subsequently found the virus in a few other breast milk samples and placental transmission. This is still an area that's very young with research. There was one case reported in 2002. There is a very large study that the CDC is involved with this year, looking at women that became infected with West Nile during pregnancy and we're following up those as we speak now. We don't have any real large amount of data on that yet, but basically we'd like to find out can the infection occur in utero and what would the outcome be.

Finally, some occupational exposure, lab workers, turkey farm workers, alligator farm workers, so people that are in places where there's a lot of West Nile virus being secreted, either in a laboratory environment or in other environments. There were several infections in that mode as well.

So the next slide, just again, just to expand on the blood donor, this has not been updated very recently, but you can see that in this past season, 2003, a lot of blood donors. In fact, I think we're approaching 1,000. We may have exceeded 1,000 adjacent to the mountain states, Colorado, Nebraska, South Dakota, and this is a result of a massive effort where the Red Cross and the American blood centers this season began screening all blood products by a molecular amplification assay for West Nile and then obviously discarding infected units.

The data, one of the values of this screening, in addition to the public health implications, is that we're getting real-time data, looking at where viremic blood donors are. So this map just shows where those were discovered this year.

So now I'll talk about, really, the bulk of what I want to cover, which is the various diagnostic assays - this is the next slide - the various diagnostic

assays that are used for detecting West Nile virus infection. The assays, as in many approaches in other systems, can be divided into serological assays and virus detection assays. I'm not going to go through all these. I just wanted to list all the ones that we're aware of here at CDC. Then I'm going to talk about how these are applied in some subsequent slides.

But, as you can see, the main frontline tests are the IgM and IgG ELISAs that were first developed here at CDC. Subsequently, these have been slightly modified and are now being marketed by Focus and Pan Bio. To my knowledge, both of those companies have FDA approval for their test kits. There are some other assays listed below there and I'll be happy to answer questions about those later, but I won't really go into those.

For virus detection, there is a CDC real-time florescent or TaqMan assay that's been developed here that's being used by a lot of the state labs. There is also a commercial version of the TaqMan and another type of test called TMA. Now those are the tests that were developed specifically for screening blood products. Again, I have listed all the different various virus detection assays, all the way down to things like virus isolation and antigen detection.

So let me go to the next slide and talk about the way that we test, and the algorithm that we use. From this table, basically what I have tried to do is divide the kinds of testing that occurs into surveillance as opposed to diagnostics. On the left-hand portion, I'm talking about the surveillance that is being conducted by many of the public health labs. Basically, if you're looking for the virus in nature, the two places that you can most readily detect virus activity is either through dead birds or through mosquito surveillance, so that's really what I'm talking about there.

I'm not really talking about live bird surveillance, where you're looking for antibody in live birds. That's really another topic, but dead bird testing and collected mosquito pools and testing those, and looking for virus in those sample types.

For bird surveillance we began by looking at tissues, looking for virus in tissues, but subsequent to that several people here at CDC and others throughout the country have found that if you do either oral or cloacal swabs of dead birds, there is a sufficient amount of virus in most of these dead birds. It can be detected without actually doing a necropsy. So, that's probably been a change over the years. Your ability to detect virus in dead birds, the sensitivity will drop a little bit if you don't test the

tissues, but birds in general, if there's enough of them in an area that are dying, you'll pick up the virus eventually, so those are the options you have. You can either test tissues or you can test swab.

The tests that we use both for looking for virus in mosquito pools or in these oral swabs or in tissues, the most sensitive tests that we found are the molecular amplification tests, either TaqMan, RTPCR, or the NASBA test. I'll, again, talk, in some more detail, about those tests.

Other things that are available are just standard RTPCR, where you would run …gel at the conclusion. Isolation in viro cells is an option. There is also something called the VecTest that some of you may be familiar with, which is really a dipstick antigen detection test. As you drop out of the molecular amplification assays and go into things like RTPCR and isolation and the VecTest, sensitivity does decrease a little bit so just bear that in mind. So, again, those are the tests that we primarily use for detecting the virus in these environmental samples.

Now, if we go over to the right-hand part of this table for human diagnostics, our frontline test is looking for antibody. I have a slide that I'll show you in a minute explaining why we look for antibody as our

primary test. It's basically because by the time people are clinically ill, in most cases, you cannot detect virus circulating in serum. So, the antibody test is really the preferred and most sensitive approach to looking for West Nile infection.

The sample types are either serum, plasma, CSF. Again, our frontline tests are the IgM ELISA, because it's the acute antibody and we want to look for acute infections. We have an IgG ELISA and we have plaque reduction neutralization, which serves as a confirmatory test, as well as a specificity check on the reaction.

So let me jump to the next slide, and this is what I was just referring to. This is a summary of what we've been able to put together from looking at a lot of infections, as well as, again, the blood donor community, which has been a valuable resource to us to try and put together a theoretical understanding of what's happening.

This is pretty standard for many acute viral infections. There is a period of viremia. In the case of West Nile it really proceeds clinical illness. So the duration of viremia is really, at this point, not very well defined, but we do know that by the time people present with clinical illness, especially

central nervous system disease, that in most cases, by the time they present with illness, you really cannot detect virus by even the most sensitive test. We've done several studies. In serum it's somewhere around 10% of the time you can detect virus at clinical illness. So it's not a very useful way to look for viral infection.

However, virtually everyone is IgM positive by the time they present with central nervous system illness, so the IgM test is obviously the preferred test for human diagnostics. IgG antibody, which really corresponds with neutralizing antibody, appears shortly after IgM, usually within about seven days.

The other thing to look at here is the viremia is relatively low. The highest copy number sample that we've detected is around 250 plaque-forming units for ML.

So the next slide shows our algorithm for our serological testing. In our first, we start with human serum or cerebrospinal fluid and we do the IgM ELISA for both West Nile and St. Louis encephalitis. I'll explain in a minute why we test for both of those. Then there is the IgG ELISA, which is available. Again, I'm going to make a few comments about the utility of

that test. But essentially if it's negative we're finished with the sample, if it's been appropriately timed. If it's a sample that's very acute, we may ask for a follow-up so we can do acute and convalescent.

So if it's positive, we then have to confirm that it is West Nile and not another flavivirus, because of the cross-reactivity. We use the plaque-reduction neutralization test with a minimum of two viruses for St. Louis encephalitis, and West Nile. I'm going to have a slide in just a few here that'll talk about the cross-reactivity and why we need to do the neutralization test.

So on the next slide the question is, well, why run the IgG ELISA? What kind of information can you get from it? Actually, it's not the most useful test when it comes right down to it. There are certain situations where it's very helpful to run the IgG. Basically I'll jump right to the bottom here. We use it here for early season testing where West Nile has not been identified in a particular geographic area and any other special case, things where we might suspect a secondary infection, or anything that's out of the ordinary.

But once West Nile has been confirmed in a particular geographic area and there have been a number of human cases, we really drop the IgG test, but I've listed in the bullet some of the reasons why an IgG test is valuable.

The first is there are going to be secondary flavivirus infections and you can use the IgG test, for example, if you have an acute specimen, a day-one specimen, and we've had these, that are IgG positive, IgM negative, and then a week later IgG discontinues to be positive; IgM is now positive. So what we've been able to show in those cases is that this person had a preexisting flavivirus infection and then got a West Nile infection on top of it. So the IgG is valuable in that case.

The others are listed there and it helps us confirm IgM results, and it also can tell us about, again, a previous flavivirus infection. Sometimes people will have IgG positive, IgM negative, and on the follow-up will remain that way. All that tells us is that they had a flavivirus infection at some time in the past that's really unrelated to whatever clinical condition they're experiencing. So those are some situations where you may want to run the IgG test. But, as I mentioned, we drop it once we get to a situation where we know there is a lot of West Nile activity in an area.

So, on the next slide is just an overview, a picture of how this IgM ELISA works. I think probably everyone is familiar with the basic IgM capture approach. We … plates with an anti-human IgM. We capture M in the next step. We then add recombinant, West Nile recombinant antigen, and then we follow that. We have patient serum, then the antigen, then we follow that with our perox based labeled conjugate.

The next slide talks about how we process the results. We calculate P over N, which is the optical density of the patient serum divided by negative control serum. This is a fairly standard approach in terms of defining positivity. In our standardized assay when we have P over N of greater than three, we consider that positive, less than two is negative, and the two to three range is equivocal. What we do with all of the equivocals and positives is we do follow-up testing.

Come to the next slide. This is just to show you the kind of results that we get in performing the IgM ELISA on - these are all four confirmed West Nile cases. As you can see, their P over N is, again, greater than three is positive, for West Nile they're all fairly high, 16, 17, and so forth. But you can see that there are other positive results with these other

flaviviruses that we're testing, for example, Japanese encephalitis, St. Louis encephalitis. Every one of these samples is positive not only for West Nile, but also for those other two related flaviviruses.

This demonstrates why we have to do the neutralization test in certain cases, because, based on the ELISA alone, we can't be absolutely sure that it's West Nile. Some of this has been modified and I'm going to talk about that in a slide or two about how we can actually use P over N value to predict whether it's West Nile.

This is actually the result of the poly - the test itself uses a monoclonal antibody and is set up to be very specific. What we're seeing here is that in the polyclonal humeral immune response that people have to West Nile, the antibodies that are produced in infected individuals will cross-react with these other viruses. So we need to do a follow-up test.

Let me jump to the next slide. Again, this is a typical result that we would get. Now when you look at this from the far right you can see the neutralization results. The two things that are critical for us in confirming a true West Nile case is that in the acute serum we have a neutralization titer of one to 80; convalescent serum is one to 1280. So we're seeing a

large rise in the neutralizing antibody titer between acute and convalescent. So that tells us that this is a West Nile infection.

The other thing to look at is the difference in titer between West Nile and SLE. We use the cutoff of four-fold difference. A four-fold difference in titer, or greater, tells us what the infecting virus was. So this is, again, our typical West Nile case. When we have all of this data in front of us, it's pretty clear that this is a recent West Nile infection.

But one of the things we observed - and I alluded to this - is that you can use the P over N value from the IgM ELISA in and of itself to be predictive about whether this is a West Nile infection or not. Denise Martin here published a paper back in 2002 showing that in primary West Nile cases the P over N, when you compare West Nile to SLE, it's two to five times greater than SLE.

Now we've expanded on that. In the next slide you can see that in this past season we looked at a much larger number of IgM-positive samples. We broke this down. We basically wanted to see at what point can we have - what degree of confidence can we have at each of these data points as to whether this is West Nile or SLE. So, as you can see in the top row,

when SLE is actually greater than West Nile, you can see that the higher percentage of them are SLE cases than West Nile.

But, as you look at the radio of West Nile to SLE activity, as it goes from two to three to four and so forth, the percentage of West Nile cases increases and the percentage of SLE cases decreases. So that by the time if your ratio West Nile to SLE is four to five times greater, 97% of those are West Nile cases and less than one percent are SLE cases.

So what this tells us is that if you've confirmed West Nile in a particular area and you do the IgM ELISA for West Nile and SLE in the same run, in the same day that you're doing this ELISA, by comparing these P over N to West Nile and SLE, you probably don't need to do the neutralization test on every sample, because if the difference is like four to five or even three to four, you can be reasonably sure that this is a West Nile case and not an SLE case.

So this is the way we actually do our testing here now. Once we have detected a lot of West Nile activity or some West Nile activity in a particular area, if we get a very clear-cut difference between the West Nile

SLE and IgM test, we won't do the neutralization test on that unless there are some unusual circumstances as to why we need to do it.

Let's go to the next slide. Very quickly, it's one of the problems of the IgM ELISA, just to point out; again, this is probably not new information to any of you. Basically, in one study that was conducted here in conjunction with New York State, you can see that the IgM to West Nile appears to persist for quite a long time. You can see in the 300- to 400-day range post infection, about 50% are still IgM positive with P to N that are reasonable, from three to 6.5.

Now this was a small study, and one of the things we're trying to do now is look at a much larger number of samples. We're looking at some of the blood donors and we're going to try and answer the question, you know, is this a universal observation? If you look at a large number of West Nile-infected individuals, do we see the persistence of IgM beyond one year?

Obviously, if that becomes generalized observation, that has significant implications for diagnostic testing, because it would be theoretically possible to pick up an IgM positive in 2003 that may have been a 2002 infection.

The next slide, actually, is some data that we picked up, showing exactly that. You can see here we had an acute day-seven and a day-25 specimen. One of the things that should strike you as unusual is, if you look between the acute and convalescent, the IgM P to N stays about the same. Everything looks fairly stable. When you do the neutralization test, again, the titer does not change at all. So that's a clue that this may be not a recent infection.

In fact, one of the things we've developed here is an IgA ELISA, which is really identical to the IgM test, but instead of capturing N in the very first step, you capture IgA. Following the subsequent protocol is the same. But Jane Johnson here is in the process of publishing this work where what she's showing is that, in a reasonable sample number that she's looked at, I think about 70 or 80 samples, that IgM does persist.

However, there are no IgA positives after day 51. So it may be that IgA is a better marker of acute infection. So it's a test that we hold in reserve here. We don't screen every sample with it, but we're still evaluating how useful this test may be for determining acute infections.

So the next slide is to point out another issue that we occasionally, in fact, it's fairly rare so far, of secondary flavivirus infections. Historically this has been a big problem in other parts of the world where viruses like dengue circulate, but without going into a lot of detail, basically if you have a flavivirus infection followed by another flavivirus infection, it's very difficult to determine what that second virus is.

You can see, looking at these two cases, the problem is that in a secondary flavivirus infection there is this broad neutralizing antibody response, high titers to a lot of different flaviviruses, and, in fact, the highest titer that you obtain in that secondary infection is often not to the recently infecting virus. In many cases it's to the first infecting virus. So it's very difficult to figure out what's going on in a secondary infection. It has not typically been a problem in the U.S., because there's been minimal flavivirus activity, but I think this is going to be an issue as the virus persists here and as the virus moves into Central and South America.

So, the next slide is just a very quick summary that talks about what we've learned from our serological test, how quickly IgM appears, the relative reactivity between West Nile and SLE, and I won't really go through

these, but this is for your reference. It's, again, just a summary of the issues with serological testing.

The next slide talks about reagents and availability. As I mentioned, the IgM and IgG kits are now commercially available from Focus and Pan Bio. They've been FDA approved. West Nile antigen, if you choose to put together your own in-house test, some state labs do this. You can get the antigen from Focus. We no longer distribute it here, because we are in, to be honest, I don't understand all the details. It's something to do with a license agreement that once we license Focus to product it, we can't give it away for free.

So Focus has that antigen. They're selling it this year. We don't know if they're going to continue to see the antigen indefinitely, because they now have an FDA-approved kit. My understanding is they're going to continue to sell it for at least a couple of years.

Hennessey Research is another company that's licensed. I think we've actually licensed the antigen to a number of companies and we hope that other companies will produce it and sell it. The SLE antigen still comes from us, but we're not far from having a recombinant antigen, which will

then be turned over to commercial entities so that they can produce it and distribute it. The conjugate and coating antibody for the IgG test are still available from us. At the bottom I've listed the Web site. We now have a Web-based ordering system for reagents, as many of you probably know.

I'm just going to skip this next slide, because I can tell that I'm running a little bit short on time, but just to point out that we still do training and technology transfer here. We have a proficiency panel project where we send out a proficiency panel every year. I think, again, most of you have participated and are aware of it. But if you're not, contact us and we can include you in this year's panel.

Some future directions on the next slide. We're trying to automate as much of the ELISA as possible and we're looking at things like reagent stability, incubation times.

I'm going to talk now, on the next slide or two, about our Luminex assay, just to talk about what we've accomplished here and where we hope to go with it. So the next slide is just an overview of, again, it's a microsphere-based assay. Most of us know it as Luminex.

Jane Johnson here at the CDC has worked very hard on this and has presented some of this data already. But, again, the advantages, as shown on the lower left-hand side, is time, really that we can do this test much quicker than the IgM ELISA, which really is almost a two-day test.

This is a very quick test and you can multiplex. You can take one sample, react it with several different antigens and look for antibody reactivity to several different viruses in the same test sample. So there are a lot of ways that this could be accomplished.

You can couple the antigen directly to the beads or you can capture … like we do in our ELISA, but it turned out that the way that it seemed to work best for Jane here was she actually coupled a monoclonal antibody to the beads that the 6B, 6C one is a flavivirus group reactive monoclone. So what we can do then is we can actually capture different flaviviruses on different colored bead sets. So we can have a West Nile bead set, an SLE bead set, and perhaps down the road something like a dengue bead set, and on and on.

That's one of the nice things about Luminex is you have different colored florescent colored beads. Now these would be associated with particular

viral antigens. We can react our patient serum and we deplete the IgG out of that serum, because we're trying to develop specifically an IgM assay. If you put the sample as is, with these beads, most of what's going to bind is IgG. So we have these little columns that can deplete IgG from the sample and then we're looking just at M.

In a single reaction, we can look at relative reactivity of a patient's antibody to West Nile and SLE and we hope to accomplish the same thing that we've done with the ELISA, which is, again, to look at whether this is West Nile or SLE based on the differences in reactivity.

So the next slide is just some raw data showing that in the first two rows these are West Nile confirmed samples. You can see the reactivity, the florescent activity to West Nile is much, much greater than to SLE. Conversely, when we look at samples three and four, when they're SLE samples, the reactivity is much greater to SLE.

We're actually designing a system where we're going to have a computer-generated analysis of all the data, so we won't even be looking at numbers when we get this all complete. Jane has looked at, I think, over 1,000 samples to try and validate this approach. In the end, the computer will

tell us whether it's West Nile or SLE based on an algorithm that's going to be developed here.

So now the next slide, and the last portion of the talk, is on molecular-amplification assays. Again, this is looking for virus in things like mosquito pools and dead birds, as well as there are situations where we do look for virus in human clinical specimens, things like tissues from fatal cases. You know, I mentioned that we don't often find West Nile virus in serum in infected people.

However, in fatal cases, 100% of the fatal cases we've looked at, we have found West Nile virus in those tissues. So we do use it for human clinical samples as well. But, again, we're going to focus primarily on TaqMan and NASBA. Those are, by far, the two most sensitive tests. When we compare what's out there, we found that both TaqMan and NASBA are very, very sensitive and easy to use in the lab.

So the next slide is just a reminder about some of the safety issues. Now that we're looking for virus in samples, it is a BSL3 virus, so when we do things like ELISA, even though there is relatively no virus in the serum

samples, we treat them in the biosafety cabinet until we get to a point in the test where we know there could not be any infectious virus there.

But basically for any test where we're replicating live virus, like the neutralization test or virus isolation, we do the entire procedure in a BSL3 environment. Things like PCR where, at the initial stage, we're adding a lysis buffer that does actually inactivate a virus, we do that first step of inactivation in a BLS3 environment. Then we can transfer and do the rest of the RNA extraction in a BSL2 environment.

The next slide shows our sample preparation flow for all of our molecular-amplification assays. Basically we've got two types of samples. What we've chosen to do is we use a uniform RNA extraction kit from Kiogen. The starting material needs to be a liquid. Kiogen and other companies do make kits specifically for tissues and things like that, but we decided here that it would be easiest for us to use one type of RNA extraction kit for every sample, so it starts with a liquid. So in the case of things like serum or plasma or CSF, we can just to the extraction from that sample.

If we have things like mosquito pools or tissues, what we do is we create a liquid. We create a homogenate. The other reason for that is we can then

use that homogenate to isolate virus in viro cells. So, that's our standard approach.

If we have a solid sample like a mosquito pool or tissue, we create a homogenate. We can either do that manually on the bench top or we can use, if we have a lot of samples, we can use this instrument called the mixer mill from Kiogen. I'm sure there are other varieties available, but basically we combine the tissue sample with some beads. It's like a vortex mixer. Then we create this homogenate. We take the lisate. We treat it as a liquid and we extract RNA using either the bench-top extraction kits or, if we have a lot of samples, we have a bio robot that automates that whole protocol. In either case, it's the same chemistry whether we're using the robot or we're doing this on the bench top. The final step is we have RNA then available in water for us to do our molecular tests.

Our TaqMan testing algorithm, we extract anywhere from 100 micro liters of samples to greater than one milliliter. Really, that's been the only modification of our test over the years. We still use the same primer sets that were published years ago. We still use the same cutoffs, pretty much, but one change that we have looked is, as we've gotten into the arena of looking for very low copy number samples in the blood donor community,

we've altered our extraction so that we can extract RNA from even greater than one milliliter of starting material, so that increases sensitivity, obviously.

Our cutoff value for being positive is a crossing threshold of less than 38 to 45 – 45 is equivocal - and all positives and equivocals are then repeated with the second primer set using RNA that's been extracted from the original sample. We arrived at that cutoff basically by doing replicate testing of a large number of samples. We do 20 replicates. What we look for is where we lose 100% positivity.

Basically when you dilute a sample out, you get to a point where it's 100 every sample. Every replicate is positive. You get to a point where maybe 70% are positive, 50%, and so forth. So we've chosen here our 100% cutoff. So basically, if we have a sample with a CT of 37.9, we know that it would be positive 100% of the time. Some other labs will use a 50% cutoff. So, if we did that, our CT cutoff may be as high as 40, because in that range half the time we'll get a positive. Half the time we'll get a negative.

So the next slide, which I'll skip, is just what TaqMan RTPCR is, and it's been around long enough that I think most of us are familiar with it.

The next slide shows some data on the right, but basically where our sensitivity detection limit is currently. Our envelope set, in our hands and in the hands of many others that have used it, appears to be the most sensitive. We can detect down to about 0.1 PFU per mil or, if you prefer to think in terms of copy numbers, it's about 40 copies per mil. Our other confirmatory sets are not quite as sensitive, but they're listed there anywhere from 80 to 160 copies per mil.

The next slide is just to point out that we have an internal control that we include, and we do this because we want to make sure that if a sample is negative that it's negative because West Nile virus RNA is not there and not because there has been some type of inhibition. Again, this is a common practice in the arena of molecular testing.

As you can see, in the upper panel there are four positive samples. Those are our positive controls. This is actually a 96… plate of mosquito pools. From the lower panel is our internal control result. You can see that

whereas above the only thing that was positive were the four controls, when we look for our internal control every sample is positive.

Approximately, they're all positive to the same degree, which, again, tells us that the amplification conditions were fine. So all the negatives in the upper panel for West Nile are true negatives.

So the next few slides are just a quick look at NASBA, which we've explored here. It's basically, for those of you not familiar with it, it's an amplification method that does not involve cycling with heat and so forth. It's an isothermal reaction. Everything is done at 41 degrees.

The other key difference between this and RTPCR is that RTPCR we start with RNA. We make multiple double-stranded DNA copies. In NASBA you're starting with single-stranded RNA and the final product is a single-stranded RNA as well. It uses different enzymes, very robust amplification, and in some ways it's a little bit faster than TaqMan, because you don't have the cycle, the stepping through thermal cycles.

So the next slide, there are a couple of different ways you can detect single-stranded RNA that's been amplified by NASBA. You can either

use what's called electrochemiluminescent reader. This whole approach, by the way, is developed and marketed by bioMerieux, so if you want to do the ECL reader approach, they supply that.

The other option to detect the single-stranded RNA is to use a molecular beacon, which is a florescent primer with a quencher and a reporter at opposite ends. In the native configuration it forms a stem loop, so there is quenching of the signal. For that primer to bind to the RNA, or when it does bind to the RNA, it actually linearizes; separate the quencher and the reporter, and you get florescent. You can use either of those approaches for NASBA.

The next slide is a picture of what our West Nile probe looks like. The next slide is a comparison of TaqMan NASBA with ECL and NASBA with molecular beacons. We've done this not only with West Nile, but with other arboviruses as well. Really, the take-home message is that we rarely see a difference in sensitivity between TaqMan and NASBA.

Our molecular beacon NASBA is really not where it ought to be. Other people that are using molecular beacons for NASBA, I've seen sensitivity that really is not that much different from ECLs. I think that's really the

difference that you see here is more of an issue with us rather than the test, but really, in terms of molecular amplification, in our experience, TaqMan and NASBA seem to be about the same.

There are some differences in setup, differences in time that it takes to set these reactions up. People need to decide what would work best for them, but essentially I think, theoretically and in practice, their amplification is about the same.

The next slide is just a very quick diversion into a molecular epidemiology in terms of we have an ongoing project here where we're sequencing the entire genome of these West Nile virus islets that we obtain. The key thing that I wanted to include this for is to point out that the strains circulating in the United States are undergoing very, very minimal nucleotide changes.

It's important for us in terms of molecular testing, because obviously if the virus is changing significantly, primers and probes would be expected to fail eventually. But according to what we've done so far, we're not seeing really any significant change in the nucleotide sequence of these viruses.

You can see a little clustering by year. The ’98, ’99, and 2000 islets are kind of together, and the 2001 and 2002; there's even a 2003 in there.

So the other thing is we’ve actually looked at all the sequencing we’ve done. We’ve compared that back to the primers and probes that we’ve published, which I think amounts to a total of maybe six primer … nucleotides altogether. We’re not seeing any mutations in the regions where our primers and probes are.

So the next slide is just a quick look at viral isolation. We continue to attempt that here because we want viral islets. It’s not really the greatest diagnostic test, because, again, it’s not the most sensitive when you compare it back to molecular amplification, but we still do it, because we really want islets.

You can see the kind of interesting pattern. Nineteen ninety-nine through 2002, or through 2001, we rarely, or we actually never, isolated the virus here. It wasn’t for lack of trying. We actually worked very hard to try and isolate virus. This is just a reflection of the fact that by the time people are ill, they’re really not viremic anymore.

In 2002 and 2003 what we saw were the detection of the virus in the asymptomatic blood donors. Again, these are individuals who, at the time of donation, were weeks away from perhaps any clinical illness. They were really in that peak viremia phase. So when you have those types of samples, we've been able to obtain numerous islets; I mean more than we even could imagine we'd want to do anything with. It really becomes the virus. In a sense, the virus does grow very, very well. It's very easily isolated if you have the appropriately timed specimen, which now is being provided through the blood bank community.

So the next slide is just a summary. Again, the most sensitive tests we have here for detecting virus are TaqMan and NASBA. By the way, NASBA is really just another name for TMA, which is transcription-mediated amplification, which is the platform that GenProbe uses to screen blood products. So it's a test that's been proven and is out there. We use internal negative and positive controls, obviously, in our tests.

Again, the final point about the strains in the U.S. are not really changing very much. I do have a note here. When I mentioned that we had looked at the primers and probes that we published, we also included some other published primers and probes. That's why there are actually nine that

we're comparing. There was one mutation in one of the ones that's been published in the literature.

The next slide, because of time, I'll just skip and let you read that. I think it's fairly straightforward in terms of what we're describing here. The next slide is just a look at some of the things we're looking at right now. One of the things that we're trying to do is compliment our TaqMan assays. We're trying to also look at some consensus-based assays where we have broadly reactive primers that would pick up many different viruses, things like dengue consensus primers or alphavirus consensus primers and so forth.

Essentially, we can't use the TaqMan technology, because that requires a specific probe. So what we're doing is we're doing cyber green assays where we detect an amplified product and then we have do to melt curves. This seems to be working fine for us, although the sensitivity is definitely not as good as something like TaqMan.

The last slide is just to thank everyone here in the diagnostic lab at CDC, because obviously I don't do all this work myself. I play a part as much as I can, but the names listed here are all those that have worked very hard to

contribute to this data and this presentation. I'd like to, again, thank you very much and I understand that there is an option for some questions.

S. Langford — Thank you, Dr. Lanciotti. We'll now check with the operator and see if we have some time for questions.

Coordinator — Our first question comes from Susan. You may ask your question.

Patty — This is Patty at the Michigan Department of Community Health. When you were talking about why run the IgG ELISA, the statement is that IgG should be used for early season testing and/or specific cases, not during a confirmed epidemic. Does this statement apply to all of the U.S. now, except for the two states that haven't had documented West Nile cases?

Dr. Lanciotti — Well, what we're trying to say is that it's really a state lab decision. I was just trying to point out some of the reasons why. I mean some states continue to do the IgG test throughout the year. We're not trying to encourage anyone to drop the test. I mean, if you've got the resources and the ability and the sample numbers that are coming in are limited, some states are actually testing IgM and IgG all season.

I think what I'm trying to say is that if you're in a situation where there's, like for example Colorado this year, there were so many confirmed cases that it just doesn't make sense to continue to do the IgG test after a while. But in the end it's a state decision. It's what you choose to do.

Coordinator — Emily, you may ask your question.

Emily — Are the blood banks screening year-round for West Nile virus?

Dr. Lanciotti — That's a good question. You know, I'll just tell you that I don't know for absolute certainty, but my understanding is that they are. The reason I say that is I know that there is discussion about whether they should continue to do that. I just don't know what the latest decision is, but my understanding is they are testing year round.

I think there is some discussion about whether that makes sense or not, and whether they're going to have regional testing in certain places year-round, but I just don't know what's been decided, as far as the latest finalized issue.

Coordinator — Our next question comes from Christine.

Christine — You mentioned that there was an internal control that you're using for your amplified tests. I wondered what that was and how you might obtain it.

Dr. Lanciotti — That's kind of a problem. We developed that here in-house, just as a small project, and we've not been distributing that as one of our certified reagents. We just haven't had the ability to go through the things that we needed to go through to get it onboard as a standardized reagent.

We've been hoping that a commercial source would become available. I think - I have not looked into this in depth, so I don't know for sure, but I think there are other internal controls used in molecular-amplification assays that different people have found from commercial sources. I just don't know. I couldn't give you company names or anything like that. I just don't know.

But the one that we developed here is in a plasmid and it has to go through certain loopholes for us to distribute it. So we just haven't had the personnel power to actually do that. It's not available from us, but I would look to places like Ambion. I thought at one point that Ambion, which is -

they call themselves the RNA company, I thought at one point they had mentioned that they were going to produce an internal control, but I don't know the latest on that.

Coordinator Our next question comes from Marguerite.

Lita Rob, this is Lita. I had more questions on the internal control as well. Are you manufacturing it yourselves?

Dr. Lanciotti No. That's the point. We're not manufacturing it. Basically everything we use here is not available. We have a subset of what we actually use in-house. We have to go through some extra work to transfer that reagent from an in-house application to put it in a separate, what we call our reagent distribution database. The problem is we haven't made that transition and I'm not sure we're going to be able to do that.

There are several other things in that same category that we use here in-house, but we don't distribute it, just because we don't have personnel to actually do that. So that's the status of it.

Coordinator Joy, you may ask your question.

Joy I was wondering if you could clarify what you have here about doing animal necropsies in a BSL3?

Dr. Lanciotti Well, that's how we do it. In fact, of all the different samples that we work with, in all the scenarios, that's the most dangerous, in my opinion, because, for example, the infected crows are just loaded with virus, you know, ten to the eighth plaque-forming units, ten to the ninth. So we treat potentially infected birds, like crows in particular – well we treat all our birds in BSL3, but I was trying to say that my opinion is, of all the things that you'd want to make sure that you do in a BSL3 environment, it would be working with the dead birds, because they have more virus than probably any other sample you'll work with, including mosquito pools and other issues from humans and so forth, which will have relatively low amounts of virus. The crows and other infected animals have so much virus that BSL3 is a requirement.

Joy What about the VecTest? Do you guys do that on a BSE?

Dr. Lanciotti We do part of it, until there's a detergent that's added, and I don't know exactly. I don't remember exactly, but at some portion you add a lysis.

It's not the same lysis buffer as in the nucleic acid test, but it's a detergent buffer. So we do that test like the molecular test where we add the detergent buffer in a BSL3 environment.

When I say BSL3 environment, the way we do it, practically, is we have a BSL3 lab. In that lab we have a biosafety hood and we do the addition of the lysis buffer under the hood. Once it's been incubated and vortexed and mixed and everything, then we take it out and we do the continuation of that test on the bench top.

Joy What about, would it be sufficient just to use BSL3 practices, if you're not a BSL3 lab?

Dr. Lanciotti This gets into, ultimately, it's up to the individual safety officer at that lab to make that decision. We follow what's in the, what we call the *BMBL*, the *Biosafety and Microbiological and Biomedical Laboratory Guidelines*. If you read that carefully, it does say that it's possible to use BSL3 - they say it's basically possible to modify a BSL2 lab to meet BSL3 conditions and so forth, but in the end it's really a laboratory decision.

These are guidelines that the CDC and NIH have published. They're guidelines for working with these viruses, but the safety officer at your location is the one who ultimately can decide what modifications are appropriate and whether it's allowable or not.

Coordinator Kevin, you may ask your question.

Dr. Berota This is Dr. Berota, asking in lieu of Kevin. The question I have is you mentioned about the microsphere-based assay test. How long will that take before it becomes available?

Dr. Lanciotti Well, at the national West Nile meeting it was presented a lot more. In fact, it was an entire talk devoted to that. So I probably didn't do it justice in terms of how far along it actually is. It's really - we're essentially done with it. I mean it's in the process of getting published. We've looked at a very large number of samples and we're satisfied.

We're actually going to use it here this year, as a replacement for the IgM ELISA for SLE and West Nile. So that's really where we are today, that we plan to actually use it. Any state that chooses to go that route, I think we're going to help in terms of protocol. There's really nothing that would

keep anyone from doing it, other than, as you probably know, the cost of the instrument. It's not inexpensive. I think to get a machine is somewhere between $40,000 and $50,000. So we have been contacted by a few states that want to try it. So we're willing to help whoever wants to try it.

Coordinator At this time I'm showing no further questions.

S. Langford Thank you very much. If you come up with some questions, you can always e-mail those to the northeast office for the NLTN. That's neoffice@nltn.org. Dr. Lanciotti can answer those by e-mail. Again, the e-mail is neoffice@nltn.org.

Again, I'd like to remind the participants listening to our program to register and complete an evaluation form by April 9th. The directions for this are on your confirmation letter and general handout. They also were e-mailed to each site representative this morning. Documenting your participation helps us to continue to bring high-quality, cost-effective training programs in a variety of formats. When you have completed the registration and evaluation form, you will be able to print your certificate of attendance.

That concludes our program today. Our next teleconference will be on April 13th. The topic for that teleconference is State Training Coordinators: Their Critical Networking Role. The co-sponsors of today's program would like to thank our speaker, Dr. Robert Lanciotti. From the Washington State Department of Health Public Health Laboratories in Shoreline, Washington, this is Shelly Langford. Good day.