DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOOD AND DRUG ADMINISTRATION

CENTER FOR BIOLOGICS EVALUATION AND RESEARCH

WORKSHOP ON DEVELOPMENT OF DONOR SCREENING ASSAYS FOR WEST NILE VIRUS

Monday, November 4, 2002

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P R O C E E D I N G S

Welcome and Overview

DR. GOODMAN: Why don't we get started? Good morning, everyone. I want to welcome you all to this workshop on West Nile Virus blood and tissue screening pathogen inactivation. I am Jesse Goodman, deputy director of CBER.

[Slide]

There are quite a number of people I wanted to thank for putting this meeting together. I think I left my list over there, but particularly Hira Nakhasi, from the Division of Emerging and Transfusion Transmitted Diseases, and Joe Wilczek, who put a tremendous amount of effort into this, and then the other people, Maria Rios, Indira Hewlett, Robin Biswas, Mahmood Farshid and Carolyn Wilson. Again, I thank everybody for putting this together in what for the government is a very short time period, probably for anybody for a meeting of this type.

I would also like to thank the co-sponsors with FDA, the Centers for Disease Control, NIH, I believe that also NIAID has contributed, the Health Resources and

Services Administration, the Office of the Secretary, the Office of Public Health and Science. Everybody has been very supportive of getting folks together quickly to move forward with this problem. Then, I thank all of you for coming.

[Slide]

Just to give a very brief overview of where we are going here, as everybody knows, West Nile virus transmission by transfusion and transplantation is a reality and it is something that we are all taking quite seriously. I think it is important to state that the degree of risk is still unknown, and you will hear some discussions of risk estimates and certainly an update on the various investigations.

In the face of a continuing epidemic in this country the need for donor testing is anticipated. There is a continuing desire to have capability for screening available under IND by next summer.

This is both real and this is also a test of our system. It is a test of flexibility and agility for the blood industry, for the diagnostics industry and certainly for the FDA. It is a test and a case study with lessons to

be learned. Many people in this room, far more than me, have learned from previous issues of infectious disease testing and HIV and hepatitis, that there are lessons of the past that everyone has learned. I would also like people, as we go through this, to learn lessons from the present. These are relevant to future emerging infectious diseases, of which there shall be more, and also the unfortunate potential for bioterrorism.

[Slide]

I do really believe that so far the response has been very gratifying. There has been a strong collaborative and rapid flow of information among all the parties. I think that although nobody is ever quite satisfied with it, as many people say to the FDA, if everybody is mad at you you are probably doing the right thing. So, I think there has been an appropriate balance in this communication in the face of a rapidly evolving situation and a knowledge base, and I particularly want to thank our colleagues at the CDC and particularly Lyle Petersen and Mary Chamberland who have worked with us very closely on this. The communication in difficult

circumstances has been extraordinary, and also the blood industry has really come through in this respect.

All of those same people have come through with what I think are prompt and precautionary actions in a difficult situation, everybody doing their best. There have been, as I said, good inter-agency activities and public-private partnerships.

[Slide]

So, we need to move forward and we are mostly here for information sharing and discussion. This isn't a decisional meeting or a consensus meeting; it is a meeting to update each other from a variety of perspectives and help each other move forward. As you will see from the agenda, it is quite full of diverse areas but they all fit together to, I think, form what needs to be an ongoing public health response.

We are going to review West Nile biology and recent events. We are going to hear about test methodologies. We are going to review issues that are very specific to an area we really haven't considered much before--cells, organs, tissue transplants. And, I would just like to mention that while this is also going, on HRSA

regulates the organ transplants and FDA has put forward a plan for a framework to more effectively regulate tissues. The tissue industry is a growing industry meeting a tremendous medical need.

We are going to hear diagnostic industry perspectives and results. Many of you here are from the diagnostic industry and we recognize that you bring critical expertise and ability to get things done here. We are going to hear about the related field of pathogen inactivation but specifically oriented towards West Nile virus as this is another strategy. We are going to hear from FDA about potential regulatory issues and pathways to try to make this more streamlined, to the extent possible. And, we are going to hear from the blood industry and others about what kinds of issues are anticipated in implementing screening.

[Slide]

Some of the kinds of questions that I think are pretty obvious but that we will consider would be what are the most promising tests? Are there special issues for tissues, cells and organs, and I believe there are? How can we move forward with the best kinds of tests? How can

we better understand risk? What is the potential role of pathogen inactivation? Are there gaps here? Are there unmet scientific and resource needs that need to be addressed to move this faster or more efficiently or more effectively?

I think a bigger, longer-term question is how can we accelerate development of robust platform technologies with the ability to rapidly alter the agents screened? Again, we are not necessarily going into that issue in detail here, but I would say that I and our colleagues at the FDA will really appreciate input and creative thinking about this issue because we sort of don't want to go through the same thing every time there is a new agent.

[Slide]

Some examples, just a few, of specific questions and issues would be what kind of sensitivity are we looking for? This is a very different situation than with the other infectious disease agents we have screened for. One I would like to raise relatively early in all this is should these tests be West Nile virus specific or cover multiple agents? We have had in the past in this country epidemics--St. Louis encephalitis. I think that Dr.

Petersen will mention that. We have a global public health threat from Dengue fever. It may be possible to design strategies and have them in place that would protect from all these agents, and is that something desirable and feasible? Again, can blood screens truly rule out tissue infection? The answer may be no but we may then not have any real other choices.

[Slide]

There are some policy questions that are a bit broader that, again, we can't finish with today but which I think really need to be on the table. Again, we really want outside input on this. This shouldn't be any kind of unilateral decision-making.

What about seasonal and regional application of screening tests? Can that ever be made to work? What is going to be the threshold of risk for testing for this and future agents? This is something that we may wish to bring to our advisory committees and again seek input on. And, how will the blood system deal with the economics of screening for this and future agents? Again getting back to the platform technologies, are there ways to make this more economical?

[Slide]

Finally, I would just like for people to think about bringing up a couple of sort of longer-term picture issues. As you can tell from how I am pitching this, we really need to be ahead of the curve. I think we acted very quickly here and by past standards maybe we are ahead of the curve, and some people may feel we are ahead of it now but I am not really sure we are, or that we have a paradigm that gets us there. You know, how can we be evaluating and getting tests ready before they are needed so they can be there exactly when they are needed?

One very promising example of a technology is microarrays or pathogen chips. We could have a hundred or more pathogens on a chip, potentially have blood amplification in one reaction using random primers, etc. and potentially detect multiple pathogens, some of which we have no concern about at the moment but others which we are all familiar with.

So, one idea which I hope isn't the Titanic but I think one needs to think about, for example, would be combining donor screening with surveillance, having a situation, which in a sense we do now. We obtain valuable

data about hepatitis and HIV from donor screening. It is a specific well population, but can we obtain information and a "heads up" ahead of time about future threats by such multi-pathogen detection? That is using blood screening as another public health sentinel system for both natural and potentially intentional disease.

[Slide]

Anyhow, I think that many of you are going to touch on these areas in your presentations. We do want input and we want people to follow-up with FDA. I know the other federal agencies feel this way for their parts in the puzzle. I think we, so far, have done well and we can succeed in this and future efforts to address these infectious threats.

Again, I thank you and all the coworkers, and really am eager to hear all your presentations. Again, welcome.

DR. NAKHASI: The next speaker is John Finlayson. John will talk about the history of the testing.

Historical Perspective of Donor Screening Testing

DR. FINLAYSON: Good morning again. This talk should really be given by Jay Epstein, however, he is in

Geneva and when we learned that he would be in Geneva it seemed logical that Jesse Goodman could give this talk. But it seems to have fallen to me so you, molecular biologists in the audience, recognize that we are operating here on a two-codon code--

[Laughter]

--with a limited wobble hypothesis. That is to say, this talk can be given by anybody whose initials are JE, JF or JG. Presumably the one in the middle has the lowest energy state so I hope I don't fall asleep during the presentation.

[Laughter]

Maybe I should say I hope you don't fall asleep during the presentation.

[Slide]

This is supposed to be a historical perspective of donor screening. Quite clearly, as you all realize since you are in this business, in the time that is available to me I can't possibly go through all of the history of how we got to where we are today. However, as we used to say, ontogeny recapitulates phylogeny and, fortunately, if we look at the status quo we can get a

pretty good idea of how we got here and the sequence in which we did.

[Slide]

Let's take a look at the first slide. Here we have the tests that are required or recommended, and you recognize these as being recommended or required for the screening of blood donors; those recommended or required for the screening of plasma, I should say source plasma, donors are a subset of these; and they are arranged in the order of implementation. If we were to have put the dates of implementation on these, we would see that there would be a very large gap between number one and number two and a very substantial gap between number two and number three, and the rest of these would be very closely, or relatively closely spaced together in time. Note that this is the order of implementation, not the order of recommendation. Were we to list these in the order of recommendation we would have to slice out the test for anti-HBC and reinsert it down here.

[Slide]

Are there any other tests? Yes. Here are a couple of tests whose implementation is voluntary. Now,

voluntary means different things to different people, like, "John, you will voluntarily give this talk."

[Laughter]

What we mean here by voluntary is simply that these are used not at the behest of the FDA.

[Slide]

Then, as we come into the modern era we have the NAT assays, the nucleic acid technology-based assays. You see they are sort of grouped into three groups here if this is a group of one--I guess you can have a group of one; mathematicians would permit that. These are licensed tests which are essentially universally employed. These tests are selectively employed and you have heard about them a number of times at Blood Products Advisory Committee meetings and you will undoubtedly hear about them again at Blood Products Advisory Committee meetings. We have the hepatitis B virus DNA test which is also essentially universally employed for screening of source plasma donors but which is still considered experimental.

[Slide]

One of the questions that people have wrestled with a great deal over the years, indeed, over the decades,

is that of at what point do we implement a new test? Many people have put a great deal of thought into this. I do not mean by any stretch of the imagination that these are the two papers that stand out head and shoulders above the others, but simply that these are representative of the thought that people have put into this. Clearly, people were thinking about this before 1990 and people were thinking about this long after 1993.

[Slide]

One can think about all the good things that a test does for one, but we can ask the question are there any downsides to initiating a new test? You perhaps remember this excitement a little over a decade ago. Both time constraints and modesty forbid me to go through all the arguments in here, but suffice it to say that in addition to those arguments, we could say, as far as downsides go, that there can be a negative economic impact. This is, of course, not something that the FDA is supposed to think about but it is one of these elephants that never goes away.

One can also say there could be donor loss. Well, clearly if a donor is infectious you want to lose

that donor, or at least you want to lose that donor during the time that he or she is infectious but, as we all know, sometimes donors will overreact and self-defer just in anticipation that they might come out reactive in a particular test.

Now, are there any quick generalizations that we can make about the time at which one would implement a new test? And, the answer to that question is probably no because every test has its little nuances but, certainly, one can say a few things that would stand scrutiny. First, if you are going to implement a new test you have to have a test to implement. The next profundity will be uttered at exactly 9:14!

[Laughter]

Second, you have to know something about the test, about its characteristics, about its performance. Then, you should have a feeling of comfort--that perhaps is the best word that I can use--that there is going to be some positive benefit for implementing the test. Again, benefit can be defined in a number of ways but we are talking about benefit from the standpoint of the view of public health. And, one has to be comfortable that the

adverse effects, if any, of this test that one is implementing do not outweigh the positive benefits thereof.

[Slide]

Now, are there some particular difficulties? In other words, which tests have given us the most trouble with the decision to implement or not to implement? Well, those of us who labor in the trenches sometimes feel that the most difficult tests are whatever ones we are working on right now, however, it might be interesting to see what Tom Zuck said in his review. He said the most difficult tests to deal with are those that offer only incremental improvement in the safety of the blood supply. In order to make this not too busy a slide, I truncated his statement but in the interest of telling the whole truth I should go on to say that what he said was those that offer only incremental improvement in the safety of the blood supply and whose increment is so small that it cannot be measured.

That is, of course, part and parcel of the sort of thing that we are considering today, what do we know about these tests? As Jesse Goodman said, which are the appropriate tests? And, what are the other considerations that go along with them?

I think you will agree that Jesse Goodman could have given that talk and Jay Epstein could have given it if he were here. I venture to say that 40 percent of the members of this audience could have given it. However, this was supposed to be a talk about a historical perspective so in the last two or three minutes let me give you something that within this field I will call, for want of a better term, pure history, if there is such a thing as pure history.

Exactly 33 years and three days ago I was attending a meeting of the National Research Council. It was taking place at the National Academy of Sciences building on Constitution Avenue, in downtown Washington, D.C. and the subject was considerations in the implementation of tests for hepatitis. Foremost among these was a test for what in those days we were still calling the Australia antigen. This was the second day of a two-day symposium. The first day had been devoted to scientific presentations. The second day was devoted to panel discussions. The first panel discussion was on the subject of--are you ready for this?--sensitivity and specificity. The second panel discussion, however, was

clearly the main event because the subject there was the impact on the blood banking community of the implementation of these tests. The panel members were all distinguished blood bankers.

However, even though they were all distinguished blood bankers, some were more distinguished than others and, clearly, the senior statesman among these was Aaron Kelner who was at the time the head of the New York Blood Center. So, when he took the microphone there was an excitement that went through the audience. There was a certain electricity in the air. In other words, it was rather like a Michael Jordan press conference. Everybody is gathered to see what Michael has to say.

Of course, it also was very much like a Michael Jordan press conference in that when Michael gets up to talk all the sports writers in the audience already know exactly what he is going to say. They have their stories written and they are just waiting for him to say them so they can file them. See? So, everybody knew that Aaron Kelner was going to say, "this is a very imperfect test." He was going to say that there can be negative units that slip through and they can cause hepatitis B and that is

going to cause a great deal of consternation among the public and ourselves. It is going to give a false sense of security that we don't know how to deal with or even to recognize a false-positive. That it is going to cause chaos in the field because we haven't implemented a new test for a long time, and he was certainly right because it has been 30 years since the syphilis test had come to be. And, there was going to be this big economic impact.

Everybody knew he was going to say that. So, what did he say? He said in effect that for the last day and a quarter we have been hearing particulars directed to the question of whether or not we should implement this test and I say that is the wrong question. The question is not whether we should implement this test but when, and I say the answer is now. For high historical drama it doesn't get any better than that.

[Laughter]

Who knows? Maybe we will have our own drama over the next two days. So, let the play begin.

DR. NAKHASI: Thank you, John. That was a wonderful historical perspective and you did an excellent

job. Even though Jay is not here, he would have said that, and this is an excellent presentation.

Now we should start the scientific sessions, and the first scientific session is on West Nile biology and epidemiology. We have two excellent speakers today who know West Nile through and through and have been in the news lately, for the last couple of months. First I would like to invite Dr. Margo Brinton. She is a professor in the Department of Biology, Georgia State University, Atlanta, Georgia. She will talk to us about the West Nile biology, all you need to know.

I. WNV Biology and Epidemiology

West Nile Biology

DR. BRINTON: First of all, I would like to apologize for my voice. I was in Washington about a week and a half ago for an NIH study section and picked up a bug during the time I was here.

[Slide]

The other thing I would like to do is thank the organizers for inviting me to speak about my favorite virus, West Nile. I started working with West Nile in 1968 as a model flavivirus for studying genetic resistance in

mice. I chose West Nile because it grew well in mice and also it was considered a very safe flavivirus to work with. During the years in between then and now I have often been told that the virus I work with is fairly obscure. This is often granting agencies, and if I would work with a virus that was more relevant I would have a better chance of getting funds.

[Slide]

Obviously, that has all changed now and West Nile is definitely the virus that is going around and, although The New Yorker portrays it like this, it is obviously much different than that and here is an actual picture of the virus. It is a small enveloped virus, spherical, about 15 nanometers in diameter. It contains on the envelope the E protein and the M protein, and then there is a single capsid in multiple copies which makes up the core of the virion.

Some very interesting structural studies recently have shown unusual features of this virus. One is that the envelope proteins form dimers, head to tail dimers, and that they sit very flat along the membrane. They don't stick up as spikes as is seen with many other viruses. The

other is that the interaction between the membrane proteins is what enforces the icosahedral symmetry on the virion, not the capsid proteins. So, both of those are unusual features in the structure of the virus.

[Slide]

Most natural infections begin with a peripheral infection, usually by a mosquito bite, and there is initial replication at the site of inoculation. It is thought that that is probably mainly in dendritic cells. The virus then spreads to regional lymph nodes where it continues to replicate. The virus is in the lymph and then spreads from the lymph to the blood. From the blood it spreads to additional peripheral sites such as the spleen, the liver and the lungs and replicates in those organs as well. Then, in some cases, it crosses the blood-brain barrier and then replicates in brain neurons. There are certainly virus and host factors which determine the level of virus replication.

[Slide]

Looking at a recent analysis of different West Nile strains, first what is obvious is that when West Nile strains are compared on the basis of their sequence

homology they can be divided into two groups, called lineage I and lineage II. But within each lineage they vary quite a bit in both their neurovirulence and neuroinvasiveness. So, there are lineage I viruses which are not very neuroinvasive, and in a sense not neuroinvasive in mice at all and still can replicate in the brains of these animals if inoculated directly, but usually it requires a higher titer of virus so that the LD_{50} is a much higher titer than with some of the more virulent ones. If you compare the ones from the U.S., they certainly fall in the category of the ones that are more virulent and neurovirulent.

[Slide]

There are host factors as well, and just comparing the viremia titers of different species of birds, you can see that the titers range from very low to very high. The exact host factors that determine the level of virus replication are not well determined and certainly haven't been studied in birds. There have been some studies in mice, which I will refer to briefly later. But there is quite a range and, in general, the lower the

titers in the blood, the less chance there is for severe disease to develop.

[Slide]

Having said that, even with individuals that develop a low level viremia it still requires a functioning immune system in order to survive the infection. The different parts of the immune system are all important in helping the host resist the infection. In the innate response the interferon pathways are quite important. Interferon has been shown to be able to induce a PQR RNase L-dependent pathway and also OAS RNase L-dependent pathway as well. Both of these can certainly inhibit West Nile virus infection.

However, interferons induce a large number of proteins and for most of these we don't really know whether they have antiviral function and, if so, what those functions are. So, I think this interaction is far more complicated than we appreciate at this time.

It is also being found that there are ways that some of these pathways can be induced even without interferon. For instance, the nucleic capsid protein of

some types of flaviviruses can actually induce the expression of OAS genes.

It has also been suggested from a number of studies that there may be the existence of interferon-dependent alternative pathways, and these may involve some of those additional proteins that I mentioned.

[Slide]

The humoral response--it has been shown that antibodies to West Nile are directed mainly to the E protein, to the non-structural protein NS1 and the non-structural protein NS3. However, low levels of antibodies are found to all of the other viral proteins. The cytotoxic T-cell response is directed toward epitopes in a number of proteins, including NS3, E, NS1, NS2A, 4A and 4B. All three of these types of responses are really necessary for recovery, and the higher the viremic titer the more competent these responses have to be for the host to survive.

[Slide]

The genome of the virus consists of a single-stranded RNA. It is a messenger RNA. It encodes a single open reading frame that produces a single polyprotein.

This polyprotein then is processed into the final proteins by the NS3 protein which contains a protease and also by cell proteases in some cases. There is a precursor of the M protein that is in virions inside cells, and then as the virion matures and exits the cell that is cleaved and then the virion maintains the M protein in the membrane and the pre part of it is lost.

The proteins in the non-structural region--the NS1 protein is not a part of the replication complex but has been found associated with replicating RNA and there is evidence to suggest that it somehow regulates RNA replication, but exactly how it does that is not known. The NS3 protein, as I mentioned, has protease activity. NS5 and NS3 also have one of the capping enzyme activities. The four proteins listed as 2A, 2B and 4A, 4B are proteins that are thought to function as membrane anchors, holding the replication complex on the endoplasmic reticulum membranes, but these proteins probably also have additional functions that are not well characterized as yet.

[Slide]

The genome RNA has, in addition to the coding region, non-coding regions at both the 3' and 5' end and

these have characteristic conserved RNA structures at each end. The sequences of these structures are not well conserved, but the structures themselves are well conserved among the flaviviruses and it is thought that they play important roles in the regulation of translation and transcription.

[Slide]

To just briefly go through the replication cycle, the virus attaches at the membrane of a cell. It is likely that it attaches to specific receptors. Those have not yet been characterized but it is thought that they are highly conserved because West Nile can infect so many different species of hosts that it is likely that it is a highly conserved protein. But it could be another type of molecule, other than a protein.

Once the attachment occurs, the virus is taken in through an endocytic pathway and then is released. It is uncoded and the capsid is released and then the RNA is released. The first thing that happens is translation so that polyprotein is produced, and then it is processed to the final virion proteins. Some of these proteins are the non-structural proteins involved in RNA replication. So,

the incoming genome then becomes a template for RNA transcription, a complementary strand called the minus strand. Then that strand, in turn, becomes the template for new genome RNA which is then packaged together with the structural proteins in the endoplasmic reticulum and the Golgi regions of the cell to produce new virions, which are then transported to the surface of the cell in vesicles and then the virus exists the cell. So, the entire replication cycle takes place in the cytoplasm of the cell.

[Slide]

During this replication cycle the virus has to interact with many different host proteins. I have sort of divided them into three classes. The first class are those which the virus requires for its replication. So, those would be, for instance, at the beginning of the cycle. If there is a protein that the virus interacts with, then that receptor would be required for the replication of the virus. But there may be additional proteins that are required through the entry process as well. This virus does not shut off host translation and so it is likely that there may be regions of the viral RNA that interact with proteins, and that these enhance the translation of the

viral RNA over cell RNA. There are certainly interactions between viral non-structural proteins and the ER membranes and protein introduction to the ER membranes. These are important for assembling the replication complexes on those membranes.

There are RNA-protein and protein-protein interactions which are involved in regulating and are possibly also required for the assembly of replication complexes. Those also are occurring in conjunction with the endoplasmic reticulum membranes. Then the various molecules that are made, the RNA and protein molecules of the virus need to be transported to the place where the assembly is occurring, and it is likely that cell proteins are involved in the transport of these molecules and also as chaperons during the assembly process. Finally, during the exit of the virus from the cells it is likely that cell proteins are probably facilitating that as well.

[Slide]

Because the viral RNA is messenger RNA, it most likely forms a closed loop conformation like cell RNAs have been shown to do. In the case of cell RNAs, proteins bind to the poly-A at the 3′ end interact with proteins that bind

with the cap at the 5' end. This interaction between the 3' end and 5' end enhances translation and helps to facilitate the recycling of ribosomes on the messenger RNA.

For viral RNAs which don't contain a 3' poly-A, and that is true of the West Nile virus RNA, other kinds of protein-protein interactions have been found to occur, and these are just two examples of that. In this case a different cell protein is binding to the 3' end but not to a poly-A. In this case it is a viral protein binding, and in each case these interact with one of the capsid binding proteins.

So far with West Nile we haven't found any protein-protein interactions between proteins binding at the 3' end and the proteins binding at the 5' end, but there is good evidence that there are RNA-RNA interactions which could bring the two ends of the RNA together.

The RNA of the flavivirus competes for three functions in the cell. As I mentioned, it is a template for translation; it is a template for transcription and replication, and then it is also encapsulated into virions. So, these three functions have to be regulated so that they are occurring in the proper time and at the proper rate.

[Slide]

This is just a little more realistic picture of what the viral RNAs look like. At the top, here, would be the virion RNA, and then the complementary strand here showing these conserved structures. There are not only secondary structures, but there are tertiary structures here which form what is called pseudo-knots. There are additional ones that have been predicted here, and it is very likely that the interaction of these structures with each other and the interaction of these structures with cell proteins are very important in regulating transcription and translation.

Our lab has been involved in detecting these proteins and analyzing them, and recent data suggests that these interactions are definitely critical for the functioning of the virus, but exactly what these proteins are doing during the replication is still not known.

The sequences shown here in red are sequences that are involved in that very long 3', 5' interaction. This is over 11 KB. This is a very long RNA, yet, these sequences are holding the 3' and 5' ends together.

I mentioned that many of the processes carried out by the virus are occurring in association with the endoplasmic reticulum membranes. This just shows a nuclear region here, and these are cytoplasmic regions. The dark spots are the virus. It is known that translation, polyprotein processing, RNA transcription and also virion assembly are all occurring in association with these membranes. In order to make this environment one in which the virus can carry out all of these processes, the virus signals the cell to proliferate these membranes and also rearranges these membranes. Exactly how the virus does that is not really known.

[Slide]

It is known that the capsid protein, the NS4 protein and the NS5 protein can go to the nucleus for a virus that is replicating in the cytoplasm. When some virus proteins are found in the nucleus, that suggests that they are there in order to recruit some components from the nucleus or to signal some type of gene regulation in order to change the environment of the cell.

So, this is an area which is just beginning to be studied but I think it will be a very interesting area. We

don't know the protein partners that these proteins interact with or exactly what the roles are. There have been some studies that indicate that when cells are infected with West Nile the expression of some proteins, for instance ICAM-1 and MHC-1, are up-regulated. There is also some indication that NS1 can function on the surface of the cell as the beginning of a signaling pathway. So, there may be a number of ways that the virus infection signals the cell to make the changes that are needed for the replication of the virus. The other thing that has been observed is that infection can stimulate cell replication, but exactly how this is happening is not known.

[Slide]

There is a consequence for changing the cell environment, and that is that when the cell notices that the cytoplasmic environment is changing it has various defense mechanisms that come into play. Generally these are called ER. The infection is inducing an ER stress and that can lead to apoptosis pathways as well as to interferon type defense pathways.

This is just a pathway here that has been recently published for a related virus, bovine diarrhea virus, and during a pathway like this you have proteins that are made which retard the development of apoptosis, and later on you also have proteins that lead to apoptosis.

It has been shown that West Nile can cause apoptosis in cells using a Bax-dependent pathway. Exactly what the components are that lead to that pathway are not known, but it is very likely that when it does happen in infected cells, until 72 hours there are ways that the virus has for inhibiting the development of this pathway at least during the early part of the replication.

So, it is thought that the interaction between the virus components and the components of the interferon pathways, and also the interferon-induced pathways and the apoptosis pathways, are probably complex and that there are virus proteins which are helping to first inhibit or delay these responses and then later on the cell may be able to overcome these mechanisms that the virus is using.

[Slide]

As part of this whole process, there is also the stress granule formation and then, as I mentioned, here is

one of the interferon pathways, and a number of these pathways will converge on single molecules. I think probably a lot of the research in the next several years will be on trying to sort out all of these interactions and trying to figure out how these interactions affect virus production, and which ones are the most important in determining how much virus is made by a particular host. If there are changes in any of these interactions, either a change in the virus or a change in the host, then it could result in a different level of virus being produced. So, this may give us a new understanding of exactly how these factors regulate the level of virus in different host individuals within a population, as well as in different species.

Just finally to mention that recently in our lab we have identified a mouse gene which is a type of OAS protein and differences in that protein can certainly change the level of virus that is made by a cell. So, a change in a single component can definitely change the level of virus replication that is produced by a cell.

As I mentioned at the beginning, lower levels of virus produced by a host usually are the main determinant

in whether that host survives, and higher levels of virus are usually not able to be contained by the immune responses of the host and can lead to severe infection and even death. So, I will leave it there and if you have any questions I will be happy to answer them.

DR. NAKHASI: The next speaker is Dr. Lyle Petersen. He is the deputy director for science at Division of Vector-Borne Infectious Disease, CDC. He will talk to us about the current status of West Nile virus disease and transmission cases in the U.S. and risk from other Japanese encephalitis family of flaviviruses.

Current Status of the WNV Disease and Transmission Cases in the U.S. and Risk from Japanese Encephalitis Family of Flaviviruses

DR. PETERSEN: Good morning.

[Slide]

The topic of my talk is West Nile virus in North America, an update on transfusion transmission. What I will do this morning is first talk a little bit about the clinical epidemiology of the virus, then move on to what has been happening with the spread of the epidemic this

year, and then move on to the organ donation and transfusion-related infections.

Pictured on this slide is a picture of Culex quinquefasciatus, which is the southern house mosquito which is the principal vector of West Nile virus in the southern United States, and it is shown three times its normal size.

[Laughter]

[Slide]

The incubation period of West Nile virus is not precisely known but generally falls within the range of 2 to 14 days, with most infections probably falling at the shorter end of this range. However, one interesting thing that we found is that in many immunocompromised people who have gotten West Nile infection the incubation period seems to be towards the longer end of this range, which is completely opposite to what one might expect.

Advanced age is the primary risk factor for severe neurological disease and death. By far, this is the most prominent risk factor. We also know that immunosuppressive drugs and hematological malignancies are probable risk factors for severe disease and death. We

have observed this both in this year's epidemic in conjunction with the transfusion-related cases, but also in experimental infections of humans in the 1950's it also showed that hematological malignancies, particularly lymphomas and leukemias, were very prominent risk factors for death. There is approximately ten percent mortality among persons with meningoencephalitis, and the long-term morbidity among people who do get neurological disease is quite substantial.

[Slide]

What do we know about how many people are actually getting infected? What we have done so far in the United States is four serosurveys, the most prominent being in 1999 which was centered in the epicenter in Queens during that outbreak, which showed that 2.6 percent of the entire population of the study zone in Queens has gotten infected with the West Nile virus.

Three other serosurveys were done in Staten Island. In 2000 there was a small epidemic that occurred and a household-based serosurvey on that island showed that about 0.5 percent of the population had gotten infected,

with lesser prevalence in areas which had less meningoencephalitis cases reported.

[Slide]

From those data, plus serosurvey data from Rumania, we showed this pyramid in which about 80 percent of the people who get infected with West Nile virus have no symptoms at all. About 20 percent of the people do develop what we call West Nile fever, which is a febrile illness of about three to six days duration, and less than one percent or about 1/150 total infections result in meningoencephalitis. This number of 1/150 is a very robust number and we have observed this in a number of serological surveys both here and abroad.

[Slide]

What has been happening with the epidemic this year? We have a national reporting system called ArboNet which tracks humans, birds, mosquito pools and horses, and these are the data from 1999 through 2002. As you can see, in 1999 there were four states affected, going out to 43 states plus the District of Columbia.

What has happened in humans is quite interesting. In 1999 there were 62 human cases with six deaths. In

2000, despite the fact that the virus had actually spread throughout much of New England, there were only 21 cases and two deaths and about half of these occurred on Staten Island. In 2001, despite even larger spread of the virus, there were only 66 humans affected, with nine deaths and so far, as of August 25, this year we have had 3391 humans reported, with 188 deaths.

These numbers, here, reflect primarily cases of meningoencephalitis since that is what we focus our surveillance efforts on. This year, of the 3391 persons reported, about 80 percent have meningoencephalitis. If you recall my last slide which showed that about 1/150 persons develop severe neurological disease, this number translates into a number much greater than 300,000 human infections occurring this year alone.

[Slide]

This map shows the spread of the virus. In 1999, the states affected are shown in red. Actually, this is a misleading slide because the whole state is covered in but the extent of West Nile virus activity was a very small area around New York City, except for one dead bird found in the State of Maryland that year.

In 2000 virus had spread throughout the upper eastern seaboard and by 2001 the virus had spread throughout the eastern United States. In yellow is shown the spread as of 2002, and you can see that it has basically gone from coast to coast. California is a little bit misleading since there has only been one human case in California and that was a woman in Los Angeles who, interestingly, worked for an express mail delivery company. We have found no other evidence of West Nile virus activity in California. This woman had not traveled so we are not sure how she got infected. Washington State--there was one dead bird found up there, in that corner of the state, but that really is the western extent of known West Nile virus transmission. So, as you can see, in four short years this virus has gone from here all the way over to here.

[Slide]

This is an epidemic curve of the epidemic this year. The epidemic--and this is mostly meningoencephalitis cases--started back here in early July, actually late June. There was one case in June which was the first case this year in the southern United States. In grey is the southern U.S.; in green is the northern U.S.

Now, the cycle of this epidemic peaking in late summer is due to the fact that this is really a virus of birds, and there is a bird-mosquito cycle that sort of winds up throughout the year and eventually, as the season goes on more and more birds get infected; more and more mosquitoes get infected and after a point in time there are so many mosquitoes infected that humans become at increased risk for getting infected and that accounts for the late summertime peak of human incidents.

The other thing you might note is that the peak in the south occurred before the peak in the north, which is accounted for by the earlier emergence of mosquitoes in spring.

[Slide]

Transfusion transmission of West Nile virus has always been a consideration because there is a transient viremia after infection and, as I showed you earlier, most persons actually are asymptomatic. So, before this year occurred the risk was viewed as small but not zero. The risk was small because there is no chronic carrier state and no cases of West Nile virus transmission, or any other Japanese encephalitis complex of flavivirus had been

reported in previous years or in endemic countries. The risk of transfusion-related transmission is related to the prevalence of viremia among donors which is the incidence of infection times the duration of viremia.

[Slide]

So this raised some concern about is there a cause for concern about blood transfusion? This cartoon says, "it's okay, Gus, you tested negative for the West Nile virus."

[Slide]

Earlier this year Brad Biggerstaff developed a model to estimate the risk of transfusion-related transmission and I will attempt to go through this model. For more details of the model, it is published in the August issue of Transfusion.

The first step of the model is to determine the incidence and temporal distribution of viremia in the population at large. First we start out with the incidence of meningoencephalitis cases reported through ArboNet or our national surveillance system. We have national data by county of all the meningoencephalitis cases that have occurred.

If we assume the onset of viremia is one to five days before the time of symptom onset, which is what most of the literature seems to indicate, and the total duration of viremia has a mean of 6.2 days., with a range of 1-11, and this figure is derived from experimental infections of humans done in the 1950's, taking these two assumptions plus this, using a Monte Carlo simulation, we estimated the temporal viremia distribution in the population at large. As I mentioned to you earlier, there are 140 more infections for every case of meningoencephalitis reported, so you assume the incidence of infection in the whole population is about 140 times the incidence of meningoencephalitis. So, all of this gets you the incidence and temporal distribution of viremia in the population at large.

[Slide]

Step two in developing this model is to determine the incidence and temporal distribution of viremia in the donor population or the people from which blood donors are drawn. We assumed for the model that the incidence in donors was similar to the incidence in the population at large. We know from our serosurveys that actually the

incidence of infection in the population is independent of age, which makes sense since everybody can be exposed to mosquitoes. So, this assumption is probably a valid one.

Step three is to account for exclusions. We took a conservative approach and we assumed that 32 percent of the viremic donors would be symptomatic and would be deferred. This number comes from population data from the New York City serosurvey done in Queens.

Finally, to estimate risk we assumed 100 percent transmission rate from viremic donors to recipients. In the paper in Transfusion the estimated risk was 1.8 to 2.7 per 10,000 donations in Queens during the New York City epidemic in 1999.

[Slide]

We have extended this model to data from this year, and this is what we got. I would like to emphasize that these data are very preliminary but I think the numbers probably end up in the right ball park. On this slide I indicate some locations. The lower 48 states is indicated below, the counties from which these estimates were made, and these are the average risks estimated per 10,000 donors and the maximum risk during the epidemic per

10,000 donors. For the entire U.S., the average risk per 10,000 donors was about 0.33, with a maximum risk of about 0.96. You can see that in selected cities which were hot spots for the epidemic the risks were much higher.

[Slide]

This is the temporal distribution of the risk. This is corresponding to August 29, about here, which was the peak of the epidemic. As you can see, for St. Louis the peak was actually earlier than in some of the more northern cities, which corresponds to the fact that the epidemic was earlier in the southern United States than the northern United States.

The other point that I would like to bring out of this slide is that the risk of transmission according to this model was highly time limited. From the peak of about August 29 till two weeks later the risk was already about half.

[Slide]

To date we have 47 possible cases of transfusion-related transmission reported from August 28 to October 26. Of these 47 investigations that we have embarked on this

year, 14 were not transfusion related and 33 are still under investigation.

[Slide]

All of these investigations began with the case of the organ donor transmission. Briefly, what happened with this person, this was a young person who got into an automobile wreck, was hospitalized for two or three days, received blood products from 63 donors, and donated her organs to four recipients. Now, a blood sample obtained at the time of hospital admission, before any blood products, was West Nile virus PCR negative and IgM negative. She got the 63 blood products over the next two days and on the day that her organs were harvested blood samples were PCR positive, culture positive and IgM negative. Her organs went to four recipients, two kidneys, liver and heart. Three of these people developed meningoencephalitis and one developed West Nile fever.

[Slide]

This incident raised the specter of transfusion-related transmission. To date, out of all these investigations that we are doing we have six that we feel very confident now were related to transfusion. Patient 1

was a postpartum woman who developed West Nile virus meningoencephalitis after receiving blood products from 18 donors following obstetrical complication. She lived in an area where there was active West Nile virus transmission going which raised the question of whether this was transfusion versus mosquito borne. However, a recovered unit of plasma from a PCR positive donor of a unit given to this woman grew out West Nile virus.

Patient 2 and 3 I will go into more detail in a minute. Patient 4 was a patient who had a prolonged hospitalization, for more than 60 days, before developing West Nile virus meningoencephalitis; had a donor that was PCR positive which was subsequently shown to seroconvert. So, this person really had no possibility of mosquito-borne transmission. Patient 5 and 6 I will detail in a second.

[Slide]

This slide shows patients 2 and 3. Patient 2 was a recipient of a liver transplant. Patient 3 was a patient who received blood transfusions postpartum. These two people had a common donor. The donor donated around August 15 and two days later developed fever, weakness and rash. So, the donor actually became sick after the time of

donation. Patient 3 received two units of red cells from this donor from September 2 and 3, and about ten days later developed onset of symptoms. Blood that was available five days later--her CSF was IgM positive in a sample taken five days later. The donor was subsequently shown to seroconvert.

[Slide]

These are two other patients, both of whom have cancer. Both of these developed West Nile virus meningoencephalitis. The donor for these two persons had fever, headache and eye pain developing about five days before the donation and developed a rash two days after the donation.

Patient 5 received red cells. Patient 6 received FFP from this donor. Patient 6 was interesting because serial samples were available from this patient. Before the FFP a serum sample was IgM negative and PCR negative. The FFP was transfused on October 6. Two days later the patient developed fever, and a sample taken seven days later was IgM negative and PCR positive. Subsequently, seven days after that, the serum sample was also shown to be IgM positive and PCR positive.

[Slide]

In summary, out of these six cases, the illness onsets were from August 1 to October 9. The days from transfusion to illness onset ranged from 3-13, with a median of 7.5. Red cells were implicated in three of these; platelets in two of these; and FFP in one of these.

[Slide]

For the four implicated donors of these six persons, the dates of donation ranged from July 22 to September 6. The symptoms of these people--two donors had symptoms two days post donation. One donor had symptoms five days pre to four days post donation. One donor had symptoms three to ten days pre donation. The maximum interval from donation to transfusion for platelets was five days, which corresponds to the shelf-life of those platelets. For red cells it was 26 days, and for FFP 44 days.

[Slide]

From these four donors, which all were shown to seroconvert which were shown to be TaqMan positive at the time of donation, as I mentioned, all four of these people had illnesses shortly before or shortly after the time of

donation. They all had low levels of viremia, less than 20 PFU/ml. For other investigations we have TaqMan positive donors identified. However, on follow-up they were IgM negative. We are not quite sure what this represents.

[Slide]

In summary, the six cases provide evidence for West Nile virus transmission via blood transfusion. The donations all tested positive by PCR. West Nile virus was isolated from a unit of FFP. The donors had seroconverted after donation, and the donors had West Nile virus compatible illness before and after donation. So, I think all of these facts provide pretty strong evidence that West Nile virus is, in fact, transmitted via blood transfusion.

[Slide]

There are a number of other unanswered questions. One is a better definition of the clinical course of West Nile virus infection and viremia. Second is to define the scope and magnitude of transfusion transmission; to determine the prevalence of viremia in donors; the rate of transmission from viremic donors and associated risk factors; look at the seroprevalence in frequently transfused persons; and also to look at transmission via

other flaviviruses such as St. Louis encephalitis and Dengue.

[Slide]

So, the question is really where are we at right now in the spectrum of West Nile virus illness in the country. One thing that we haven't discussed very much is the relationship of St. Louis encephalitis and West Nile virus infection but these two viruses appear to have a very similar ecology and probable behavior. So, if we want to predict the future maybe we should look at the past, and one way to do this is to look at what has happened with St. Louis encephalitis in the last 34 years.

As you can see, this chart shows the incidence of meningoencephalitis due to St. Louis encephalitis virus by year, and you can see that St. Louis encephalitis causes periodic outbreaks which are very difficult to predict and varying in location. In 1975 there was a very large outbreak of St. Louis encephalitis in the Midwest which caused about 2000 cases.

[Slide]

If you start comparing St. Louis encephalitis in 1975 with West Nile virus in 2002--these are maps showing

the incidence of meningoencephalitis per million population and you can see that both of these epidemics centered in the Midwest.

The other thing I would like you to take note of is this map which shows the incidence of meningoencephalitis for West Nile virus. What we would estimate is that the incidence would directly correspond to the risk of viremia among the donors taken from those areas. You can see that the high risk donors would be those predicted to be in the Midwest this last year.

[Slide]

I would like to end on this slide. This is the carrier of the West Nile virus and this is the carrier of West Nile virus hysteria!

[Laughter]

DR. GOODMAN: First of all, thanks for those great talks. We have a crammed schedule here and we are way behind already but I think we could take one or two questions if they are really good ones. If they are not, I am going to laugh at you! So, please, come up with one or two good questions, or were those talks so clear that there

are no questions? Yes? Please, when you get up identify yourself so if I want to take retribution I can do it!

Questions

DR. KLEINMAN: Steve Kleinman, from UBC. I have a question for Lyle. The serosurvey data that you showed from the 1999 and 2000 series seemed to be very sparse actually. I mean you have estimates of 2.6 percent but only 800 people were tested. So, they must have wide confidence intervals. So, my question is have there been similar serosurveys done this year? If so, do we have any meaningful information and do we have large enough sample sizes to really pin this thing down?

DR. PETERSEN: The answer is household-based serosurveys are extremely difficult to do, as you might imagine, particularly in New York City. So, it is a monumental task and one of the limitations is that you just can't get huge sample sizes out of a household-based serosurvey. So, the confidence intervals are wide, as you pointed out.

A serosurvey was done last week in Louisiana, in an area just north of Lake Pontchatrain, where we estimate that the seroprevalence in that population should be about

20 percent. So, I think out of those data this year we will get much more precise estimates both of the asymptomatic to symptomatic ratio but also the prevalence at least in that population.

DR. BUSCH: Mike Busch, both for Lyle and Dr. Brinton. What do we know about the distribution of virus in blood, especially is the virus strictly in plasma or might there be a cell-associated component? Secondly, the infectious titer of virus, do we have any understanding as to the concentration or dose of virus required to transmit, and whether that dose is reflected in probability of disease?

DR. BRINTON: It has been estimated that you need 10^5 PFU/ml in the blood in order to have transmission. Obviously, statistically you could have less than that and have the mosquito just by chance pick up the virus.

DR. BUSCH: Is that dependent on the inoculum size?

DR. BRINTON: Yes, it is a very small drop of blood.

DR. BUSCH: So, if you put a blood unit in a much smaller concentration it would likely transmit.

DR. BRINTON: Obviously, the larger the volume, the more chance of transmission. As far as tissue associated, certainly the virus in the periphery is replicating in tissues. Then, when it gets to the brain it is in neurons so there would be virus in tissues as well as in the blood.

DR. BUSCH: How about leukocytes?

DR. BRINTON: There is certainly evidence the virus can replicate in macrophages.

DR. PETERSEN: There is one piece of data from these experimental infections of cancer patients in the 1950's which showed that inoculum size actually had no relationship at all to the clinical symptomatology.

DR. NAKHASI: Lyle, this question is for you. We know from the last epidemics in West Nile virus throughout the world in 1974 in South Africa and in 1976 in Rumania, what would you predict will happen in the future? I am asking you to look at the crystal ball. Are we over the epidemic now, or are we going to have future epidemics? Also, because this virus, as many of you have told us, is changing, you know, the virulence has changed from what we have seen in the past versus now, what are we predicting?

DR. PETERSEN: Well, one thing that Rob Lanciotti, who is here in the audience, has done is look at the genetic variability of the virus here, in the United States, and has shown that over the past years the virus has remained very genetically stable. So, it is unclear whether the virus genetics are going to change anytime soon that would cause a change in virulence.

About what we might predict in the future, I think the best model is St. Louis encephalitis virus in which we would predict that the incidence will remain fairly low in most years, as it has in previous years here in the U.S., with periodic outbreaks of varying sizes in various parts of the country, and these outbreaks will be very difficult to predict. The one caveat is that if you look at St. Louis encephalitis in birds versus West Nile virus in birds, West Nile virus produces a much higher viremia, several orders of magnitude higher in most species of birds than St. Louis encephalitis, indicating that the potential for more outbreaks or higher incidence throughout the country is probably there.

So, if I had a crystal ball I would say the incidence of West Nile virus is going to be higher on

average than St. Louis encephalitis and there will be periodic outbreaks, sometimes very large as occurred this year.

DR. GOODMAN: One last question.

DR. TABOR: Ed Tabor, from FDA. This is for Dr. Petersen. Of the six cases that are confirmed transfusion-transmitted West Nile virus infections, how many of the donors had symptoms at the time of donation? In the morbidity and mortality weekly report it looked like at least two of the donors had symptoms before and after donation. But is it possible that some of the donors had symptoms at the time of donation and that they slipped through the currently implemented screening process?

DR. PETERSEN: It is possible that they slipped through the donor screening. Now, as you mentioned, a couple of these people clearly did not develop symptoms until after the time of donation. A couple of people did have symptoms before the time of donation, and whether or not they felt okay at the exact moment they donated I don't know. It is difficult to tell from the donor histories. We have gone back and talked to these people again and again and tried to elicit good histories out of these

people, but the symptoms that many of these people had were rather minor and it is difficult to really determine how symptomatic, if at all, they were at the time of donation, at least on the day of donation.

DR. GOODMAN: Well, we will have more opportunity for discussion. Since Ed brought that up and Lyle commented, I think it is very interesting that it is a very small N, four. Of four donors in these proven cases, none of them were the majority of individuals who we believe get West Nile and were totally asymptomatic. That is very interesting because we were quite skeptical certainly of how efficacious either pre or post donation illness screening would be, and at least in these four cases it could potentially have been efficacious. So, whether there is something different about symptomatic donors that makes them more likely to transmit disease such as level of viremia, although the preliminary data doesn't indicate much about that--so, I think it is something to keep in mind as the investigations of the other cases go on and we learn more.

We can move on with the next session which Maria Rios and Bob Lanciotti, from CDC, will oversee. We will

just start right up with Susan Wong, from New York State, who will be talking about some of the antibody assays that they have developed. Susan? Thanks very much, Susan.

II. Methodologies for Detection of WNV and Flexibility for WNV NAT Cross-Reaction with Japanese Encephalitis Family of Flaviviruses Detection of Human Antibodies to WNV with a Recombinant Antigen Microsphere Immunofluorescence Assay

DR. WONG: I would like to thank the FDA for this morning for the opportunity to be able to present to you a new immunoassay which detects antibodies to West Nile virus that was developed over the last several months at the Wadsworth Center.

[Slide]

I plan to tell you first what properties I consider important for a diagnostic serology assay for West Nile virus, and then what other properties might be desirable in an assay that is used to test for antibodies to West Nile in the blood supply.

[Slide]

Certainly, the quality of the antigen is very important for any sensitive and specific immunoassay. With respect to testing for antibodies to flavivirus, it is very important to have a highly pure antigen in a native confirmation and, hopefully, with the glycosylation as would occur in vivo. Many of the antigens which are currently being used have been polyethylene glycol precipitated and acetone extracted or lyophilized. Such procedures have the potential to partially disrupt this fairly complex tertiary structure of a homodimer of the West Nile envelope protein on the surface of a cell. Moreover, many of the antigens currently being used have some contamination from other cell proteins or from growth medium, leading to a phenomenon known as non-specific binding. It is also important to have a high affinity detector antibody, and also to have a signal amplification mechanism so that you can have a sensitive assay.

Certainly, West Nile virus is not the only flavivirus which may be encountered in donors to the blood supply in North America. Before West Nile virus had arrived in North America we had encountered, as you have heard, previous outbreaks of St. Louis encephalitis virus.

It is also possible that individuals who have been on their winter holidays in the Caribbean returned to the United States while they were incubating Dengue virus.

Certainly, an antigen that is predictably cross-reactive to these other flaviviruses could actually be advantageous for screening the blood supply. It is also important that we have an accurate test and that we have a long shelf-life of the antigen reagent under normal storage conditions to decrease the problems with maintaining quality control in an assay. Fewer technical steps and a small number of reagents also leads to a better assay. We want to have low variation and a defined specificity of our assay in other infections and diagnostic conditions.

[Slide]

This slide shows an SDS polyacrylamide gel of four different product lots of recombinant West Nile envelope protein. This recombinant West Nile envelope protein is a proprietary product and is expressed in a eukaryotic cell line, purified by column chromatography.

[Slide]

Our antigen was covalently linked to the surface of fluorescent polystyrene microspheres by a two-step

carbodiimide process. The assay protocol included a 30-minute incubation with a serum dilution, then filtration, washing and a microfilter plate, followed by a second 30-minute incubation for the fluorescent anti-human antibody. After more filter washes, the bound signal was interrogated by a laser on a Luminex bench-top flow cytometer, and we obtained a quantitative result.

Fifty micrograms of pure antigen, when coupled to six million beads, which is our normal lot for making antigen beads, provides enough reagent for 2500 patient test results. For comparison, the standard CDC MAC ELISA and IgG ELISA currently being used in most public health labs take 24-48 hours to obtain the results and, moreover, the laboratories have to frequently make coated plates every single week, which leads to increased problems for us with performing the assays.

[Slide]

The test protocol that we developed initially was to test for total antibodies with a polyvalent detector antibody. Our strategy to detect IgM was actually to deplete IgG with a goat anti-human IgG and retest the sample. One value here that you haven't heard yet this

morning is the P to N ratio. The P to N ratio is the patient's optical density against West Nile antigen, or the patient's fluorescence against West Nile antigen measured over the negative control serum being tested against West Nile antigen. In our assay, a P to N ratio of greater than 4 indicates evidence of flavivirus infection at an undetermined time, and P to N of less than 4 is no evidence of past occurrence of infection.

So, our current test protocol is to test for polyvalent total antibodies. Then, if greater than 4 to test with an IgG depletion step. If the P/N stays greater than 4 we say current or recent infection, that is, IgM has been detected. If the P/N has now decreased to less than 4, it means that the sample was mostly IgG and that is an indicator of past infection. However, for confirmatory testing samples are referred for cross-neutralization plaque reduction titers.

[Slide]

Our assay has shown very good linearity over two logarithms in dilution series, for example shown here, on four positive samples. This indicates that our assay has a relatively broad dynamic range. Our chosen starting

dilution was 1:100 because we had shown in other studies that frequently if you started at 1:25 the other serum proteins were inhibitory of the sensitivity of the assay and, moreover, 1:100 dilution gives near optical median fluorescence intensity, which is the fluorescence measurement on the surface of each bead when interrogated by the laser and 100 beads are counted.

[Slide]

Certainly, when we made a product lot of antigen and we used our positive control serum which gave a median fluorescence intensity of 7000, when we tested every day for approximately four months the value was still 7000. Our antigen on the surface of the beads is stable for approximately four months. However, we did some thermal denaturation studies to see how rapidly it would degrade at 25 degrees centigrade when the antigen was left on the bench and when heated to 37 and 50 degrees centigrade.

You will notice that the antigen-coated beads were actually fairly stable when left on the bench at 25 degrees centigrade--but certainly by having an antigen with a long self-life that decreases lot-to-lot variation and

your ability to maintain good quality control from assay to assay.

[Slide]

We were challenged by a blinded serum panel of 19 human sera from CDC. After we had performed our test and sent the results back to my colleagues in Fort Collins, it became apparent that our polyvalent assay to total antibodies with microsphere immunoassay, which is the black solid bar, had correctly identified all seven negative sera. This grey bar is actually the West Nile MAC ELISA, IgM capture ELISA as performed at New York State and at CDC, which is the striped bar. You will notice that one of those seven negative sera for West Nile actually was falsely positive in both New York and in CDC with the IgM capture antibody.

You will also notice, moreover, that the total antibody microsphere immunoassay was able to detect as positive above our cut-off of P/N of 4 10/12 sera from patients with confirmed flavivirus infection by plaque reduction neutralization. Moreover, you will notice that we have in seven of these 12 sera a considerably higher signal to background level with the polyvalent microsphere

immunoassay compared to the IgM capture ELISA and the IgG ELISA.

The two sera that we missed I was told were collected on day zero onset of infection and day one after onset of infection. I am convinced that if we had received another sample within a few days we would have, indeed, identified these patients as well.

I think it is important also to point out the ability of this microsphere immunoassay to pick up antibodies to Dengue. We had a very, very significantly strong signal in the sera from patients who had Dengue. Perhaps this is associated with the high viremia that can occur in Dengue patients.

[Slide]

We also challenged this microsphere immunoassay with a panel of sera from employees of the state health department who had received the Japanese encephalitis vaccine. These samples were received in our laboratory blinded, and after unblinding it became apparent that we had correctly identified the four new employees, who had not received the vaccine, as negative, and in the employees where we had a sample prior to vaccination to compare it

to, those employees who had a neutralizing titer that developed as determined by plaque reduction neutralization testing, there was a significant boost in the antibodies to Japanese encephalitis virus as detected with this microsphere immunoassay. So, not only does the microsphere immunoassay detect antibodies to West Nile, St. Louis, Dengue but also to Japanese encephalitis virus. We also did an IgM depletion step to repeat this assay as an IgG version of the assay. The data is not shown here.

[Slide]

Certainly, one of the things that is very important is to compare a new assay to what is currently available. This was a very early experiment, about two weeks after we set up this assay. It shows a comparison of about 101 samples of our polyvalent assay or total antibodies detected compared to the West Nile IgG ELISA using a CDC reagent and format. In this instance our correlation gave a value of 0.92 and a slope of 2.09

[Slide]

It was very important, however, for us to be able to develop an antibody test that could pick up IgM because IgM is a measure of infection. The top curves are the

ELISA with an IgM antibody capture ELISA, the CDC reagents as performed at New York State. The bottom curve is a series of the same five samples of a West Nile encephalitis patient, going out to 260 days, and that is the IgG ELISA curve. Notice that the IgG ELISA curve never got higher than about 7 or 8 P/N. The bottom curves are from the microsphere immunoassay. The top curve is the total antibodies picked up by the polyvalent detector antibody.

You will notice that the peak of antibodies detected in this assay was at about 58 days after onset, which correlates with the peak of the IgG reactivity in the IgG ELISA. You will also notice that we did an IgM depletion. The curve parallels the total antibody curve, again with the peak of IgG in the 58-day sample of the samples we tested.

However, when we did IgG depletion, you will notice that the peak of IgM reactivity as detected with a polyvalent conjugate and with an IgM specific conjugate gave almost identical curves and the peak of reactivity was on day 17 after onset, as shown above in the samples tested with the IgM antibody capture ELISA. So, this was one of the examples of the protocol that we can actually get

sensitive detection of IgM with both the polyvalent antibody conjugate and the IgM conjugate.

[Slide]

One of the things that is very important when one is developing a new assay is to make sure that the people performing the assay can replicate the work of one of their other laboratory technologists performing the test. This shows the cumulative inter-operator specificity study with our West Nile microsphere assay performed by two different technologists in my lab independently on the same day. You will notice that we had correlation on the results of 0.995 when 91 samples were tested on a plate, with a slope of 1.125. So, this is very good inter-operator performance for lab techs working at the bench.

[Slide]

Of course, one cannot develop a diagnostic assay, nor a test for the blood supply, without understanding the specificity of performance of your assay. We challenged our assay with sera from a variety of infectious disease conditions, such as Lime disease, granulocytic erlichiosis, syphilis, HIV, herpes simplex virus, EB virus, CMV infection, and also from patients with indicators of

autoimmunity, such as patients with high antinuclear antibody titers and rheumatoid factor.

Unfortunately, we did find one really surprising outlier. Greater than 50 percent of the sera from confirmed cases of syphilis actually related to our West Nile envelope protein that is very pure recombinant. This was a big surprise and I am certain that none of us want to put on our questionnaires to encephalitis patients have you ever had or do you currently have syphilis. However, it is important to know that we reflect these positive sera to the CDC IgM antibody capture ELISA and, indeed, found out that the same thing happened. This phenomenon was primarily a phenomenon of IgG reactivity, not IgM, however it was very reproducible and we don't know the reason for it. My colleague, Dr. Ray Koski searched the T. pallidum genome compared to the envelope protein gene sequence and we could find no significant areas of similarity. So, I did, indeed, notify my colleague at Fort Collins that IgG to West Nile envelope protein does not necessarily mean past flavivirus infection; it could mean past syphilis or current syphilis.

[Slide]

One of the things that I did was try to develop a cost estimate of what it costs us to perform the new microsphere immunoassay. Every day we have to spend about $6.74 to do the daily QC. About once every four months it costs us about $57.37, excluding staff costs, just to covalently link the antigen to the microspheres. However, to actually perform the test where we can put 94 samples on a plate, we can do a patient polyvalent result at a cost of 24 cents and a patient IgM result at a cost of 24 cents. So, we can get patient total antibodies and IgM at a cost of about 50 cents. This is excluding, of course, the cost of antigen. However, it costs us about $4.84 to get an IgM result with the CDC assays and about $5.25 to get an IgG result. So, we can decrease by 95 percent the cost to New York Health Department when we implement this for patient testing.

Our time commitment in terms of technologist time is about four hours, start to finish, with 94 patient specimens on a plate. That means that we can do polyvalent screening in the morning and turn around in the afternoon actually to tell which patients have IgM antibodies.

[Slide]

Certainly, we have done parallel testing with the sera that came into the Wadsworth Center in year 2002. To date, 669 sera have been tested by the polyvalent microsphere assay and where they were positive, and we tested them by the IgM version assay and we compared these data to the results from the IgM antibody capture ELISA and the IgG ELISA. As anybody doing West Nile testing this year knows, data analysis is incomplete because convalescent sera and plaque reduction neutralization test results are pending on many of the patients who actually were positive in August, September and October in New York State.

Moreover, my institutional review board approval is for blinded testing. So, whenever I get a result that is positive in my assay and which is negative in the CDC assay I am not allowed to request the follow-up serum.

[Slide]

However, some preliminary results from our parallel test are that on 669 sera there is approximately 94 or 95 percent concordance between our new, more rapid assay and the assays currently being used. There are approximately 43 sera where we are hoping that we do get

follow-up sera, perhaps clinical information, neutralization testing results. Of these 43, 29 are sera that are positive in the microsphere immunoassays while negative by the CDC assays.

One of the larger areas of concern is that with the IgM antibody capture ELISA approximately 1.1 percent of these samples have to be reported out as non-specific or "I don't know" because of high background binding to what is called the control antigen, which is a tissue culture supernatant. In the IgG ELISA 16.7 percent of the sera, or 112 out of 669, are reported as non-specific. Certainly, in New York State I recognize that if you ever send out to a clinician a result of a non-specific laboratory test he is very unlikely to give you the follow-up sample that, for example, would have been useful for plaque reduction neutralization testing.

[Slide]

So, I think we have some advantages with the relatively new assay we have developed at the Wadsworth Center. Our pure antigen on beads is stable for months. The microsphere immunoassay has a higher throughput than the ELISAs, with 94 patients per plate. We have a low

specimen volume requirement. We use 10 mcl of serum to do our initial dilution. And we have a spinal fluid version of the test, that I can't talk about today, which uses 30 mcl of spinal fluid. We have relatively good precision and accuracy, sensitivity and quality control.

I forgot to mention that in our covariate studies for intra-assay variation we have CVs of approximately seven percent. Our inter-assay CVs are approximately 15 percent on positive samples. We have a low reagent supply cost compared to enzyme immunoassays. As I mentioned previously, a little antigen that is pure goes a long way in terms of producing patient results.

[Slide]

Some further advantages that should be considered are the fact that our assay seems to be predictably quite cross-reactive to the other flaviviruses that would be important for monitoring the blood supply. However, if we consider the other flaviviruses which may be encountered in North America, then perhaps by putting NS1 or pre-M or M or some other flavivirus protein on a different bead in this Luminex technology we could multiplex and actually get a more specific result for an individual flavivirus without

having reflex to neutralization testing. Moreover, the assay could be multiplexed with antigens for other arbovirus infections.

The turn-around time of our assay certainly would be good for organ donor investigations in a cadaveric donor setting. Certainly, the cold ischemic time for solid organs prior to transplantation, I believe, is somewhere between 24 to a little bit over 24 hours and we can turn around the result in three hours.

Certainly, many transplant labs have on board a flow cytometer and often it is used for T and B cell cross matches. So, the tech could actually do these assays while they are doing the T and B cell cross matches in the lab. Also, the technology with antigen on beads can be adapted to other platforms by changing the bead or the detector.

[Slide]

The nucleic acid tests and the serologic tests are complementary. Serology, when it is sensitive, can approach the viremic period decreasing the window between when viremia is present and when IgM or IgG are detected. Sensitive serologic tests would give confidence to findings

of nucleic acid testing, especially if pools are tested in nucleic acid testing.

[Slide]

My future plans are to, hopefully, characterize ranges for an indeterminate zone for polyvalent and IgM microsphere assay; set up ranges for our spinal fluid assay for which over 600 samples have been tested to date; and go live with applying with our new testing protocol for residents in New York State, hopefully, in the near future. I think it would be helpful if we could work together with people in the audience to make the technology available for screening, for diagnostic testing and also for surveillance applications.

[Slide]

This assay could not have been developed without a fairly unique and very productive collaboration between the biotech industry, academia and public health at the state and at the federal levels. My colleague, Dr. Ray Koski is in the audience today. He and Michelle and Kali actually produced the antigen used in my assay. The plasmid and the construct were designed by Dr. Fitrig and Tina Wong at Yale University. Valerie and Rebecca did all

the work. Dr. Laura Kramer's lab did the neutralization testing, and Karen kept all of the rest of the diagnostic work ongoing while we developed this assay. Kathy Kellar, at the CDC in Atlanta, advised us on how to use the Luminex effectively. Dr. John Roehrig, Bob Lanciotti and Jane Johnson, I thank them for very kindly providing the challenge panel to me, and Dr. Tony Marfin, at Fort Collins, for saying, "Susan, don't give up" during minutes when it was frustrating to work with the technology. So, thank you very much.

DR. RIOS: Thank you, Dr. Wong, for a beautiful presentation. I would like to call Robert Lanciotti, from CDC. He is at the Division of Vector-Borne Infectious Diseases and he is at Fort Collins. He will be talking to us about serological and molecular applications for detection of West Nile infection.

I would just like to make an announcement. When you hear the beep, you have five minutes to finish up your presentation. We have a very tight schedule and we would like to keep up with all the presenters. Thank you for your consideration and cooperation.

Serological and Molecular Amplification Assays

for the Detection of WNV Infection

DR. LANCIOTTI: You could also just tell me to shut up and sit down, and I will be happy to do that.

[Laughter]

[Slide]

What I would like to do this morning is talk to you about the kinds of tests that we use at the CDC in Fort Collins. As you can see, I am with the Arbovirus Diseases Branch in the diagnostic and reference laboratory in Fort Collins.

First of all, I would like to mention that these are the recommended tests or the tests that we use at CDC. As you can see, basically we divide serology tests for human serum or CSF.

[Slide]

I am going to talk about the ELISA testing that we do, and Dr. Wong has already mentioned the IgM and IgG ELISAs that we currently use. But I would just like to point out that our nucleic acid-based tests are not really the tests of choice for human serum and/or CSF. We have found over the past three years that a positive result by PCR in CSF or serum is quite meaningful, however, a

negative really doesn't mean anything, and this gets into the whole discussion of the duration of viremia and the titer of virus in serum and in CSF and there are many times when there are true West Nile cases and we are not able to detect West Nile virus by PCR. So, we don't really rely on the nucleic acid tests when we are dealing with acute human serum and CSF. So, for serum and CSF we rely primarily on the serologically-based tests, the ELISA and the plaque reduction neutralization.

When we get into discussion of tissue and fatal cases, tissues, whether they are from humans or from the dead birds in our surveillance or mosquito pools, in all of these environmental samples we find that the nucleic acid-based tests are, in fact, the most sensitive. So, again, for the environment testing of mosquito pools and dead birds, and so forth, the real-time PCR tests are our tests of choice. We can process many samples that way.

We also do virus isolation on all these samples, and we found over the past three years, looking at thousands of samples, that the TaqMan test or the NASBA test is, in fact, more sensitive than virus isolation. There is a point at which we can still detect viral RNA by

TaqMan or NASBA and we can no longer isolate virus. So these, again, are our tests of choice for looking at those types of samples. It is recently that we have gotten looking into human serum in a more comprehensive way by TaqMan, and I will talk about that in a few minutes.

[Slide]

So, this is our testing algorithm for serum, whether from humans or if we are looking for seroconversion among sentinel chickens. We do the IgM and IgG ELISA. If negative, we are finished. If we get a positive result, we then need to do a plaque reduction neutralization test with some of the other related flaviviruses. I will show you in a minute that there is a cross-reactivity of the immune response so we need to do the plaque reduction test.

[Slide]

Very quickly, this is what the IgM capture ELISA looks like. We coat plates with goat anti-human IgM. Then we add patient serum diluted 1:400. We incubate for an hour at 37 degrees. We then add our West Nile recombinant antigen, and the final step is that we add horseradish peroxidase labeled anti-flavivirus monoclonal antibody. So, that is our IgM specific test.

For IgG it is a little different format. We coat with anti-flavivirus monoclonal antibody. Again, it is an overnight incubation. We add our West Nile recombinant antigen. We then add patient serum and then we follow that with an anti-human conjugate that is alkaline phosphatase labeled.

You can see from these two slides, as Dr. Wong mentioned, there is overnight incubation involved. That is one of the things that we are trying to move away from in terms of developing more rapid diagnostic tests for serological purposes, to get away from those overnight incubations because the test does take at least 24 hours to complete.

[Slide]

This was also mentioned so I will just go quickly through this, the way we determine positivity is we divide the optical density of the patient divided by the optical density of the negative control. We do our assay in triplicate. Our cut-off is anything greater than 3 is positive; less than 2 is negative. Then we have this equivocal range of 2-3 and we find greater than 95 percent of the time those turn out to be negative. It is pretty

rare that something between 2 and 3 will hold up to be a positive. So, we repeat all of our equivocals.

[Slide]

This is to demonstrate the cross-reactivity that everyone that is familiar with flaviviruses would be aware of. These are four West Nile confirmed cases. You can see that the P to N to West Nile is, in fact, highest. However, these are all positive to Japanese encephalitis, St. Louis encephalitis, Dengue, less so to yellow fever. This also reflects the antigenic relationship of these viruses. West Nile, JE and SLE are all in the same antigenic complex. So, based on the IgM test alone, it is difficult for us to interpret that this is a recent West Nile infection.

[Slide]

This is the kind of data that we like to see. This is a single patient. You can see we have two acute specimens, CSF and serum, and then a convalescent serum. If you look at the P to N in the ELISA, strong in CSF. These are all three positive. What really tells us that this is a recent West Nile infection--this is the neutralization test here across the top with all the

related flaviviruses--the highest titer in all cases is to West Nile. We also see a greater than four-fold increase in the neutralization titer between the acute and convalescent. So, this is what we would call a complete picture and in this case we can determine that it is a recent West Nile infection.

[Slide]

This is a typical West Nile serological case picked from the thousands we looked at in 2002. What we do is we run the West Nile IgM ELISA and the St. Louis encephalitis ELISA along with the neutralization, again, just West Nile and SLE. If there is travel history we will include other flaviviruses but we just include the two domestic flaviviruses at this point.

One of the points that I would like to make is that this is a trend that we see, a trend that has been backed up by a statistical analysis. Greater than 99 percent of the time the signal in West Nile is going to be three to five times greater than the P to N to SLE. So, in this case we have 12.75 as opposed to 4. This is a very common trend that we see and we can make a presumptive diagnosis of recent West Nile infection but, again, the

trend we always see here is a four-fold or greater difference between the SLE and West Nile neutralization titer, as well as a four-fold increase in titer from acute to convalescent serum.

[Slide]

I just want to talk about a couple of unusual features from our perspective of West Nile, the antibody response to West Nile infection. One of the surprises we determined following the 1999 outbreak in New York is if you look at these numbers here, basically after 200 days almost 80 percent of the West Nile cases still had IgM present and detectable in serum. Remarkably, when you get out to over a year, a year past initial infection, we have over 50 percent of the West Nile cases that still have detectable IgM in their serum. I think this has implications for discussion in terms of the value of an IgM-based test in donor screening. If we know that over 50 percent of the people are going to have IgM a year later, I think that is worthy of a lot of discussion. The numbers were small in that study but there is still I think good statistical support for over 50 percent.

[Slide]

From looking at several thousand West Nile cases, I will just summarize in these bullets, and the first point is that we find IgM nearly all the time by day of onset. This year, in 2002, going back and looking at the data, I only found two exceptions in the first 500 that we looked at. In other words, how many times do we find negative IgM on day zero or day one of onset, and then on a follow-up bleed we find out that this person actually had West Nile? It is very, very rare. We do find IgM nearly all the time by the time of onset.

We find IgG in every case by day seven. I mentioned the trend of a three to five times higher signal in West Nile cases to West Nile antigen and to SLE. I also mentioned that we know that IgM persists for greater than a year.

This is a topic that I won't really go into in any detail, but suffice it to say that if it is a secondary flavivirus infection it is very, very difficult to determine the most recently infecting virus, and we have a lot of data where we know that this is very problematic. It is going to be very difficult even with the

neutralization test to determine the recently infecting virus.

[Slide]

I will just mention that since '99 the CDC has been involved in a lot of training of the state public health labs and other agencies. When this all began in '99 there was only a very few number of laboratories that were doing testing for West Nile. So, it was obviously to our advantage to get as many labs trained as possible. We have offered training courses every year since '99. We have trained over 60 public health labs. We do a proficiency panel where we send out blind coded specimens to all these labs and we are getting very good feedback. As you can see, 100 percent agreement on the IgM ELISA this past year and 92 percent agreement on the IgG ELISA. The problem in those cases where there was not complete agreement was that they missed some of our low positive serum samples.

[Slide]

What we would like to do in the future, wrapping up this serological assay part of what I am going to talk about, we would like to go in the direction of automation. You can see here a Qiagen robot, called a robot twister in

a rapid plate. We are trying to automate as much of the IgM and IgG ELISAs as we can.

We would like to look at reagent stability. All the things that Dr. Wong mentioned are very much a concern to us. The fact that you need to coat these plates before you do a run. The incubation times are also an issue. Again, this test is a 24-hour test. We would really like to try and shorten the incubation times. We have done some of that. The signal does decrease but we need to look at that some more and see if we can come up with a rapid version of this test. Finally, I won't mention this because, you know, Dr. Wong talked about it, but we would really like to move in the direction of Luminex as well for all the obvious advantages.

[Slide]

Let me talk about the molecular side of all the testing that we do. This is kind of a busy slide but this kind of gives a history of what we have done at CDC. All of these nucleic acid tests are based on really three steps. You have to extract RNA from your sample; amplification by some method; and then detection. I will

have one slide about RNA extraction and kind of how we evolved there.

Amplification, as with the industry, we have started with standard RT-PCR, followed by agarose gels. We have really moved away from this; we have never really used this at all for West Nile, just very, very early on. The West Nile epidemic in '99 coincided with the development of TaqMan assays in our laboratory so we never really spent a lot of time doing standard RT-PCR. We have done a lot of TaqMan for West Nile. We have also developed SYBR Green consensus primers to look at multiple flaviviruses and some of the other arthropod-borne viruses.

I won't talk much about this but we have done a lot of work with NASBA as well, which is a transcription-based amplification system, looking both with electrochemiluminescence as detection or even molecular beacons.

[Slide]

Just very quickly, we have evolved in the way we process our samples for RNA extraction. We began with what I would now call some very ancient technologies, using liquid-liquid extraction, guanidine isothiocyanate and all

those sorts of things. As many of you probably know, they are time consuming and they are limited in the number of samples you can do per day.

We then moved into the silica gel-based kits. We can process more this way. In fact, we still use this technology when we don't have a very large number of samples that we are dealing with, but our method of choice now in the larger studies that we are involved in is Qiagen-9604 BioRobot, and this is a 96-well based system where we can do at least 300 samples a day, and this is really taking into account kind of a government work day of eight hours. I know that for those of you in industry, because I spent some of my time working for a private company, eight hours is not, you know, the limitation there; you could work 24 hours if you want. But in an eight-hour shift you can easily do 300 samples.

In fact, we are using this now in a very large study that is going to be discussed here I think on the blood bank viremia survey, as I call it. Everything comes in bar-coded. The samples can go right into this slot, here. The bar codes are read and it is a beautiful system because in the end the RNA is eluted into a 96-well plate.

The RNA can then be transferred directly into another microtiter plate and you can do the real-time PCR there.

[Slide]

I think everyone here is probably familiar with TaqMan chemistry. It is an RT-PCR. We need to do reverse transcription. Then we need to do polymerase chain reaction. The real-time aspect is where we have a probe besides the two amplification primers, and probes labeled with fluorescent dye, and there is a quencher and during the amplification Taq polymerase will chew the probe up so you can directly measure the increase in amplification there.

[Slide]

This is a typical result. In fact, this is from the blood study that we have looked at. Down here, in the baseline you can see actually the 96-well plate. Most of the samples, in fact all of them, are negative, except for our four standards. We include four positive controls in every one of our runs that we make at the beginning of the year and we quantitate the amount of virus. Our standard way of quantitation is to determine the number of plaque-forming units per milliliter.

So, in every run that we perform we have these four standards. How you define positivity in TaqMan is time at which the fluorescence goes above the threshold. This is the Biorad I-Cycler. The instrument determines its own threshold and then it determines the time at which the threshold is crossed. We have settled on a cut-off for determining what is positive at 37 for two reasons. One, we found that among samples that we know are negative we see this occasional situation where something will just barely drift over the line after 37. The other is looking at replicates we find that we cannot reproducibly get a signal. If we get a signal of 38 or 39, you repeat that. It may be negative; it may be 38 or 39 again. So, we are trying to stay within the range where the assay is reproducible.

[Slide]

This is a study that we do every year when we make our positive controls. We look at the detection limit of the assay in terms of our standard quantitation method, which is the number of plaque-forming units. We take the same serial dilutions. We put them on vero cells and we

count plaques. It is pretty straightforward. We take the same samples and extract RNA and perform the TaqMan PCR.

There are so many ways you can define these things. I am just going to settle in on the number of plaque-forming units per milliliter. We have two primer probes that we use in our West Nile assay. We actually have three but we spend most of our energies on these two. One is in the envelope gene; one is in the 3' non-coding region. You can see that there is a difference in sensitivity, about a three-fold difference. We have transferred all this technology. Basically everybody I have talked to has noticed the same difference between these two primer probes. For comparison, we also have synthesized a set that has been published by Dr. Lipken's laboratory. You can see that those primers are really somewhere in the middle between these two.

[Slide]

If we talk about copy number, which in some cases is more relevant to what many people in the industry look at, I have looked at two things in our lab. Both are plasmid and just a double-stranded DNA as a target. These are quantitated by optical density and also running an

agarose gel with standards. You can see in this case that we can detect five copies of a plasmid containing the envelope gene. This is with the envelope set which is the most sensitive set. This is not a concentration; this is actually the number of copies in our test volume of 5 mcl. So, five copies here. When we do the same kind of experiment with a double-stranded DNA molecule, not a plasmid, we are at 12 copies.

Dr. Kramer is going to talk, I am sure, but in her laboratory's publication it looks as if they are talking about 37 copies of single-stranded RNA. So, I think this is fairly good agreement because we are really talking about two different kinds of amplification. Here, this is just PCR. Here we are talking about including the RT step since RNA is the starting point.

[Slide]

The other thing we do in our lab is we have an internal positive control. This, again, is the same figure I showed you a minute ago, looking at a whole 96-well plate of samples. At the same time that we have the West Nile primer probe set, we have added an artificial RNA molecule and we have a primer probe set that is labeled with another

fluorescent dye called JOE. You can see that the only thing here positive are the positive controls. Down here everything comes up as we would expect. This is to ensure that there was efficient amplification in all wells.

[Slide]

This is just another way of looking at the data, comparing TaqMan. I haven't gotten into this much, but we have developed nucleic acid sequence-based amplification for West Nile, and this is typical of what we see. The sensitivity is about the same. On one day NASBA might be better, on another day TaqMan.

[Slide]

Specificity, just to point out that the TaqMan and NASBA assays are specific not only for all West Nile strains here, but in the case of our envelope it is actually specific for a particular phylogenetic clade of West Nile viruses, and no reactivity with other arthropod-borne viruses.

[Slide]

Dr. Petersen mentioned this. We have looked at the complete genome of West Nile strains in the U.S. since '99. We have nearly completed a 2002 strain and we are

finding the same thing from year to year, just very minimal, 15-20 nucleotide changes from year to year and most of those silent. In fact, with the 2002 strain we have only seen one amino acid difference when you compare it back to the '99 strain. So, they are very genetically stable viruses.

[Slide]

I will just summarize what all this is showing us about viremia and, again, Dr. Petersen talked about this but we know from everything we have done so far that viremia in humans is very low. If you take all of the positive TaqMan, positive human sera from this year, the range is between 100 to 150 PFU/ml with an average of 18 PFU/ml.

I will just point out this should initiate a lot of discussion about NAT testing in pools. People ask me about that all the time. I think that if we diluted 1:16, of all the ones we looked at this year we would lose about half. Half of them would no longer be positive. Viremia is short lived, and this is another point that we have a considerable amount of data on where we have looked at a lot of West Nile cases from this year from which we have

day zero or day one onset of symptoms, and in that situation it is less than five percent of the time that we can detect West Nile virus. In fact, this year we only detected 2 out of 500 or 600 that we looked at. So, by the time there is onset of symptoms there is not really detectable viremia.

The other point is that there is a very, very short window when you are going to detect both IgM and West Nile virus by these nucleic acid tests. Again, this year we only found four that fall into that category where they are IgM positive and they are TaqMan positive as well. There are other studies out there, studies that we were involved with this year in Louisiana where we went looking for viremia. We went to clinics. My colleagues at CDC did this study, looking for viremic people and what we found was a fair number of IgM positives but not a single viremic case.

[Slide]

This is how I envision things based on the data. We have viremia that precedes onset of illness, and then the appearance of IgM at onset, and then this very, very short time period where you can detect both IgM and virus.

[Slide]

The same thing applies to TaqMan. We have transferred this technology to all the state public health labs. We got pretty good agreement, not quite as good as with the ELISA. There are issues in the state labs, false positives, as you would expect with any amplification assay. Some labs had problems with false positives. The other extreme is failure of some of these labs to detect our lowest positive. We sent a very, very low positive and some labs didn't get that.

[Slide]

This is my last slide, just to acknowledge that I have a great group of people that I work with at the CDC, and this work represents a collaborative effort between all of us. Sometimes people are surprised; they think the CDC has hundreds of people working on this. This is it.

[Laughter]

Two of the main people that do the TaqMan testing, and I have done a lot of it this year because one of the main people got pregnant and had a baby. Anyway, I want to acknowledge them for their hard work and support at CDC, and thank you for having me this morning.

DR. RIOS: Thank you, Rob. We would like to break now for ten minutes only, if you don't mind. We will be back at 10:35. Thank you.

[Brief recess]

DR. LANCIOTTI: We need to begin to make some attempt to get back on schedule. Our next speaker is Dr. Robert Myers, from the Maryland Department of Health. He is going to talk about assays that they are using in their lab. Dr. Myers has been a great support and colleague to us at CDC. He has been one of the labs that we have relied on for second opinions and for follow-up testing, and so forth. Dr. Myers?

Utilizing Nucleic Acid Amplification Technologies in Arboviral Testing Programs: Practical Experiences from the Maryland Public Health

DR. MYERS: Thank you.

[Slide]

Basically, as Rob mentioned, the CDC has rapidly transferred their developmental technologies to public health laboratories and we basically validated and implemented them. We are very grateful to them. Our

technological capacity has increased remarkably over the last three years.

[Slide]

Basically I am going to give you a very brief overview of RT-PCR technology which is the cornerstone technology for us. We are going to talk about the applications to our public health-related arbovirus testing activities. I am going to show you relative levels of West Nile virus RNA in various sample matrices--birds, mosquitoes, horses, humans. Because this is basically a blood transfusion committee, I am going to relate our experiences with handling West Nile RNA positive human specimens.

[Slide]

Over the last four years since West Nile first appeared in the country, arboviral testing programs in public health laboratories, which have languished for decades, have really taken off. I can say from personal experience that over the last three summers we have devoted a great deal of time and resources toward these testing activities.

[Slide]

These include surveillance--dead bird surveillance, mosquito pools, equine and mammal surveillance and, of course, human surveillance for encephalitis and meningitis patients, as well as doing some diagnoses.

[Slide]

Real-time PCR technology is really the cornerstone of our laboratory as far as nucleic acid amplification. It allows for the simultaneous detection of agent-specific amplification, detection of specific agent nucleic acid sequences; of course, as Rob mentioned, the specific hybridization by complementary probes and, of course, there are several reaction strategies and instruments that are used in real-time PCR assays.

[Slide]

Advantages are high throughput. We have really done tens of thousands of these assays in the last few summers. Without this technology we couldn't have. Our results turn around quickly enough so that public health intervention, so to speak, mosquito control and public education campaigns for arbovirology could really be useful. It does reduce but doesn't totally eliminate cross-

contamination of PCR products. You will see that the levels of viral RNA are very high in birds, for example, and mosquito pools and you can get cross-contamination even at the level of unamplified RNA.

Sensitivity, as you saw before, low copy numbers can be detected; multiplex capabilities, which we do utilize in our laboratory; and, of course, you can get a fairly accurate quantification over a fairly wide dynamic range.

[Slide]

TaqMan, very quickly--you see the hybridization probe, the quencher molecule, the reporter molecule. It cannot be extended by Taq. Of course, the biophysical principle is the light energy coming in to excite the reporter molecule. The wave of the energy that is transferred is given off to the quencher molecule as long as they are closely together and, therefore, no fluorescence is given off.

[Slide]

After the RT step, when you are actually doing the DNA PCR, of course, the probe binds. You get an elongation from the Taq. The probe starts to be displaced.

It is chewed up and broken up, separating the quencher molecule from the reporter molecule and now the reporter molecule is in solution and is excited and gives off fluorescence that is picked up by the instrument. Of course, the more product you have the more fluorescence you have.

[Slide]

This is some of our bird data, just giving you an idea of what a routine one looks like. This is our control. This is our lysate control to make sure the RNA extraction works properly in each batch. These are some positive birds. This is the envelope.

[Slide]

Two of our instruments--we have an ABI 7700 which is a 96-well instrument, and a Roche light cycler which is a glass capillary tube, 36 position. This is more of a work horse. It is an older instrument. It does a data dump. We have to wait three or four hours for results. We truly do get real-time results off the Roche light cycler, which we do for a lot of our confirmation testing. In addition, this summer we have gotten a Biorad I-cycler.

[Slide]

As mentioned, at the lab at Fort Collins and at our lab too, we do have a robotic extraction system. When we are doing thousands of tests, normally we can do 96 specimens in about an hour and a half. Our human work, our volume is not very high. Because we are handling high titer bird samples and mosquito pool samples, our human work is done manually.

[Slide]

This is our normal testing logarithm for PCR testing. We use two multiplex TaqMan PCRs. We use the WN-3, the most sensitive set from CDC that was developed, the internal positive RNA control, the IPC control. That has worked for us. The Patuxent Wildlife Center in Maryland was feeding heavy metals to birds and the birds were dying, and they wanted to make sure they weren't dying of West Nile, and it totally inhibited our PCR. It also controls for technicians to make sure they added RNA to the reaction mixture, reaction plate.

We also look for eastern equine encephalitis virus which is endemic here, in Maryland, on the eastern shore. We do get a few cases every decade. We also are looking for SLE. We had a LaCrosse case appear, a

serological LaCrosse case in Maryland last year. For selected mosquito pool types and also all of our human samples we also run a singleplex PCR, real-time PCR for LaCrosse.

[Slide]

All of our positives are confirmed with additional primer probe sets for West Nile. This is our screening. We use the Lifkin. There was one developed at Walter Reed, called 156, that we use on the light cycler and, of course, the WN-3 and, of course, the non-structural polymerase gene of West Nile.

[Slide]

As you know, certain bird species, particularly the corvids, are highly susceptible to West Nile. This is a very important component, our West Nile surveillance plan. Birds tend to become positive first in an area that West Nile enters. In this particular area here, in the Washington, D.C. area, this year the Virginia State Health Department picked up a bird in the Virginia suburbs of Washington in late April. We picked one up in the District of Columbia in early May. I don't think we saw mosquito pools positive in this area until July, and we didn't see

human cases probably until late July. So, really they are a very early indicator of initial activity and it is an important program.

[Slide]

This is just a summary of our testing. We have had 1800 submitted. About 1700 were suitable for testing. They come in all states of decomposition. Roughly one in three are positive, 18 different species, and about 92 percent of the positives are either corvids or raptors. In Maryland we cut off at two per zip code. The District of Columbia stops testing after they basically have ten positives, I believe, in each ward. They stopped probably in early August. Our West Nile surveillance program just stopped last week.

[Slide]

These are our average CTs or distribution of our CTs, 600-and-some positives that we had. As I mentioned, the brains are in various stages of decomposition. We have had things with maggots crawling through them and road kill, and we still can pick up and confirm levels of West Nile. As you saw before, there are extraordinarily high levels of viruses in birds, 10^{10}, 10^{12} experimentally. We

have less than pristine samples and our estimation is about four logs of RNA PFU equivalents/ml.

[Slide]

Mosquito testing is by our Department of Agriculture mosquito control. We have pools of 1-40 individual mosquitoes. We test them for West Nile, EEE, SLE and certain species for LaCrosse. They are grounded to a suspension of collagen B-bill mixer, and then we also save some of this homogenate before activation to go into tissue culture. We are concerned about emerging infectious arboviruses being imported into our country through containerized cargo or at airports. I am sure you are familiar with the malarial situation here, in Maryland and Virginia, this year. With PCR you only find what you are looking for. The tissue culture casts a broader net for us and occasionally we find things. So far they have been relatively benign, like Jamestown Canyon. We tested 7400 pools; 46 were positive by PCR for West Nile. Pools represent over 100,000 individual mosquitoes.

[Slide]

This is our breakdown of CTs, primarily Culex and also Aedes albopictus, the Asian tiger mosquito which is an

import. One of our positive pools ended by on the editorial page of Science this summer. There was an editorial comment about globalization of a pool that we found here, in Montgomery County. This is our average CT and it looks like it is about 3.5 logs. When we have done tissue culture probably 85 to 90 percent of the time we can isolate West Nile from our PCR positive specimens.

In 2001 we did find EEE on the eastern shore and Culiseta melanura, which is a swamp type mosquito that transmits to birds. We also had a positive bird in 2001. This year was a drier year. The swamps weren't as wet early in the season and we did not see EEE activity.

We did do equine testing, IgM modified CDC MAC testing. Unlike humans, the IgM response in horses tends to be fleeting. We see sometimes horses that are actually clinical and have very low levels of IgM, and then a few days later it is totally gone but it still neutralizes in a plaque reduction assay.

The concept of dead-end host, we have had five positive this season out of 21 tested, but the amount of RNA is relatively low. We are usually seeing probably less

than 1 PFU RNA equivalents/ml and that is a ten percent weight volume of the brain suspension.

[Slide]

Human arboviral testing this year, just to give you an idea of what we have done, again, we are looking for clinically ill individuals and this may skew our data a little bit because we are not seeing the subclinical infections--encephalitis, meningitis and also the West Nile fever.

We looked at 380 cerebral spinal fluid specimens that were tested. Seven of them were West Nile positive from four different individuals. For our serum testing we did acute specimens, less than eight days post onset. We got more aggressive this year as we thought potentially we were missing people in a window phase. Whereas years past basically we were looking only at CSF, we decided to really look at serum this year. We looked at 265 serum, plasma tested, and found five of them West Nile positive from five different individuals. We did have two postmortem brain specimens for West Nile testing in our laboratory. Both were positive. The level of virus is very, very low. Usually I am sending them out to the lab at Fort Collins

and saying can you reproduce this? It is pretty much at the limit of detection of our assay.

[Slide]

This is a breakdown of the CTs. As you can see, we are pretty much at the same cut-off as CDC, which is 37 or anything less than 38. The brain specimens, as you can see, run about 34, 35. These are some of our sera and these are our CSFs. We had some very high levels of virus. These were immunocompromised people. Again, we are looking at the WN-3, the most sensitive primer probes that we use.

[Slide]

This is our quantification curve that we established. This is the $\log_{10}$ of the West Nile virus. It is PFU per PCR reaction and, of course, this is the regression line and this is an approximation. I would warn you, each laboratory, when it produces a virus stock, the amount of copies per milliliter or per input PCR line could vary with defective particles, depending on how that stock is handled in the laboratory. So, we do really need some standardization in this area to make sure that we are all basically talking on the same page. Our level of

sensitivity is very close to CDC's. It is between 0.1 and 0.5 PFU/ml.

What we did, we retested all of our human samples that we found this year in the same PCR that we generated this curve off so we could account for any differences in cycling or technician pipetting per reaction.

[Slide]

This is what we saw. Our human specimens, the five sera we had, had less than 10 PFU/ml; I think roughly six was our highest there. As you can see, our CSF specimens rose from less than 10,000 to a high of almost over 11,000 PFU/ml. Again, this was an immunocompromised patient and, of course, we did run one of our brain samples and found it very, very low, probably between 0.1 and 0.2 of a PFU/ml.

[Slide]

I have broken our human specimens down by the clinical presentation to show you an idea of the viral load that we are actually seeing, the West Nile viral load. These are two West Nile fever specimens that came. They were clinical presentations, headache, fever, myalgia,

rash, and these were all acute specimens, collected usually within three to four days post onset of illness.

In our hands, these two specimens were both IgM negative by the CDC MAC ELISA, and we had relatively low viral loads, according to our calculations somewhere between 1.5 and 2 PFU RNA equivalents/ml. We got the opportunity to reproduce some of the work that Dr. Lanciotti's laboratory has done at Fort Collins, and this was comparable to the viral loads that we saw in some of the transfused units.

[Slide]

The first one was a 22-year old female, who was a veterinary student who traveled to the Midwest, in Illinois and Michigan, to collect birds to bleed for West Nile. She was a Maryland resident. On her flight back to Maryland from Chicago she became ill on a Saturday and went to her doctor on a Monday. This specimen was drawn and was handled very quickly and sent to a private laboratory from her HMO. I think that is important. The private labs and the HMOs sometimes are used to handling viral load type specimens for hepatitis or HIV and the specimens are handled very quickly and very well. Sometimes we get

specimens that are passed through three or four different hands and we don't know how the serum or plasma has actually been handled. As you see, she had a very low viral load. When we did her IgM on the serum specimen, it was negative. Seven days later it was positive. It was also positive for G and M. She has fully recovered.

[Slide]

The next one was a 34-year old healthy individual from the District of Columbia, again, ill; went to her HMO; very low viral load, originally a negative IgM response. We had to wait two weeks for this but we did see positive on that for both and eventually G; again, completely recovered.

[Slide]

We had one case of an immunocompetent person, an elderly woman hospitalized with encephalitis and meningitis. West Nile was detected in an acute specimen and it was detected in the presence of West Nile specific antibodies, but again it was a very, very low level of virus in the acute specimen.

[Slide]

This was a 76-year old woman. On the day of onset of symptoms she went to the hospital, and we did run a CM specimen and, again, we found 0.1 PFU and, as you can see, a CT of about 35, 36. We didn't have any CSF specimens on her. We did see IgM and, paradoxically, we also saw a low level of IgG.

[Slide]

Our immunocompromised patients, two leukemia, lymphoma patients and a kidney transplant patient with a possible transfusion transmission case; high persistent viral load; potentially compartmentalized. We are seeing more viral RNA in the CSF compared to serum. Again, the acute specimens in two of the three cases were IgM negative. We did see delayed seroconversion in some of these cases for several days. They remained viremic for several weeks, and there were fatal outcomes in all three cases.

[Slide]

This was a patient who was at the clinical center at the NIH, who was a 57-year old male who was a leukemia, lymphoma patient. We had a CSF on the fourth day after onset and, as you can see, it was about 300. We had almost

12,000 PFUs detected here. As you see, he retained a high level of virus in his CSF for almost two weeks. He was treated with an immunoglobulin treatment, anti-West Nile IgG from Israel, on this particular day. We got an equivocation here basically beyond our limit of detection. This would be considered positive; this would be considered negative. When we run our confirmatory primers we would not be able to confirm this particular result. After his treatment, of course, we no longer detected virus in his CSF.

[Slide]

As far as serology, we did not see an IgM response until day 37 after onset. He passed away about a week or two after this occurred. I believe he was comatose throughout this. This is the pre treatment; this was the post treatment on the same day. We started to see some G in the serum at that point. Of course, we did find G after that but that could have been due to the treatment that was administered.

[Slide]

This was an immunocompromised woman from the Baltimore area hospital. I believe she was in her late

60's or early 70's. She was found comatose at home. Initially we had, as you can see, a relatively small amount of serum but a relatively high amount of CSF. She was immediately hospitalized. The next specimen that we got again had a very high viral titer. Again, this was a leukemia, lymphoma patient, negative on both days on acute specimens for IgM; serum also negative. We didn't have a sample of serum on that particular day. She died, I believe, within ten days of onset.

[Slide]

Our third case is a possible transmission-associated case. This was her pre-transplant specimen. It was totally negative. The donor was a relative, negative for West Nile by PCR and serology. On day four we did find a low level of virus in her serum. We found a slightly lower level in her CSF. We did find in her CSF IgM positivity on the ninth day, not on the first day. We did find IgM in her serum. She was negative on her pre-test. We haven't tested these yet. I have to pull them out of the freezer to actually see what happened. She did develop an immunoresponse to the virus, at least for IgM. She did succumb and die with a reasonably high viral load.

[Slide]

In closing, real-time PCR has proved to be an invaluable tool for our arbovirus testing program. I don't think it would be successful without it. The levels of RNA detection in human specimens from non-immunocompromised patients--and, of course, the bias in this case is that we are looking at clinical people--is relatively low, less than 10 PFU RNA equivalents/ml. Due to the low levels of West Nile RNA found in the serum plasma, in essence pooling of samples for nucleic acid testing would not be an effective strategy for screening units of donated blood.

[Slide]

Just to acknowledge all the folks in my lab who did all the hard work of breaking open birds, and grinding mosquitoes and running the assays, I couldn't have done this talk without them generating the data. Thank you for your time.

DR. LANCIOTTI: We will take questions at the end of all these presentations. I would like to invite Dr. Cinnia Huang next, another one of our invaluable colleagues at the New York State Laboratory. She is going to talk

about the work at the New York State Lab and nucleic acid testing as well.

New York State Encephalitis Initiative

DR. HUANG: Good morning.

[Slide.]

I would like to divide this talk into two parts. The first part is the New York State Encephalitis Initiative. The second part is I would like to share our lab experiments for West Nile detection by PCR.

[Slide.]

The Encephalitis Initiative began in 1997. Here is the background to start the project; that is, Herpes simplex encephalitis can be diagnosed using PCR to detect a virus genome sequence in CSF and it is rare to recover the viruses from CSF by cell culture.

The central number system infection caused by enterovirus can be diagnosed by RT PCR. The result is as good as those obtained by cell culture. 23 percent of unexplained deaths reported to CDC are due to encephalitis. The etiologic agent has not been identified in 50 percent or more of CNS infections.

[Slide.]

Because of that, our primary goal is to improve diagnosis by nucleic-acid amplification technology, mainly PCR, so we can increase the success rate in determining the agent and also we will send the results back to the physician in a time frame useful for patient management. With unresolved cases, we would like to develop the archive for future study such as we will archive the specimen and we will develop the detailed case-history database and, wherever possible, will include information that might allow the GISN analysis.

[Slide.]

Up to date, we have eleven viruses in our PCR panel. They are the Herpes group. There are also Arboviruses and Enteroviruses. The virus in red, that means it has been detected in clinical samples. Because this is a CNS infection, our specimens are many CSF or brain tissue. The blue for rabies and Dengue are for confirmation tests as requested by our rabies lab or by physicians.

[Slide.]

This is a summary table of our PCR testing results between the beginning of the project in 1997 to

October 31, last month. You can see total we have about 2500 patients. The first column, the initial diagnosid, is provided to us by the physician. The two most common viruses that are detected in our panel are Enterovirus and HSV. We also detect some other Arboviruses in CSF.

One St. Louis case is a 1998. That is the import case. Overall, the percentage of positive is about 15 percent.

[Slide.]

Because we have an encephalitis project in place, after the 1999 outbreak, we quickly added the West Nile into our PCR panel. This is just to share some experiments we have on the developing work in 1999 when we have four fatal cases. It is a little bit complicated. I will explain it in detail.

In each panel, you can see there are four cases, Number 1, Number 2, Number 3 and Number 4. In each panel, there are three specimens. The first one in each panel is the CSF collected while the patient is still alive. The second one is the brain or toxic tissue. Then the third one is the CSF obtained at autopsy.

You can see, in Case No. 2, we do have an additional lane. That is the two CSFs obtained at autopsy. Autopsy has two different colors. One is clear. One has pink color indicating there is some trace of blood in the CSF. Two oligo primer pairs are used. Both are located in NS3 region. The first one is WMV3 and 4 will yield 300 base pairs of PCR band. The second one is 5 and 6. It is in the same region and it will yield 250 base pairs.

From this experiment, what we have learned is one primer pair is better than the other in terms of sensitivity. The second one is the CSF collected while the patient is still alive. We don't have good luck with no detection by PCR except for Case No. 2. That is just speculation because we only have one, the same for CSF, contains a very amount of blood. You can see, at the lower panel, at the lower bottom, we were able to detect that one by West Nile 3 and 4 primer pairs.

[Slide.]

This is the transfusion-related case. We received two serum samples collected about six days apart. In addition to the two primer pairs we used in the previous slide NS3 regions, we also added another primer pair called

WNV 7 and 8 which located in the envelope region and another primer pair in the NS5 region which is a degenerate primer. It is designed for group-specific.

We have used these primer pairs to detect St. Louis, West Nile, Dengue Type 2 and Dengue Type 3. In the data here, you can see, for Serum No. 1 collected earlier, we were able to pick up West Nile sequencing by all four oligo pairs. For Serum No. 2, which we collected six days later, and only two more sensitive primer pairs were picked up, that was 5-6 and 7-8 located in the NS3 region and enveloped region.

[Slide.]

The next one is the encephalitis case which is not involved in the transplant or the transfusion. The patient died within 48 hours after hospitalization. A full autopsy was performed and eight tissues were sent to our lab for PCR testing as well as a blood sample and CSF obtained at autopsy. You can see the tissue contents, brain stem, brain cortex, pancreas, liver, heart, lung, adrenal gland and kidney.

Using the previous four primer pairs, we were able to detect all tissues, 3, 4, 5, 6, 7, 8 and then CSF is less sensitivity as we expect.

[Slide.]

Here is another data to show the quantitation of the virus in each tissue. We used a single-stranded RNA transcript as our standout. In each tissue, we randomly cut four small pieces and we performed a quantitative TaqMan assay. In each experiment, we also include the CSF and a blood sample.

As you can see, there is a big variation in the tissue depending--we only cut a small piece, and therecord is less variation in CSF and blood sample. Overall, as we expect, kidney has the highest copy numbers.

[Slide.]

Finally, this is another encephalitis case in Year 2001. Initially, we received the CSF collected six days after onset. We performed the entire panel and it is negative for all viruses except the West Nile Virus. Dr. Susan Wong's lab also performed a serology test on CSF and it was negative.

So, after talking with the physician, we learned this patient is a lymphoma patient and we were able to retrieve two earlier specimens, a blood sample collected one day after onset and a blood sample collected four days after onset. Also, the patient died 35 days after hospitalization and we also have two more samples collected later, a blood sample and a serum sample.

As requested by the physician, the serum sample collected twenty days after the onset was sent to Dr. Lanciotti's lab for serology testing not just for West Nile but also for other viruses and it is negative.

We found the highest copy number is in blood sample collected four days after the onset. We were able to recover the infectious virus just using 10 microliters of blood sample. The results of this patient will be published in EID, the December issue. Actually, we just learned the patient is out on the CDC EID website.

[Slide.]

What we have learned in the past three years in our encephalitis project is not just for West Nile. We also learned primer selection is very important for the level of the sensitivity. Type of specimen and the

collection date are critical, especially for the detection West Nile Virus. Turnaround time using our current protocol is about twelve hours.

We have to use the Trizol reagent because we need to recover both RNA virus and DNA virus. We use a conventional RT-PCR. We don't have much experiments in the high through-put because, for the encephalitis project, we don't have the TaqMan assay available for all eleven viruses in our panel.

After the finding of the West Nile sequence in a non-neural organism, we have been asked by our administration to develop an assay for a transplant donor-screening program so we just combine the Trizol for RNA detection and TaqMan assay. We were able to reduce the turnaround time to about seven hours.

Progress is we are trying to compare the sensitivity of the TaqMan assay by using RNA assay kit and the Trizol for RNA extraction using the different types of specimens.

Finally, I would like to thank our collaborators to provide a clinical sample in the early development of

this project. This encephalitis project is under CDC's EIP Program. Thank you for your attention.

Detection Issues in Testing Tissues from West Nile Virus

DR. KRAMER: Good morning, and than you very much.

[Slide]

I am going to be giving another state perspective, similar to Dr. Myers' and Dr. Lanciotti. The aspects that I will be considering are the sensitivity of the assay, the speed of the assay, where is the virus in the blood in the animal, and stability of the virus.

[Slide]

This is the same cartoon you saw before and I hope the whole talk is not totally redundant.

[Slide]

But mostly our lab has been testing mosquitoes and vertebrate tissues for West Nile virus, and just recently we have been getting into blood products. As you have heard a number of times, the assay that we depend most on is the nucleic acid analysis where the tissues are triturated, RNA is extracted, and we use an ABI-6700

robotic workstation which then feeds into the TaqMan, and we use two sets of primer probes in our assays.

When we have tissues available, we also do frozen sections and we stain with monoclonal antibody in immunofluorescent antibody studies in order to look for the distribution of viral antigen. We look for live virus because we are looking for viruses besides West Nile. So we inoculate predominantly vero cell culture, allow viral amplification to occur, and then again stain with monoclonal antibody if we are looking for West Nile or grouping antibody if we are looking for other viruses.

[Slide]

You have heard about the primer probes that were designed by Dr. Lanciotti. We use three of them, the envelope one, the NS1 primer probe set and the 3' untranslated region primer probes.

[Slide]

Again, this is our standard curve where we show that our limit of detectability is 0.08 plaque-forming units, which is basically the same as the 0.1 plaque-forming units you have heard about in the other talk. This

is 800 so as the CT value goes down the amount of virus is actually going up.

[Slide]

This is a slide that shows our $\log_{10}$ plaque-forming unit analysis by real-time RT-PCR assay using the primer probe set to the E gene. So, our sensitivity is 0.08 plaque-forming units in 5 mcl, which is what goes into the TaqMan assay, which we translate to 16 plaque-forming units/ml.

[Slide]

When we do it per copy number--our sensitivity is 40-60 copies--we use an in vitro transcribed RNA to determine the copy number so it goes through RT and PCR which translates to about 8000 copies/ml.

[Slide]

When we calculate an estimate of ratio of particle count to plaque-forming unit, using those figures we get about 500 copies to one plaque. Now, this is not exact and we are doing this now with EM to count actual infectious particles which are then copies.

[Slide]

When we tried to develop a multiplex to do our TaqMan PCR using the E and the NS1 sets of primer probe because this way we could include the two primer probe sets in one reaction rather than run two reactions, we lose about ten-fold sensitivity so we don't generally use this, but it could be used with birds which have high copy numbers in them. In our single step it is 0.08.

[Slide]

Now I will go into some of the animal studies that we have done.

[Slide]

This is a study that we didn't do but Bob McLaine did, looking at viremia titers in crows. I will be showing you crows, mice and horses to show you the relative values of the viremias. He inoculated ten crows. This is the day post infection versus the number of crows and versus the virus titer $\log_{10}$. You can see that by day four there are 10^{11} plaque-forming units of virus in the crow's blood. If we take 10^{5} as our cut-off to infect about 50 percent of the mosquitoes that feed on them, then you can see it is there from day one to day seven.

[Slide]

When we look at levels of infections in horses--and this is work that was done by Mike Bunning, published in 2002--each number is a horse and the horses were bled two times a day from day one through day six. They were infected either by inoculated mosquitoes feeding on the horse or by a crow isolate inoculated directly into the horse. You can see how low the levels of virus are. They are nothing like the crow. In this horse the highest level of virus is on day four, 2.4 $\log_{10}$. This one never gets above 1.6 $\log_{10}$ on days five and six. So, they are significantly lower than what we have seen in the birds.

[Slide]

Now I will talk a little bit about our mouse studies. The one I will be talking about is work that predominantly Kristen Bernard did. Five-week old mice were inoculated. They were Balb/C mice. They were inoculated with 10^3 plaque-forming units. Three mice were sacrificed per time point and there were controls and there were cage mates also to look for mouse to mouse transmission.

[Slide]

The red is the serum; the yellow is the spleen; the purple is the heart; and the white is the kidney. It

shows a classical flavivirus pattern of infection where virus gets into the lymphoid tissues and the vascular system and then gets into other parenteral tissues. So, the serum peaks on day two and day three and is cleared very rapidly. So, each bar is one mouse. By day four only one of the mice has detectable viremia. At 19 hours, it is interesting because one of the mice has virus in its spleen and there is no detectable viremia in the blood. Then, eventually, the heart and the kidney come up and go out further after the virus is gone from being detectable in the blood.

[Slide]

We also wanted to look at where the virus was located in infected mice. This is a figure of the $\log_{10}$ PFU in the blood component. These were the two days of peak viremia. So, we inoculated mice and bled them on days two and three and tested the virus in the whole blood and in the plasma.

I showed a slide last week of this where we did it by RNA copy number and it looked like there was very little virus in the plasma. It turned out that was an artifact of the extraction protocol that was followed for

the plasma. So, what extraction protocol you follow is very important for viral detection. But when we did it by infectious virus it is essentially equal on both those days whether you test the whole blood or plasma.

[Slide]

We also were interested in the viral stability. This, again, is infected blood and the $\log_{10}$ PFU versus time in days, and this goes out to 14 days. We are now out to about 20 days and it hasn't dropped very much. But this is the curve at 37 degrees where the virus goes from 6.5 down to undetectable by day six, and the same thing is seen at room temperature, 28 degrees. This is the virus in the refrigerator. So, the virus is very stable and this needs to be kept in mind because of the storage of the blood components. We will just keep on going out until we can't detect it any further but, of course, we are starting with a high level of virus here and that may be a little bit of a problem.

[Slide]

Basically I will end by just saying that I think we need to maintain a global perspective. We know that

Dengue has been transmitted via transfusion, and JE, Japanese encephalitis might very well be.

The other comment I just want to make is that arbovirus cycles are very complex cycles. They involve mosquitoes, birds, other vertebrate hosts and the virus itself. So, you can't just look at the virus and worry about whether it has changed in virulence. You can have environmental factors outside of those three major components that may impact the distribution of the virus and the outbreak of the virus. So, everything needs to be watched and that is why it is important to continue following what happens in mosquito populations and bird populations, not just the virus genetics itself.

[Slide]

I also want to thank the people in my lab who have contributed to this work, Kristen Bernard did the mouse work. Mary Franklin, Sue Jones and Betsy Kauffman are responsible for overseeing all the surveillance. Alan and Greg have been doing a lot of the tissue culture work. Mike did the blood work and Kit has done a lot of primer development which I didn't really go into. Also I would like to thank CDC. Thank you.

DR. RIOS: Thank you, Dr. Kramer, for that excellent presentation. Now I would like to go to our next and last speaker for this session, Dr. Mark Manak. He will talk about controls, panels and sensitivity and specificity for real-time RT-PCR for West Nile virus.

Controls, Panels and a Sensitive and Specific Real-Time RT-PCR Assay for West Nile Virus

DR. MANAK: Thank you very much.

[Slide]

First of all, I would like to thank the FDA for this opportunity to talk about some of the work that we have done at Boston Biomedica with West Nile virus. Specifically, I will be telling you about some of the controls and panels that we are making available to study the sensitivity and specificity, and also a few words about the specific real-time RT-PCR that we have used to characterize these materials.

[Slide]

You have already heard a lot about quantitative RT-PCRs today. Ours is similar. We have developed this independently. We do use specific primers for West Nile virus which target the 3' UTR region, and they were designed

to detect all the known isolates of West Nile virus but not to amplify the St. Louis encephalitis, Dengue or yellow fever.

The assay is designed in a close to real-time fluorescent assay that is quantified either with the TaqMan system which you have heard about or else with a molecular-beacon probe, and it is specific for West Nile. We also have an internal control which monitors for nucleic acid recovery and the presence of any inhibitors which may be present in the sample.

[Slide]

The kinds of samples we have looked at include blood and plasma, as well as West Nile virus culture material in mosquito and avian brains, tissues. For the blood plasma and culture we use a collagen extraction system. For mosquito pools and avian brains we use either bead-beating or pressure cycling technology, which I will mention a little bit later, to extract the RNA. That is then amplified by RT-PCR and detected either with TaqMan or the molecular-beacon system.

[Slide]

You have heard about the TaqMan. I just want to mention a few words about the molecular-beacon. That also has a fluorescent signal and a quencher moiety in the same molecule, but in this particular case that probe is configured into a hairpin loop structure, such that the quencher and the fluorescent moiety are in very close association with each other for very little florescence until the molecule hybridizes to a specific region when it opens up and then the fluorescence is generated.

[Slide]

These are just some quantitative results. These are half-log dilutions, going from about 100 copies to 10^6 copies/ml, and you see very similar results between the TaqMan and the molecular-beacon assays. The duplicates are very closely reproducible in both systems. So, it has a very broad dynamic range.

[Slide]

For the field samples and the clinical samples that we have looked at, we always include one-log dilutions of our standard controls, and then this shows the actual field samples. As you see, they range over a broad range, from about 100 copies to about 10^7 copies/ml. There are

also some negatives in some samples that go a cut-off above a CT of 38 and those would be considered negative in this assay.

[Slide]

The samples we have looked at include avian brain samples which were supplied by Barbara Werner, from the Massachusetts State Health Department. She send us blinded specimens, five infected brains and five from normal birds, and also mosquito pools, five that were confirmed to be culture positive and five from culture negative pools. In fact, we were able to verify that all five of the positives were positive and all five of the negatives were negative in both the brains and the mosquito pool specimens.

We also looked at some human donors. These were supplied by the American Red Cross. We have looked at 100 plasma samples and 100 CSF samples and so far all have been negative by the West Nile virus assay.

[Slide]

I would just like to say a few words about how the mosquito samples and the birds were extracted. We used the pressure-cycling technology which applies pulses of very high hydrostatic pressure to disrupt cells and

tissues. This is a very rapid method that allows complete destruction and release of the nucleic acids. This is the system that was used for the mosquitoes and avian samples.

The instrument that we used for this is Barocylcer NEP2017, which is made by BBI and is capable of processing six samples in about five minutes. The samples are placed in a pulse tube which contain about 1.2 ml of total volume. The samples you see are little mosquito plates at the top with an extraction buffer. That plunger at the top moves up and down with pressure, breaking up the sample into small pieces, and the high pressure actually releases the nucleic acids and totally homogenizes the specimen.

[Slide]

These are just some examples of the data that we have obtained from those five mosquito pools and avian brains, the five positive and five negative. As you can see, the avian brains get very high titers, up to 10^8 copies/gram. The mosquito pools are quite variable, going anywhere from 1000 to four million copies/ml. The negatives were negative, which agreed with once they were decoded.

[Slide]

The West Nile virus assay that we have has a very large, broad linear dynamic range going between 100 to 10 million copies/ml. It has a sensitivity of about 100 copies/ml. It can be used for quantitation of probes and panels. In fact, this is the assay that we have used to make our reagents, which I will talk about. It can also be used for independent confirmation of positive specimens provided by other laboratories. It serves as an adjunct to the CDC TaqMan assays for mosquito pools and bird tissues. Again, it can be used for independent confirmation.

[Slide]

We have developed a couple of panels to be made available to researchers who are developing West Nile virus assays. This is a training panel, which is a quantitation panel that was made from cultured West Nile virus. This virus was heat treated and diluted in human defibrinated plasma so it resembles a normal human serum sample. We put together a 15-member panel with positives ranging from 30 copies to 10,000 copies/ml plus three negatives. These are randomly assorted and some are blinded.

[Slide]

These are the panel members; this is the actual panel. You can see there are three negatives, two copies at 10,000 copies and two at 1000 copies and three each of 300, 100 and 30. These can be used blinded, tested blinded and then decoded or looked at by the data sheet which we provide.

[Slide]

This panel can be used for assay development. It can also be used for quantitation and probing analysis when needed; for assay validation; training of laboratory personnel; and proficiency assessment.

[Slide]

In addition, we have also made a run control, which we call the Accurun 370 for West Nile virus. Again, this is also manufactured from cultured West Nile virus. It is heat treated, diluted in defibrinated plasma and the target level is 500 copies/ml. This control is suited to monitor assay precision from run to run, and also to validation of analytical runs. It is purposely set at a low level to ensure the samples that are tested are able to detect this.

[Slide]

In conclusion, the panels and controls which we made are made from cultured virus, heat treated, diluted in defibrinated plasma. They are designed to closely mimic a true serum sample. They contain a defined copy number of West Nile virus RNA, and they have to undergo all stages of analysis, including lysis and extraction, and can be useful for quantitation and method comparison.

[Slide]

I would like to acknowledge some of the people who have worked on this, Richard Schumacher helped put together the whole program. Alan Doty designed the panels. Jay J. and Xiuli Chen developed the RT-PCR assay and the TaqMan assay, and then all the testing of the clinical samples. The blood and plasma samples were provided by Dr. Chyang Fang at the American Red Cross, and Barbara Werner provided us the avian and mosquito tissue samples. Thank you very much.

General Discussion

DR. RIOS: Please don't go away. I would like to invite all the speakers from this session, Dr. Susan Wong, Robert Lanciotti, Robert Myers, Cinnia Huang, Laura Kramer and Mark to sit at the table and respond to questions from

the audience. When people ask questions, please identify yourself. Don't try to be polite, just go.

DR. BIANCO: Celso Bianco, America's Blood Centers. Most of the speakers identified the most critical issue for us in testing blood donors, the issue of sensitivity. We got a couple of recommendations against pooling samples. But one thing that I would like to hear is, for instance, Dr. Laura Kramer mentioned that the correspondence between plaque-forming units and the number of copies is probably the one that is going to define what we do because it is a more quantifiable number and reproducible between lots of assays, and all that. She said 500 copies per plaque-forming units. Dr. Lanciotti seems to have a somewhat different correlation. I am not clear. Could you help us with what the range is of copy numbers, and what would then be the lower limit of detection of the assays that you currently have in terms of copy numbers/ml?

DR. LANCIOTTI: I am not sure that in my data I even talk about the correlation between plaque-forming units and copy numbers because I have never done that kind of experiment. Dr. Kramer can talk about this as well, but

I think these are estimates because we haven't really done the really comprehensive kinds of experiments that need to be done, such as electron microscopy along with these other assays.

DR. KRAMER: Yes, I would like to reiterate that. Right now we are doing the EM in parallel with the plaque assay, and these are very rough estimates. I know the question just keeps coming up so I wanted to come up with an estimate, but the much better figure will be the one that we are getting now from the EM where we can count particles and see what flaviviruses do if some particles are complete and some particles are empty so we can get an exact estimate.

DR. LANCIOTTI: The other issue that we didn't talk too much about--when everyone talks about their sensitivity or detection limit, there is the whole issue of concentration of your sample. For instance, we have never spent a lot of time on that. We extract a certain amount of serum and we elute that in our elution buffer. So, that is a part of it. When someone can detect a different concentration, really what may be going on is they may be extracting more volume and eluting in less volume. So,

those are factors that need to be brought into the discussion.

When I talk to people who have sensitivities that are a little different than ours, it turns out that it really has to do with how much volume of sample is being extracted and what they are eluting in. But the sensitivities of the assays I think are very close.

DR. KRAMER: Yes. That is why I tried to bring it up to milliliters. I think you also have to look at whether you are using in vitro transcribed RNA or double-stranded DNA. It all makes a difference because you are including or not including the reverse transcription step where you are going to lose RNA.

DR. RIOS: I would like to comment on that too. It is very critical that when you do detection for RNA we do not consider DNA plasmid as the standard for internal control quantitation. I think you want to see how much RNA there is in your transcript in parallel with seeing how many actual virions you have in electron microscopy because you mentioned that there are empty particles there as well.

MR. PRASAD: Shiv Prasad, NIAID. This question is for Dr. Susan Wong. It seems that one of the

limitations of the assay that you presented is that it detects antibodies against a single protein of West Nile virus, and there is a possibility that some infected individuals may not make antibodies to that antigen. I think there was some data to support that in your slide showing the antibody titers in the employees pre and post vaccination with the Japanese encephalitis virus vaccine. I was wondering if you could comment on that, and are there any plans to develop an assay looking at specificity to more than one antigen in the virus?

DR. WONG: Yes, we would like to look at more than one antigen on the virus. However, the envelope protein has demonstrated that basically its immunodominance is the primary target of most of the immune response. Moreover, there could be the ability to work with fragments of some of these antigens which may be expressed if one is not looking at the confirmational epitopes. I believe the antigen we worked with had many of its confirmational epitopes exposed. There may be other antigens which follow virus processing by the immune system which are presented at a later date to the immune system that patients may react to.

So yes, indeed, that is one thing we would like to do. What I presented was a prototype assay, but I feel that there is great potential because with the Luminex technology we can actually put different antigens on different beads and actually get a total value to more than one antigen type.

DR. NELSON: Nelson, from Johns Hopkins. Relating to that question, I was a little surprised at the persistence of IgM and it may make it a problem to identify a recent infection versus somebody who was infected even a year ago. I wonder if there are any strategies that anybody has looked at, either looking at response to different antigens or Western Blot or less sensitive test, or something, to differentiate an incident acute recent infection from a past infection.

DR. WONG: I think one thing I can point to is that slide with the sequential series of five sera from a given patient. You will notice that 17 days after infection his IgM patient to negative ratio was very high. By 55 days after infection the IgG was much higher than the IgM patient to negative ratio. Therefore, a correlation of how high the IgM value is to how high the IgG value is with

a sensitive method may be able to tell you the recency of infection. Therefore, just IgM presence isn't important but the relative proportion of IgM to IgG may be an indicator of recency of infection.

DR. NELSON: The seasonality of the epidemic transmission may help us, I suppose.

DR. WONG: I hope so.

DR. NAKHASI: Hira Nakhasi. I am a little bit confused by what I heard this morning. I heard in one presentation that the viremia comes after the onset of infection and then lasts for three to four days. In another set of experiments, I heard that it is much earlier than the onset of disease. So, the question is when is the real viremia? When do we detect RNA? And, what would be the value of serological tests and what would be the value nucleic acid tests in detection of West Nile virus in the donor screening setting? Anybody can take a crack at that.

DR. LANCIOTTI: Well, I think the biggest issue is that the number of viremic humans that any of us is looking at is very small. In the entire sample pool we have looked at we have had fewer than ten positives. So, I don't think we really have the numbers of viremic humans

available to make really good conclusions, but I can only say that, for example, we have looked at I think 500 IgM positive, confirmed West Nile cases from this year, and of those, we have only found I think four that also have RNA at the same time. So, that is all I can say. If we had more numbers, maybe they wouldn't hold up as solidly but that has been our experience. We don't find IgM and West Nile virus RNA in the same specimen--you know, we hardly ever find that.

DR. GALLARDA: Jim Gallarda, from Roche. I have two questions, one for Dr. Wong and Lanciotti. You are both using recombinant antigens as a basis of your EIA. Correct? Have you looked with just the background strains that are producing the recombinant antigens without having the expression plasmid present to see if the contaminants that are going along with the assay might explain some of the reactivity? In other words, Dr. Wong, you said you have greater than 95 percent purity, which means five percent contaminants.

DR. WONG: Well, you saw the gels that my colleague, Dr. Koski, ran on four different antigen preps. I didn't see any bands visible on the slide. There may

have been something there. So, I think it is probably closer to 99 percent purity.

DR. GALLARDA: Is that Coomassie blue or silver stain?

DR. WONG: Dr. Koski?

DR. KOSKI: Coomassie.

DR. GALLARDA: So, the question is have you looked at non-expression background proteins as a source of an EIA to look for non-specificity?

DR. WONG: I have not.

DR. LANCIOTTI: That is our negative control. We have a transformed cell line and then we use the background cell line as our negative antigen.

DR. GALLARDA: So, it is the host cell line expressing the recombinant antigen as your negative control?

DR. LANCIOTTI: The host cell line with non--

DR. GALLARDA: No, I am talking about the source of the antigen for the EIA. In other words, if you have five percent contaminants, could some of those have antibodies in the patient population that are, in fact,

binding the solid phase and you detect those as false positives and misses?

DR. WONG: In the CDC version of the assay where the kos1 cell tissue culture supernatant is run as background binding antigen, that is how we derive those results, which I cannot report. Those are our non-specific binding results. That is why I feel that the material that I have used recently on the microsphere assay is much cleaner, and approximately one-third of the samples which are non-specific in the CDC assays resolve as positive in the microsphere assay and two-thirds resolve as negative in the microsphere assay.

DR. GALLARDA: Right. The second part of my question is for Cinnia Huang. I think you showed the only data in a serial bleed scenario normalized to RNA copies/ml of an individual who came up to 2.5 times 10^6 copies of RNA at day four. Did I understand that correctly?

DR. HUANG: Yes, it is about 10^6.

DR. GALLARDA: So, it is similar to what we see with HCV pre seroconversion as far as viremia prior to an immune response. I just wanted to make sure I understood the numbers.

DR. HUANG: Yes, I think you can see the whole thing in the publication.

DR. BUSCH: Mike Busch. I think this was a really important session. There was a lot of very good data. Particularly, for the first time we are seeing translation of plaque-forming units to copies at the beginning of that process. I think your comments toward the end about the volume--and I think most of the public health labs are actually processing an extremely small volume of specimens compared to what blood screening does, and the sensitivity that Laura quoted of 8000 copies/ml--you know, we are testing 0.5 ml and we are achieving sensitivities on a neat sample in the range of 5/ml. When you pool those to 16, we are seeing sensitivities of 100 or 200/ml in the individual sample.

So, I think at this point a lot of the data on negative results and IgM positives or the frequency of viremia need to be taken with a grain of salt because the assays that you folks are using, which I am sure are fine for public health diagnostic settings, are not the kind of tests that are routine in the blood screening NAT arena.

I think Mark Manak's work is really important, the ability to develop these standards. As you heard, his run standard is 500 copies/ml, which is a log lower than your folks' analytic sensitivity, and those are the kind of run standards that blood banks are used to employing. So, I think we are going to see the blood screening systems coming into this arena with much greater sensitivity.

One question for Mark, the standards which you built, which you indicated are heat treated, viral culture derived, how have they been quantified to allow you to establish your levels? Also, can the virus still be pelleted from those preps?

DR. MANAK: The quantitation is based on an external control which is RNA transcripts, which are quantitated by optical density. So, we have transcripts and it is relative to that that we get the copy number. We don't know about pelleting. We have not done those kinds of studies yet. But we did do the heat treatment and we know that the RNA is still stable in there.

DR. PETERSEN: Lyle Petersen, CDC. I would just like to go back and address the one question about the relative value of PCR-based technologies versus antibody-

based technologies for detecting these window period donors. I think if you go back and look at the data of the six confirmed cases that I presented earlier relating to four donors, all four of those donors at the time of donation, samples taken at the time of donation, were all IgM negative. All were positive via the TaqMan or had positive cultures from samples taken at the time of donation, which would suggest that the antibody development is occurring later in time than when these people are infectious.

The other comment I have relates to Rob's presentation, and Rob mentioned about the low TaqMan positivity at the time of onset, but what I am wondering is, is that really onset of disease or is that the time the first sample was taken.

DR. LANCIOTTI: Well, that is the million dollar question because when we get the samples submitted they describe day of onset. We never really know how accurate that is. That is probably more likely the day they went to the hospital or the physician. The onset may have actually been days before that. But we just go by what they report

to us, and typically day of onset I think is going to be when they finally went somewhere.

DR. PETERSEN: Because one of the things we have done this year is detailed clinical studies on subsets of patients. One of the things we found was that many of the patients, even the ones who develop meningoencephalitis have a prodromal period, sometimes lasting up to a couple of weeks. So, the actual time they present to the doctor from the time they were actually infected could be a substantial period of time.

DR. KRAMER: I might just add to that, although mice aren't the same as humans but with the mice we don't see clinical symptoms until six days after infection, yet, the viremia has already peaked and gone down by that point, on day three.

DR. NAKHASI: Hira Nakhasi again. I thought by now somebody from the tissue group or the organ donor group would have asked this question. Since they didn't ask, I will ask the question. How is the sensitivity of these tests which we heard about applicable to the tissue settings and organ donor settings? Both TaqMan and IgM?

DR. LANCIOTTI: Again, the numbers are limited. The most common tissues that we test are submitted fresh-frozen brain tissue from fatal cases so there is, again, a limited number of those. We have detected West Nile virus RNA in every one of those that turned out to be, you know, true West Nile cases where there is supporting serology. So, it is there, and Dr. Myers had a lot of data on that as well. It is very low level but it is detectable.

We have only had one other case where other tissues were submitted where we had a good representation of things like liver and heart tissue, kidney and so forth. In that one instance we detected most West Nile in liver, followed by brain and then some of the other organs. Again, that is just one case where we have had those kinds of samples submitted.

DR. RIOS: Actually, regarding testing in tissues, I think Dr. Huang had some kidney, liver and heart. I guess she got more viral load in the kidney than in the liver. So, there seems to be a discrepancy between the two of you. I don't know how that goes, but--

DR. LANCIOTTI: We just had one or two samples. We really need more numbers.

DR. RIOS: Yes, but I would like to go a little bit further with this question and ask if you guys think, or if any of you think that any of the specialized cells from each tissue would support viral proliferation, or how do you think that this virus multiplies? I would like some speculation about which cell can be infected by West Nile, and if it is the specialized cells from the tissue, the organ, how does that go?

I have a second question and you can take both, but this one is for Dr. Robert Myers. I see that you did a lot of studies in serum, and I would like you to comment on whether or not you have made a correlation between serum and plasma collected in the same sample--or Dr. Kramer with the animal studies, where the plasma and serum was collected at the same time, two different samples, and correlate the viral load.

DR. MYERS: To address your question about serum versus plasma, we get whatever they send us and usually they are requesting serology. But we look at the onset date. If the onset date is less than eight days we automatically do a PCR. They are probably not molecular biology grade samples. We do viral load testing in our

lab. I think if clinicians and laboratories would start collecting, like, in a PPT tube, I think the levels of virus that we would be seeing in some of these samples would be significantly higher. Basically we are like a reference lab. We get whatever they send us. Sometimes it passes through two and three hands before we actually get a look at it.

DR. KRAMER: Regarding the cell type, I wonder if Dr. Brinton would be the best one to comment on that, where West Nile is replicating in the tissues.

DR. LANCIOTTI: Because we don't know!

[Laughter]

DR. BRINTON: There is not a whole lot of work on that, but I mean it is certainly true that it replicates in macrophages. There is some evidence that it can replicate in some types of epithelial cells. But I don't think there have been very extensive studies in humans, especially in acute cases and in the peripheral areas. There is a lot of evidence on which neurons in the brain are infected. That probably represents the majority of the evidence.

DR. NAKHASI: I had not finished my second question so can I ask my second question?

DR. RIOS: Sure.

DR. NAKHASI: The second question is really basically about the confirmatory testing. Once we have initial testing done, would a serological test or nucleic acid test be useful in the confirmatory test? As you know, in blood screening you need a second confirmatory test. So, I would like to ask the experts what are the advantages or disadvantages of using either of the two, nucleic acid tests or serological tests.

DR. MYERS: The two West Nile fever cases that we saw were first done by a private lab and we reproduced their findings, but I wasn't too sanguine because the level of virus is very low but we did have the advantage of collecting a follow-up specimen which, in turn, was positive for IgM West Nile antibodies. I felt much more confident about our original results at that point. So, I think in some cases when you find low levels of virus at the limit of sensitivity of your test, the serology test on a subsequently collected specimen will have some advantages.

DR. KLEINMAN: Steve Kleinman. This actually maybe is a question for the panel and maybe Lyle. In the

slide that he showed for the transmitting donors, right towards the end he had a bit of a viral quantitation and I think showed that three or four of the donors had less than 20 plaque-forming units, I assume per ml. My question is do you think that is accurate because that would suggest that relatively low levels of viremia in donors can transmit? Or, is there some limitation of the specimen quality? I mean, we have quantitation of specimens that the labs receive but we don't know if that sample quality is sufficient to tell us that is the real titer. So, I guess the question is to Lyle. Do you feel confident that those were really low level viremics, or do you just really not know how to rely on that result? Or maybe Rob?

DR. LANCIOTTI: There is a variety, but some of the specimens are quite good and have been handled in a very good fashion. So, I am confident that the viremias are really that low. I think as someone mentioned, even if it was 1 PFU/ml, if you give someone half a liter I don't see any theoretical issues for that person getting West Nile because of such a large volume that is given.

DR. KLEINMAN: Right, I don't disagree. I just want to see that we are all in agreement that, in fact, we

believe that low levels of viremia can transmit, which obviously then tells us that we need assays that can pick up these low levels in screening.

DR. LANCIOTTI: I always defer back to the fact that we have a very limited number of human viremic serum samples. If we had much larger numbers I think we could draw better conclusions about levels of viremia. But based on what we have, they are all low; they are all in the low range.

DR. PETERSEN: Yes, and I would also like to add that back in the 1950's when they did the experimental studies of humans one of the observations that they noted was that the viremia levels were pretty low.

DR. BIANCO: My question is for the panel and whoever else can help. The sense that many of us have is that certainly as we try to develop these tests for blood donor screening, we are not going to have, the way you are having so much difficulty with the specimens and the serial samples, and all that would be required for what we would call an ideal test. So, my question is would there be an animal model that could be used for the development of those tests as models of viremia, models for sensitivity

maybe until we have enough access to human samples that could help us better understand that? If there is such a model, what would it be and how would we handle it? And, would you trust a test that comes out of that model?

DR. KRAMER: Rob just said the horse which has titers that seem to be somewhat similar to man, but I don't know that that is a good animal model for experimentation. Our lab is trying to develop a good mouse model. I would say it is not going to happen that quickly. I don't know if Dr. Brinton wants to comment on her mouse models but so far in our hands the titers are higher in the mice, but we do have lines that have lower mortality than the one that I showed you, the Balb/C mouse, of about 58 percent mortality. We have lines with less mortality. It is not an immediate answer to solving the problem because it takes time to develop a good model.

DR. BIANCO: Is there any other answer for the time frame that we have?

DR. MYERS: I think looking at plasmapheresed donors--we have longitudinally collected samples, and you may get lucky and find individuals who are actually subclinical and you may then get a reasonable idea of what

viral loads are in subclinical patients. Most of my data is all biased by the fact that they all have clinical presentations at the time of draw.

AUDIENCE: What about non-human primates?

DR. KRAMER: There is an old Russian study with non-human primates, and the only thing that I really remember well from that is that they theoretically have chronic infections. Although they didn't really confirm that the virus they got out at the end was West Nile but it probably was. Otherwise, I haven't really seen anything, and that is quite an old study.

MS. ZYLBERBERG: Claudia Zylberberg, from NABI. This question is for Dr. Brinton. I would just like to ask you if there is any specific receptor when the virus goes into the cell that can be detectable? Maybe it is related to the question before, but if there is any receptor that could be part of a marker for detection and quantification of the virus.

DR. BRINTON: The receptor is not known. There are a lot of people who have looked for it. There was some evidence that heparin sulfate was facilitating flavivirus entry but that is probably not the specific receptor.

Because the flavivirus, at least in cell culture, can infect so many different types of cells from so many species and also in nature infect so many different hosts, it is likely that whatever the receptor is, it is highly conserved but we don't know what it is.

DR. BURDICK: Jim Burdick, from Johns Hopkins. There are areas of the world in which West Nile virus is endemic, but presumably in those populations there are people who haven't been exposed. Is there anything we can learn about blood transfusion that must be carried out in some of those areas where it has been around for quite a while?

DR. PETERSEN: Those are actually probably the worst places to look for a couple of reasons. One is that in many places where the infection is highly endemic most of the infections actually occur in children and people are immune by the time they are adults. So, these people are, again, likely to be blood donors.

The other thing is that these investigations are really complicated to try and do. For those of you in the audience who have been involved in them, I think everybody would agree. One of the things that we are able to do

here, in this country, is we are able to get specimens from many of these people at the time of donation. This is pretty much an impossibility in most high endemic areas of the world.

The third problem is trying to eliminate natural infection. It is always an issue of mosquito bite versus transfusion. So, the level of evidence has to be pretty darned high before you can implicate transfusion above mosquito-borne transmission. In many places in the world where the infection is highly endemic it is going to be very difficult to rule out mosquito versus transfusion.

So, actually here, in this country, we are probably ideally situated to look at this problem because there is a relatively high infection incidence, particularly this year, plus a good blood collection system where you can actually go back and look at retained specimens.

DR. RIOS: Are there any other questions or comments? Please? You need the microphone, please. Excuse me, it is being transcribed; you need the microphone. You can come here. Could you identify yourself for the transcript?

MR. LYNCH: Tom Lynch, Clearant. I think there is certainly a consensus that the overwhelming majority of infections are self-limiting. I am wondering when, after the acute infection, a unit of, say, whole blood from the infected individual would be considered safe. How long a period following the acute disease even would one have to wait before there was a de minimis risk of transmission?

DR. MYERS: You don't know the viral load in the sample to begin with. I guess we can study the K rates in serum and plasma but if we are not aware of what the initial viremia actually is at the time of donation it is really an unanswerable question.

DR. RIOS: Are there any other questions or comments? If not, I would like to thank the audience and the speakers. At one o'clock we will resume.

[Whereupon, at 12:05 p.m., the proceedings were recessed for lunch, to resume at 1:05 p.m.]

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A F T E R N O O N P R O C E E D I N G S

III. Human Cells, Tissues and Cellular and Tissue-Based Product and Organ Donor Transmission Issues

DR. ST. MARTIN: This session has a somewhat different focus than this morning's discussions. It will be somewhat less data heavy and focus more on some of the special issues raised by organ, tissue and cellular transplantation, such as the implications of West Nile virus in the setting of donor shortages. Our first speaker is Bill Hobson. He is the director of the Office of Special Programs in the Health Resources and Services Administration.

Overview of the Organ Procurement and Transplantation Network

MR. HOBSON: Thank you, Laura.

[Slide]

I have been asked to provide a very general and very brief overview of organ transplantation and of HRSA's roles and responsibilities with regard to regulation of transplantation in the country.

First of all, HRSA has a dual interest in West Nile virus in regard to our transplantation activities, both soft tissue and bone marrow transplantation. In addition, HRSA provides direct funding for services for over 20 million Americans, most of whom are low income. Included in that group are all of the HIV-infected individuals and those with HIV AIDS. So, we have a particular interest in that a lot of the clients of HRSA-supported programs are probably at risk for West Nile virus due to nothing more than housing conditions, as well as a large number of immunocompromised patients.

[Slide]

I will just start by providing you with some general information. What I would like to do is to provide an overview of the organ procurement and transplantation network and an overview of the national bone marrow donor registry program.

[Slide]

The statutory basis for our federal involvement at HRSA stems from the National Organ Transplantation Act of 1984, as amended in 1988 and 1990. This created the organ procurement and transplantation network. That is a

network of all of the organizations involved with organ transplantation in the country. In addition, it created a scientific registry for transplantation recipients that gets involved with keeping data related to organ transplantation. We also provide grants to organ procurement agencies.

In 1984 we were charged with evaluating unrelated bone marrow donor transplantation and the feasibility of establishing a national registry for unrelated bone marrow donors. In 1988 we did establish the national bone marrow donor registry program, and I will provide you with a little bit more information on that and one of the next speakers will go into that in more detail.

Also, within the National Organ Transplantation Act of 1984, it prohibited the purchase of transplantable organs so this was actually the legislation that made the selling of transplantable organs illegal.

[Slide]

This is just a brief overview of where we sit within the Department of Health and Human Services. Under the Health Resources and Services Administration, we are one of several offices within the office of the

administrator of HRSA. The name of our office, again, is the Office of Special Programs. Within that, all of our transplantation oversight activities are housed in the Division on Transplantation and that unit is directed by Ms. Joyce Somsak who is here with us today.

[Slide]

HRSA's Office of Special Programs, under the Division of Transplantation--once again you see that the organ procurement and transplantation network is really administered by a grant that we make to the United Network of Organ Sharing which is known as UNOS. Once again, the scientific registry collects data both on donors and recipients, and then we have our national bone marrow donor program registry. All of our activities are generally involved with coordinating organ and tissue donor activities, and we also assist with the Secretary's initiative to promote organ donations.

[Slide]

The United Network of Organ Sharing is the organ procurement and transplantation network prime contractor. They handle most of the activities in this area. They maintain the organ waiting list and match patients to donor

organs. They administer the board and the different committees that we have in the organ procurement and transplantation network. They coordinate the collection of pre and post transplant data and facilitate policy development. At the bottom here, you have the URL for the web site that you should note as a source of information on organ transplantation. Most of the major information you want to access is available on that web site.

[Slide]

If you take a look at the whole organ procurement transplantation network general membership, it includes 257 transplant centers, about 59 organ procurement organizations, 158 histocompatibility laboratories, a number of voluntary health organizations and 10 general public members and 26 medical scientific organizations. So, as you can see, this is a very broad network that essentially includes a number of organizations, spread both geographically as well as by type.

[Slide]

If you take a look at the type of transplant programs we have here, here are some numbers basically that will give you the number of actual transplant centers that

we have for kidney, heart, pancreas, liver, heart/lung, lung, intestines and pancreas islet cell transplantation.

[Slide]

This is a chart to give you a perspective of the major increase that we have in the number of patients that are waiting for organ donation at this particular time. You will notice that, in the pink or rose colored line, the wait list is climbing at a very steep angle, much steeper than essentially the availability of organs both from cadaveric donors--you will notice the other line is for cadaveric organ transplants. We do have some growth in the number of transplants but the major growth that we are seeing right now is in the number that are waiting for transplant organs to be made available for transplant.

[Slide]

This is a chart that gives you an idea by soft tissue organ of how many patients are waiting on the waiting list. You will see that the first column there is for registration and the second one is for patients. You will notice that the number for patients, 80,103, is slightly higher than the number of registrations. The difference in these two numbers is that in some areas, some

organ procurement designated areas, you can list twice at different transplant centers. So, you have a slightly larger number and you have the number of registrations always slightly exceeding the actual number of patients that are waiting for organ donations.

[Slide]

This is a summary of the transplants facilitated by the organ procurement and transplantation network in calendar year 2001. You can see that we have a total of 24,110 transplants performed. So, with that, you can see that there are roughly about 2000 per month and you can see the breakdown of the different types of transplants that were provided during that year.

[Slide]

The regulatory framework for our activities is really housed in 42 CFR Part 121. That set of regulations was issued on March 16, 2000, and it is those rules that you would refer to if you are looking for the regulations that govern organ procurement and transplantation in the country. In addition, these regulations set our the OPTN board configuration, its membership requirements and basically other designated transplant program requirements.

Also identified are the specific data collection and reporting responsibilities and, last, the ACOT stands for the advisory committee on organ transplantation. We have a national advisory committee that has established reports to the administrator of HRSA, and through the administrator of HRSA to the Secretary on organ transplantation.

[Slide]

This is a schematic that basically outlines the policy development process that involves numerous committees and the OPTN board of directors. I think that it is important to note that with regard to medical care we follow very closely the guidance that has been developed by CDC with regard to the testing that is involved with organ transplantation.

[Slide]

We also administer the national bone marrow donor registry program in our Office of Special Programs. That is directly operated under contract with the National Marrow Donor Program, NMDP. We have outlined in the second bullet here the testing that is basically required with regard to bone marrow donation.

[Slide]

In terms of the history of the national bone marrow donor registry, it was set up in 1996 when the federal oversight was really changed from NIH to HRSA. In the Reauthorization Act of 1998 there were several specific requirements that were set forth that we have been working under since that time. One of those is development of quality standards and procedures.

[Slide]

For the National Marrow Donor Program network in the past year we have listed for you here some of the basic statistics. There are 149 transplant centers; over 4.6 million volunteer donors; 16 cooperative registries; 91 donor centers, 7 that are international. A number of the matches that are made currently for a bone marrow donation are made on an international basis.

[Slide]

In addition, we have 4.6 million volunteer potential donors in that system. Here, you have the number of transplants since 1987, 14,684, to give you some dimensions about how much bone marrow transplantation is really occurring in the country.

[Slide]

Here we are providing the URLs for the organ procurement and transplantation network once again and for the national bone marrow donor program registry where you can get additional information.

To date, we have had basically one confirmed case where we have had West Nile virus implicated in an organ donation. We have had four cases that resulted from the transplantation from a single organ donor. You saw the slides earlier today that outlined that particular situation.

You also basically heard Dr. Huang point out that in some of the tests that have been done you have a higher level of virus in some of the tissues than actually in blood. So, we have reason to believe that there can be West Nile virus transmission in the soft tissues of an organ, however, that is not proven thus far.

We have several other cases, most of which have been reviewed this morning, where we feel that organ donation has been implicated in West Nile virus transmission but none of those has been confirmed except the one case of a woman in Georgia where we had four organs that were transplanted and essentially four individuals

that received those organs all wound up being positive for West Nile virus. That is the only instance where we have documented the transmission of WNV through the transplantation of organs and kidneys.

We have most of the staff of our programs here, and I understand that we may be taking questions a little bit later. So, if you have specific questions with regard to organ transplantation or any of our oversight activities, we can take those up when we get to the question and answer session. Thank you very much.

DR. ST. MARTIN: I should have mentioned that we will be taking all of the questions at the end of the presentations so hold your questions if you can. The next speaker is Dr. Richard Freeman. He is associate professor of surgery at Tufts New England Medical Center.

Organ Donor Screening/Donor Acceptance in Extra-Renal Transplantation

DR. FREEMAN: Thank you very much. It is a pleasure to be here and, as was stated a minute ago, I am kind of going to change the whole tenor of the meeting considerably. There are a lot of ways I am going to do that. I am going to tell you first of all that I am not a

molecular virologist and I am not an epidemiologist. I am not a state public health expert and I don't work for the CDC or for the FDA.

[Laughter]

[Slide]

I am a transplant surgeon and I am the guy who gets the call in the middle of the night from an organ donor coordinator who gives me a clinical history and some serologic data on a potential organ donor. I take that data, as do all my colleagues who do the same thing around the country every night, and we have to synthesize all that down to a very simple resolution, yes or no; that is what it comes down to. It doesn't matter how many plaque-forming units there are or what the viral copy numbers are. It doesn't matter whether it is a beaded antigen assay or coated plate assay. I have to decide based on the data that is there and Jim and my colleagues do the same thing; it is not just me. Based on the data there, yes or no. It is just that simple.

I am going to focus my comments on the so-called life-saving transplants but, in fact, when you talk about quality of life all transplantation is life-saving and the

data is out there for every form of transplantation. But it is even more acute in these life or death cases so I am going to focus most of my comments on liver transplantation because that is where my expertise is but a lot of what I am going to say applies to heart transplantation as well.

The last thing I want to do, I want to emphasize to you that this is always a risk-benefit analysis that we do, and we are going to weigh the risks of doing something bad to the recipient with an organ versus the risks of something bad happening to the potential recipient who doesn't get an organ.

[Slide]

As Mr. Hobson just showed us a little while ago, the number of people who are waiting on the list is increasing. About one person every 13 minutes dies because there isn't an organ available for them in the U.S. In liver transplantation it is over 1000 deaths a year on the waiting list and overall it is in the range of 5000 deaths a year. So, people die who don't get them and I am going to try to emphasize that for you.

[Slide]

So, from my point of view, I am not talking about the blood donor pool, I am talking about solid organ transplantation--why screen? Why do we bother screening? Well, there are a couple of reasons. One, we need to assess the probability of transmission from a donor to a recipient. We need to limit the exposure of naive recipients to the lethal or life-threatening infections. I would like to point out that the word there is "limit." That doesn't mean we don't transmit infections and, in fact, I am going to show you data down the line here that we transmit viral diseases every day on purpose, knowingly, because the risk-benefit is in favor of doing so.

So, one of the things we are concerned about in the organ donation realm is that transmission of infection is not necessarily unacceptable. We want to know about it, for sure, and that is where these assays become important. We want to make informed decisions for our patients and we want to be able to inform our patients about the risks that we are going to take with potential infections, not to mention other donor characteristics. But it doesn't mean we are not going to do it. In fact, we do this routinely. We direct knowingly infected organs to recipients.

Hepatitis C, hepatitis B and CMV are the major examples that I am going to talk about, but we do this knowingly all the time.

It is also important to keep in mind that donors that are infected by whatever means, viral or otherwise, potentially their organs will not work so well either and we need to take that into account when we are assessing the risk and benefit for doing transplantation with a given donor to a given recipient.

[Slide]

When I get that call in the middle of the night, the coordinator does take a clinical history and assesses for risk factors including travel, and they now talk about CNS symptoms but, as somebody mentioned earlier, these are very non-specific in the case of West Nile virus and it can be very difficult to assess. These people are brain dead so by definition they have CNS symptoms.

[Laughter]

So, it can be very difficult to tease out symptoms specific for West Nile virus or some other encephalitis versus brain death. They pay very close attention and follow the CDC guidelines for high risk

behaviors. I put mosquito bites there to emphasize the fact that it is going to be very difficult in the case of West Nile virus for these clinical screening factors to have any use for us in organ donation and in blood donation as well.

[Slide]

We had a lot of talk today about all the various assays, and so forth. The goal, from my point of view, is that I need a rapid, sensitive and specific result indicating an active infection or the presence of a transmissible agent. Now, in the case of hepatitis B, in the case of hepatitis C and CMV, those viruses that I told you that we intentionally transmit lots of times, most of the time the donors are not viremic. You go back and you test their blood and there is no virus to be measured. We are using these serologic antibody and antigen tests because we know that the diseases can be transmitted to the recipient in the cases where there is no viremia. So, from my point of view again--it may be an issue for blood donation but from my point of view any exposure, any evidence of exposure, whether it is IgG, IgM, viremia or

whatever, indicates the possibility of a transmissible infection.

We used to think that hepatitis C and hepatitis B only resided in the liver years ago but, in fact, we know that we have transmitted these diseases with organs and with tissues and with blood in cases where no liver was involved. The fact of the matter is that these viruses reside in the macrophages, in the white blood cell population, and just about all the tissues everywhere. We saw some data earlier suggesting that that is the case for West Nile virus too. So, I think the issue of whether the virus resides in one tissue or another, from my point of view, is moot. If there is any indication of infection by whatever means, then that donor is potentially going to transmit the virus, and I think that is the way we need to approach this at least, again, from my point of view.

The difference with organ transplantation is the decision-making, the clinical decision-making. That yes or no answer is compressed time-wise into a very short period of time. It is not so much how long the organs can be preserved. We like to do liver transplantation within 12

hours and we like to do heart transplantation within six hours of removal of the organs.

But the problem is that you have to think about the donor situation. A family has just lost a loved one. The hospital operating room timing needs to be considered. There are lots and lots of things that are pushing to get this organ donor procurement process under way, finished and done. The organs have to be placed in the appropriate recipients. But generally speaking, test results need to be available within six hours. So, anything that is longer than that is not going to be useful to us. We would like it even shorter than that but six hours is what I would say is the time limit.

We also want to be able to have backup testing for the equivocal and positive results to confirm things down the line, and we routinely do that now. But these are things that are done after the fact.

[Slide]

Currently all organ donors are screened regardless of the risk factors. As I mentioned, diseases have been transmitted knowingly and unknowingly, and this is an incomplete list of all the documented cases that have

been out there. We screen for all of these things--well, most of them now with tests.

Currently the tests that are required under the OPTN policy that Mr. Hobson outlined are these that you see here. These are required and the policy requires that the test be FDA approved. I am going to show you a little bit of sensitivity and specificity data in a minute about these tests.

[Slide]

My own organ bank and many organ banks around the country screen for additional viral diseases, as you can see here, particularly the EBV virus which is very prevalent in almost all adults; 98 percent of adults are infected with EBV and it is transmitted. It is particularly a problem in giving organs to children who have not been exposed because then they are at higher risk for development of post transplant lymphoproliferative disease, a malignancy of the lymphoid system. Again, we knowingly, intentionally do that. We put those children at risk for the development of that disease because the benefit outweighs that risk. I want to emphasize that.

We also screen for anti-hepatitis B surface antigen. Again, we want to know if the donors have been immunized before. There is a lot of controversy about hepatitis B core antibody and the use of IgM tests. You know, we do the confirmatory tests for positives and these are just molecular tests that our organ bank employs. Interestingly, the HIV P23 antigen technology now has evolved so that test is available within six hours and we do that in equivocal HIV serology tests before organ donation proceeds.

[Slide]

It is important to note that the current policy states that organs from HIV or HTLV-1 positive donors are, quote, unsuitable for transplantation. However, there is a medical emergency escape clause in that policy that says that for emergent life-saving organs it is okay to use these; it is permissible to do this provided that the recipient has informed consent. Again, this issue is extremely important and is one of the reasons why the turnaround time on test results is critical.

[Slide]

The factors that affect transmission to organ recipients have to do, as I have emphasized, with timing of the tests, how fast you get them, and also where were the tests drawn relative to transfusion of blood products and the resuscitation of the patient? There have been several cases where the antibody screening has been negative because the blood was drawn in the middle of a trauma resuscitation where lots and lots of intravenous volume and blood products have been administered to patients, and all you are really doing is measuring the resuscitative fluids levels of antibodies, not that patient's level of antibodies. So, the timing of those tests is important.

We heard a lot about the windows of false negative, false positive, windows of viremia, development of IgG, IgM and so forth. Those can all play a role in giving false-negative results in potential donors. The specificity and the sensitivity of the test can also affect whether a virus is transmitted or not. Also, the virulence of the organisms. That is one of the reasons why we knowingly accept transmitting a lot of these viruses because we know the virulence is there but it is controllable. I will show you some data about how we do

that now. Finally, we treat donors and recipients to diminish the risk of transmission or the consequences of infecting a recipient.

[Slide]

These are the tests that our organ bank, the New England organ bank uses, up in Boston. It shows you the product name and the sensitivity and specificity. You can see that even for hepatitis C the sensitivity is not super ideal and we do have false negatives and false positives, and these organs are sometimes discarded because there is no rapid confirmatory test. But oftentimes we go ahead and say, well, there aren't any other risk factors. The recipient can't wait; we are going to use this organ and pay the consequences. We tell the recipients that. In fact, we tell all recipients that all the screening tests for blood donors and organ donors are not perfect and that they may face the question of whether they are willing to accept an organ from somebody who tested positive, with a test that may be imperfect, or who tested negative but, in fact, was positive after the fact. So, patients need to be informed of this but it doesn't mean that it can't be done.

[Slide]

These are the other tests that our organ bank uses that are not required testing but, again, you can see that some of these sensitivity and specificity levels are not yet fully documented even but we are still using these tests to screen donors. Again, this is to give us educated guesses about risks and benefit for individual donors in transplantation.

[Slide]

We have talked a lot about the consequences of infection but what about transmission to organ recipients? The recipient, obviously, can get infected; they can lose their graft or they can die. Those are the main factors. So, we have to assess every time, as I mentioned, what are the donor and recipient risk factors, the mortality risk for heart and lung candidates.

I am going to show you some data that we have looked at very carefully under the OPTN policies about a score that defines the risk for liver recipients while they are waiting on the list, very precisely defines their risk of dying within three months so we can assess that. That has to be weighed against the donor factors and the

possibility of graft failure or mortality of the recipient due to donor factors.

And, it is not just infection risk that we have to evaluate but the organ quality itself. How injured was the organ during the brain death process? How old was the donor? There is data that suggests that organs from different races don't function quite as well as other races. Gender makes a difference. The cause of death, whether it was traumatic or intracerebral bleeding also can make a difference in the organ function afterwards. There is even data to suggest that somebody who is a smoker, because of oxygen transport not being as good, will result in poor organ function afterwards.

There are other donor co-morbidities, incidence of hypertension, diabetes and many other factors in the donor himself, plus the infections. So, all of these donor factors--and, to be truthful, the infection risks are part of but certainly don't play the major role in the evaluation of these organ donors.

[Slide]

This is the MELD model for the risk of mortality while waiting for a liver transplant. This has been

validated in several studies. We published a paper just recently for where this graft came from but, basically, this score on the bottom is calculated based on laboratory tests of candidates waiting for liver transplants, and it defines your mortality risk within three months if you don't get a transplant. The PELD score is for pediatric patients and I am focusing mostly on adults here, but you can see that if you have a MELD score of 35 your chance of being alive three months later is 22 percent without a transplant. So, your mortality risk is 78 percent. You are going to die within three months. So, we have to take that into account when a donor is being referred to us for a patient who has that kind of mortality risk.

[Slide]

I am just going to go through these cases that have been documented where virus has been transmitted unknowingly and knowingly, and I want to show you the data that is out there just to give you a flavor of what kind of risks we are talking about.

We saw lots of slides about media hype and West Nile virus, and it is true with HTLV and HIV as well. I think it is important to look at actually what has happened

out there. Now, HTLV is a virus that has been implicated as a cause for hematologic malignancies. There has been a lot of concern about this when these reports first came out. It is well-known that it can be transmitted with blood transfusion or with organ donation, and there was a lot of concern about inducing malignancies in organ recipients.

As this data was coming out, in the process of the testing getting implemented and so forth, actually 22 organs were transplanted. The policy was developed that said that those organs were unsuitable for transplantation, but I searched the literature again--this is an article from AIDS from 2001, and I could not find a documented case where HTLV-1 or 2 was transmitted from an organ donor to an organ recipient of any organ type where a hematologic malignancy was identified in that recipient. Currently those donors are unsuitable for transplantation by regulation.

Hepatitis C-positive donors--there is approximately a 50 percent transmission rate. These are patients who are serologically positive and, again, the test is not 100 percent sensitive or specific. Several

studies have looked at the patient and graft survival rate for recipients who receive a hepatitis C-positive liver who are either hepatitis C positive or negative. That is, the donor is either positive or negative and all the recipients are already positive for hepatitis C, which is the most indication for liver transplantation, and there is no difference in the outcome. So, transmitting hepatitis C from a donor who is infected to a recipient who is already infected doesn't change the recipient's outcome relative to getting an organ that was not infected with hepatitis C. But, again, you know that those recipients have a mortality risk without the transplant.

[Slide]

A very interesting issue, patients who are positive for hepatitis B surface antigen by definition have some form of active infection. It has been thought that hepatitis core antibody positive patients have cleared their infection and this is simply not true. Results have shown that the transmission rate is much higher from organ donors than from blood donors, in some reports as high as 90 percent. The fact that the recipient has anti-hepatitis B surface antibody may protect the recipient. We try to

inoculate all of our recipients with the vaccine prior to transplant but, unfortunately, in liver failure patients the vaccine is not so effective.

Interestingly, and this goes back to my previous point, the absence of hepatitis B viral DNA in the serum of the donor does not preclude transmission of hepatitis B infection to the recipient. So, viremia is not a necessary precondition for transmission.

[Slide]

We routinely transplant CMV-positive organs into CMV-negative recipients. It used to be, when I first started in this field, that there was about a 30 percent mortality rate if you got a CMV infection and about 50 percent of patients got some form of viremia after transplantation. But effective prophylaxis has been developed so we actually have a treatment for the recipients. We can treat those recipients now and even though 50 percent of them still get viremic, the mortality rate is down to two or three percent from CMV disease. If we prophylaxed every recipient though it would be extremely cost inefficient. It would be way too expensive to do. So, donor screening has allowed us to effectively target

the treatment of those recipients and still transplant those infected organs and actually infect those recipients with the organs because we have accurate screening that allows us to target the prophylaxis.

[Slide]

Now we get to the more emotional infections. In the MMWR, back in 1987 there was a case reported of an HIV-positive donor transmitting infection to organ recipients. The initial donor was negative by EIA but retrospectively was strongly positive. The reason he was negative was because the donor got 56 units of blood in the process of the resuscitation. That is my point about timing. The post donor transfusion sample though was positive as well.

One renal recipient got the organ, was Western Blot positive but has not developed any sequelae that we know of at this time, although the virus is still detectable. The same is true with one liver recipient. The heart recipient from that donor died intraoperatively or a day or two after the heart transplant procedure. The other kidney was not transplanted because it became known that the donor was HIV positive. Again, these organs are now labeled as unsuitable. I would put to you that they

may be unsuitable for lots of patients but currently, in the current era of our better treatment for HIV, that may not be the case.

[Slide]

Lastly, the West Nile virus case that we discussed. One recipient died. Two other patients got severe encephalitis and required mechanical ventilation. Neither of them developed high levels of antibody afterwards but the other two patients, from what I understand, are recovering.

[Slide]

How do we assess these risks and benefits? Well, currently there is a move to start doing organ transplantation in HIV-positive patients. In fact, 40 percent of HIV-positive patients are also hepatitis C positive, and hepatitis C is not easily treated these days and we are going to be facing liver transplantation for those patients in the future in a big way. There is already a national trial under way to look at the results in those patients. But I would put to you that having an absolute regulation that says you can't transplant HIV-positive organs is not effective at least for those

potential recipients, especially with the data we have available now.

For HTLV the same thing is true. Maybe if you have a high MELD score we should not worry about HTLV infection because the risk of malignancy, at least in the documented cases that we have there, seems to be quite low. The risk of dying without a transplant is quite high, especially for the high level patients.

Hepatitis B--we routinely transplant core antibody organs into naive recipients now because we have effective drugs to suppress viral replication and effective treatment modalities to suppress viral replication and these are well-documented studies.

As I mentioned, HCV-positive organs are transplanted routinely in HCV-positive recipients. The question then is what about West Nile virus? How are we going to handle that? We know that patients can die. That has happened. What is the morbidity and mortality?

[Slide]

You have seen this before. We have to assume that West Nile virus is endemic. What was interesting on the graphs I saw was the analogy to the St. Louis

encephalitis outbreaks. Are we seeing an upswing in the curve of West Nile virus, or is this it and it is going to go down to be the way St. Louis encephalitis is now where it hasn't happened or we haven't had an epidemic in a while? I don't know.

[Slide]

The other thing that is interesting is the seasonal nature of this. I talked to Barbara Werner, in the state lab in Massachusetts, and the latest case that was seen in Massachusetts so far in a bird was in November. Barbara says they found some mosquitoes that have survived the winter in Massachusetts, so I am sure they can survive the winter further south.

The other question is age relationship. Is that a risk factor? If an older person has encephalitis, are they more likely to have West Nile virus than somebody who doesn't? And, what about the other risk factors? Again, I mentioned how unlikely it is that these are going to be helpful for us.

[Slide]

Transmissions can cause recipient death and historical risk factors are not likely to cull out the high

risk donors. But the exclusion of potentially infected donors by history would severely limit the donor pool, blood and organs but inclusion of all West Nile virus patients would likely lead to more recipient problems.

There are tests out there that are being used, and I think the goal should be that we should have a serologic test by next season at least for screening purposes for organ donation. Thank you very much.

DR. ST. MARTIN: Our next speaker is Dr. James Burdick. He is professor of surgery at Johns Hopkins Hospital.

Donor Acceptance in Renal Transplantation

Viral Disease Transmission in Transplant Recipients

DR. BURDICK: I would first like to congratulate the CDC, HRSA, FDA and all the units in HHS that have put this together. I think it has been excellent. Rich and I have discussed how much we have learned and I am very confident that this is going to lead to rapid improvements in what all potential patients in this area need. So, again, I thank you.

I also ceded a little bit of my time to Dr. Freeman because we are talking to a large degree about the

same thing. So, I will repeat some things and take much of what he said as sort of a basic underpinning for what we are going to be talking about in terms of kidney transplantation.

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What I want to do first is talk a bit about the realities of kidney transplantation, which are similar to but a little bit different from liver transplantation, and then give you a couple or three slides on my sort of sense of where we are right now and where the problem that is presented by the West Nile virus issue interfaces with the renal transplant community.

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You have seen a couple or three examples of what the problem is. This just illustrates it from the kidney point of view. The number of patients on the wait list for kidneys continues to rise more and more asymptotically. You can see that the cadaver donor population has been almost stable, and one of the things that is happening is that there is some increase in the number of living donors.

I would like to reassure HRSA that we all agree that buying and selling organs is illegal. In fact, this

is about the only funny thing I have to show you today and, in retrospect if you think of it, it is not so funny, but it is the National Enquirer's attempt to make something entertaining, but it is not reality. Seriously, in blood banking the issue of selling blood has been, I know, an issue and I just reassure people that we don't need to worry about that in regard to the topic at hand in the foreseeable future.

What we do need to worry about even in kidney transplants is that we are in the process of risk management. We cannot make the process of risk zero and serve this population waiting for transplant even in the case of kidneys.

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There are two things I want to mention about improvement in our ability to get organs to patients. One is the increased use of living donors that I mentioned. This really started with the understanding that you don't need a really good tissue match, shown initially by living donors from spouse to spouse and now it has become much more common in living donor transplantation in general.

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The particular issue with regard to West Nile virus is emphasized on this slide from some work done at my institution by Dr. Montgomery mainly and the team that works on this in a multi-specialty way. This involves the problem which is to have a living donor which could be used but can't be because of a positive cross-match. By intensive plasmapheresis and IVIG it is possible to take patients with high antibody levels, high percentage of PRA, and after using the pre and post treatments using plasmapheresis and IVIG, result in a good postoperative outcome, median follow-up of 22 months, creatinine of 1.2. These are essentially patients, 50 or 75 percent of whom would have rejected their donor if this treatment hadn't been done. So, I think this represents a very promising new horizon in transplantation.

The issue for us is that plasmapheresis, as some of you may know, tends to deplete everything floating around in the blood, including clotting factors, and these patients need an operation during this treatment process. So, a lot of fresh-frozen plasma is used and that is where the problem with West Nile virus comes in.

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So, living donors are one way to increase the pool of recipients. The other way is to use cadaver donors that have what are called expanded characteristics. There are two or three things to note. In the first place, Dr. Freeman said that heart and liver are life-saving but really other organs may be, and this is very strikingly illustrated in the data I am showing you here from Dr. Ojo and colleagues in Michigan.

If a group of patients who receive a transplant are compared, in terms of a cohort with the right waiting time and the right characteristics otherwise, with a group of patients who are in the same boat, are on the list but by happenstance don't get the transplant, you can see that the risk of death increases to over three-fold in the first few weeks or days because they have an operation etc., but it drops precipitously. Looking at the ideal cadaver donor recipient by three months, a little over three months, the chances of dying are less. So, in essence, this is a life-saving process even though it is just a kidney transplant.

Even more important here is that we can take donors that years ago we wouldn't have used, that are hypertensive, that are older, and that may have other

characteristics that make then less than ideal and, sure, those recipients don't benefit quite as much but well within a year they are also benefiting from the mortality benefit.

So, we are talking about being able to expand what we do partly with, for instance, older donors and, as we have heard, older donors are a little more likely to have problems with West Nile virus. So, these are all things in the back of the minds of those making these decisions in the middle of the night that Rich mentioned with regard to kidneys.

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He told about how we do this so I won't spend a lot of time on it but just mention and reinforce what he said. If the donor is CMV positive we rarely pay any attention to it. If it is RPR positive every patient gets the equivalent of a dose of penicillin and we don't worry about it. We do a PCR to confirm a negative hep C antibody. It is not required. We generally try to avoid transmission in this group, in the ways that he mentioned a little bit more carefully than with livers, because the

life-saving characteristics are a little bit less but the principles that he talked about really are the same.

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I thought you would be interested in an estimate of how we do it, so this is a look at the transmission rate for kidney recipients, looking at their hep C antibody at one year post transplant. These are recipients who were negative at transplantation. Now, it is about a two percent transmission rate on this slide. It is worth showing you this but you may stop and think, well, this isn't a very good way to look at it.

There are two sort of general problems. One is that you have to remember that exactly when the negative was done is a question. It may have been months before the actual transplant. We don't do these on a daily basis on everybody. Then, obviously, this is at one year so there has been lots of opportunity afterwards.

The other thing is the data in the UNOS database, the OPTN HRSA UNOS database, are really unparalleled with a national, very well established and complete and validated database, but they are not perfect, and there is noise in here that I suspect, by virtue of tests that weren't done

or that were reported incorrectly, probably swamps out the exact percentage. It must happen occasionally. It is hard to be sure that it happens very much. I am quite sure it is not two percent, and all of those factors go into that but certainly we do a very good job.

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How would we go about applying these principles to kidneys? Well, donor history, as has been pointed out, is pretty useless. That is an important part of what we do when we get these calls. We hear quite a bit about the donor but, unfortunately, the lurid stories that we sometimes hear that might allow us to rule out other infectious sources really aren't very valuable. A mosquito bite pales by comparison. So, it is really a little hard to know.

The one thing that I think we are going to wait with bated breath to hear more about is the interesting point that has been made in a few things that have been looked at where you could tell something about the donor. The transmission seems to involve symptomatic donors at much a higher incidence than the usual incidence of symptoms when somebody gets infected. So, it may be that

symptoms will do a little bit better screening than we think. The American Association of Organ Procurement--I think you are going to be hearing from one of their representatives today or tomorrow--have unexplained encephalitis as a rule-out but, of course, that is a problem since only 1/150 who are infected get encephalitis.

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Serological screening--we really have about a three-day turnaround, even though it sounds like it ought to be a little faster. There are false positives. The persistence of IgM is a real issue. With a living donor we probably could utilize present technology and do it a few days before the scheduled transplant but, as Rich mentioned, for kidneys and all organs the organ procurement organization needs about a six-hour turnaround and we don't have that at present.

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One thing that I have found some cause for comfort is this computer-modeled analysis of the index cases of the epidemic in Queens in '99. You have probably all seen this. These bars represent the period of time that the patients were viremic. Each dot is a patient. By

the time you get about half way into the epidemic many of the patients that were viremic here are already no longer viremic and between that very short interval and the low transmission rates that have been noted and the seasonal character of it, I think all of these factors mean that the risk to an organ transplant recipient, given reasonable precautions, is minimized.

That is very different, for instance, from hepatitis C. If this were a hepatitis C line it would start here and it would go on forever. So, once the serology is there the donor is a potential risk. Even if you know that this has happened really the only question is whether you are really close to that event or not, at least in the vast majority of cases.

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So, for kidney transplants infection from a donor, it appears that maybe it is in cells other than floating around in the blood although it is still possible because we can't get every bit of blood out of a transplanted organ. The transmission would essentially be by blood that is residual in the organ and not in the organ. The presence in blood is not a great concern in

kidney transplants in general because you usually don't use a blood transfusion for a kidney transplant, although for special cases you may need it. But the plasmapheresis issue that I mentioned for using a large amount of FFP is really a particular concern.

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We need a rapid turnaround assay, as was mentioned. That is really the thing that would be nice. In fact, if we had that for a way of looking at IgG over IgM to give us a sense of the temporal situation, that is really what transplantation needs I think. There has been talk about sensitivity of the tests, and so forth, but really the sensitivity of these tests is very good. What we really need is the temporal relationship because that is what would tell us whether we have a viremic or infected organ that is likely to be transmitted because of the very short interval of infection that occurs.

Right now we are attempting to think about, if this happens again, use of immune globulin. That may help; It is not clear it does. I was delighted to hear about vaccination earlier today, and I think for living organ donors and maybe some others that may be something the

transplant community will think about more. I certainly need to look into that.

So, that is sort of an overview of the similarities and differences from our point of view in terms of the kidney transplant field, and I thank you for your attention.

DR. WILSON: Thank you very much. That was very nice. Both Dr. Freeman and Dr. Burdick gave us a nice overview of how we deal with the risk of West Nile virus in the setting of organ transplantation.

Our last few talks are going to switch gears a little bit to the areas of tissue and cellular transplants. The first talk will be from Dr. Dennis Confer who is the chief medical officer of the National Marrow Donor Program.

Marrow Donation/Transplantation Issues

DR. CONFER: Thank you very much. I would like to thank the organizers for inviting me to talk today.

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As was mentioned, I am from the National Marrow Donor Program, and we do hold a contract to operate the national bone marrow donor registry, a contract that was

recently renewed with our organization by the Health Resources and Services Administration.

In my talk today I am going to not talk solely about unrelated donor transplants, but I am going to approach this really from the standpoint of what I believe may be the concern for all hematopoietic cell transplantation and concern about the West Nile virus.

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These are some annual numbers to give us an idea about the numbers of patients who may be at risk for infection with West Nile virus or similar agents. There are about 17700 hematopoietic cell transplants annually in the United States, and this number is similar to the total number of organ transplants which you heard earlier, around 24,100, in the same ball park as the number of new cases of leukemia annually, around 31,000. All of these numbers really pale in comparison, for example, to the number of new cases of cancer, over 1.2 million new cases annually.

Among the hematopoietic cell transplants, about two-thirds of them are autologous transplants. These are transplants in which the recipient is also his or her own donor. So, the recipient provides the blood stem cells

that are later transplanted back in following some type of high dose chemotherapy. The reason I point this out is because these transplants are much less immunosuppressive than allogeneic transplants. The allogeneic transplants number around 6700 annually, and about three-quarters of these use a matched sibling donor. So, these are brothers or sisters who are HLA matched with the transplant recipient. About 1800 unrelated donor transplants are performed annually in the United States and, as I indicated, the NMDP facilitates the vast majority of these.

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Given these relatively small numbers, I think it is reasonable to be somewhat alarmed that out of the 33 cases we heard about earlier under investigation as possible transfusion transmitted West Nile virus, 12 of these are hematologic disorders, including six hematopoietic cell transplants, five organ transplants and, in contrast, only three patients with other types of cancer. So, what this suggests or the question that is raised is really what is the risk of West Nile virus in the setting of hematopoietic cell transplant or in the setting of solid organ transplant?

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We head these numbers this morning. In the normal population, the risk of infection with West Nile virus is potentially on the order of 1/1000 if, indeed, there were over 300,000 infections in the United States this past summer. Among the infected, symptoms occur in about 20 percent. We have heard that this is a number where there may be a significant margin of error. Meningitis and meningoencephalitis, however, occurs in only 1/150 and the lethality of the infections is rarer still.

I submit that we have no idea what these risks are in the hematopoietic cell transplant recipients. However, it may be that they are substantially higher, and it may well be that HCT recipients are much more likely to be infected and that those who are infected will almost all develop symptoms and could develop CNS disease and there could be a high rate of lethality. So, I think we don't know the answer to this but I think it is concerning nonetheless.

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Why are HCT recipients more likely to be infected than the rest of the population? Well, certainly

hematopoietic cell transplant recipients can get mosquito bites just like everybody else. If you are from Minnesota like I am, everybody gets mosquito bites. In the spring the lakes thaw around April 15. The first skier is out on the lake the day after the ice goes out and usually the first mosquito bite is the day after that. So, it is a very long season up in Minnesota.

But the hematopoietic cell transplant recipient has other sources and chief among these is the hematopoietic cell progenitor product itself, the stem cell product. Now, in contrast to solid organ transplant, this is effectively a blood product. It is collected from the blood stream or from the bone marrow space and I think it in all likelihood represents the same risk of infectiousness as blood does. So, the other potential risk is from blood products themselves.

Among these three sources of risk, I believe the risk is certainly greatest from the blood products. That is simply by sheer numbers. Transplant recipients require a lot of blood support. This is predominantly in the form of red blood cells and platelet transfusions. They are initiated early in the post transplant period and may

continue for weeks or months after the transplant. I think probably depending on how the transplants are conducted, whether you use single donor platelets or pooled random unit platelets, there may be anywhere from 20 donor exposures on a minimum to well over 100 donor exposures for these hematopoietic cell transplant recipients. So, in comparison, the donor product itself is just another donor exposure. So, the majority of the risk I think lies here.

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I have tried to show that on this slide, which is my attempt to show a calculation of the risk of receiving at least one infected unit versus the number of potential donor exposures. I have done this for three different levels of infection. So, this is three units per 10,000 infected; ten units per 10,000 potentially infectious; and 30 units per 10,000 potentially infectious. I think what this shows is that although these are in the single digit risk levels, they are, nonetheless, substantial once you start getting up into 40-60 donor exposures.

We heard data from Dr. Petersen this morning that at least in some regions of the country the rate of potentially infectious individuals was over 10 maximum

points, granted, for short periods of time. But if these infections are serious in the hematopoietic transplant recipient, if they carry a high lethality, then almost any risk of exposure that exceeds one percent is probably way too high.

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What I wanted to talk about now is turning attention to the HPC donor. I think to summarize the first part of the talk, what we really need in hematopoietic cell transplantation is screening of blood donors. But if we have that, or once we have that, what will we do about the hematopoietic progenerator cell donors themselves?

I think it is probably not a big issue for autologous transplantation. You could get into arguments about whether if you collect autologous cells at a time that a person is viremic and then infuse them after high dose therapy they are at risk for reestablishing infection. I don't think we have any idea about that, or whether they would have sufficient antibody to neutralize that virus. But I think it is probably not an issue so much for autologous transplant, although it is not entirely clear.

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I do think that allogeneic transplants, however, present a greater problem for several reasons. One of them is the way that we have learned to do allogeneic transplants in the world. This is pretty much the model that is used. It is really sort of a "just in time" model of transplantation. By definition we always count the day of transplant as day zero, and the days leading up to transplant are the minus days, minus seven, etc. The model that is used in the United States and throughout the world is that the actual collection of donor cells occurs on or the day before the transplant. So, the goal is to collect cells and infuse them fresh into the recipient on the day of transplantation.

This creates a problem because during the week typically prior to transplant these recipients are receiving high dose chemoradiotherapy in order to prepare them for transplant. This therapy by itself, in the absence of transplant, is lethal in the vast majority of cases. There are these newer non-myeloablative strategies that are coming along but, still, for practical purposes once this therapy is initiated you have to do a transplant. What that means is that donors are cleared prior to

initiation of the recipient prep, either immediately prior to initiation or in the weeks or days preceding the initiation of recipient prep.

While it is possible that you could test a donor at some point close to transplant, getting the results of that test is not going to be particularly helpful if the test is positive because really you have committed to performing the transplant on the day you start the recipient's preparative regimen. We could easily test donors back here or question donors back here about symptoms but when we do that we are obviously creating a fairly significant window during which time the donor could be exposed to West Nile virus and become viremic by the day of donor collection.

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An additional problem that comes up concerns the donors of peripheral blood progenitor cells. Traditionally, bone marrow was the first hematopoietic stem cell source that was used for transplant, but currently at least 50 percent of the allogeneic transplants in the United States are performed using peripheral blood progenitor cells. In order to do a peripheral blood

progenitor cell transplant, the donor himself has to be mobilized and this is done by administering hematopoietic growth factor, most commonly recombinant human GCSF given subcutaneously once or twice a day in the five days prior to the actual collection of the blood stem cell product. So, these donors begin mobilization shortly after the recipient has initiated their preparative chemoradiotherapy. We do this with both related donors and with unrelated donors.

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The reason this is a problem is because these donors develop symptoms consistent with West Nile virus. This is data from the NMDP on just over a thousand peripheral blood stem cell donors. What you can see here is the frequency of symptoms. The single most common symptom experienced by almost all donors is bone pain, but look at myalgia, headache, malaise, insomnia, nausea, sweats, other flu-like symptoms, anorexia, fever, chills. I mean, these people have the symptoms of West Nile fever. So, it is impossible really to know during the mobilization phase whether these people are also experiencing symptoms from a virus infection.

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So, what if we said, well, let's collect and quarantine the stem cell product? We know that we can freeze these hematopoietic cell products and then thaw them on the day of transplant and infuse them. So, perhaps we could evoke a model like this where a donor is cleared, and then the donor is collected. This looks like a bone marrow collection since collection occurs right after clearance as opposed to PBPC which would require this mobilization phase. But collect the donor, then freeze the product and then, in this model, you could test and wait for a test to come back. Once that test has come back negative, you could initiate the recipient's preparative regimen and thaw the product and infuse it on the day of transplant.

This is technically feasible and possible. This is only possible if we have a test because in the absence of a test we would really need a much larger window here if we were looking for the development of symptoms. However, as has been pointed out repeatedly, this is probably not reasonable because only a minority of people will develop symptoms. So, waiting for symptoms to develop when they

may not, even in the face of viremia, is illogical. So, this model probably goes away entirely.

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We are left with the model on the preceding slide if there is serologic testing available or other virus detection testing. But I don't think this eliminates some significant problems with freezing and quarantine. One is that in the processing associated with freezing, it reduces the content of hematopoietic progenitor cells. So, if we did this we may face an increased risk of non-engraftment, repeat transplantation, etc. and mortality related to poor graft function simply as a result of having frozen the cells. There has never been a comparison of cryopreserved transplant versus non-cryopreserved so we don't know what cryopreservation does to the overall effectiveness of the transplant.

Also, this strategy may delay transplant for urgent patients. That is really the whole idea behind the "just in time" philosophy. Once you have cleared the donor, if it is a very urgent transplant you can start the recipient prep the next day and give that preparation and then do the transplant. There are, frankly, many patients

who have life-threatening leukemias, unstable leukemias, lymphomas and other disorders for whom delays of even a week or two are not allowable and would have negative impact.

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Finally, although we don't like to think about the cost alone, this would add significant expense to the allogeneic transplant procedure in return for eliminating only a modest amount of the risk.

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What about cord blood? This is my comment about cord blood, "what about cord blood?" I don't think we know anything about whether the cord blood units that are currently being collected and frozen away in the United States contain in some cases West Nile virus or not. We don't know about transplacental passage. We know that many of these cord bloods are contaminated with maternal blood at the time of collection and that, by itself, may contaminate units. So, I submit that this is a big black box and I don't have any inkling of what to think about it.

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So, what will the National Marrow Donor Program do? We are going to question donors about West Nile virus before donation and/or before their mobilization procedure. We have drafted questions and are in the process of trying to figure out how to disseminate those out into our community, our network.

We will also add instructions post donation for the unrelated donors to report new onset symptoms so that if a donor develops symptoms that could be consistent with West Nile virus infection we can follow up on that and perhaps have at least some opportunity to consider an intervention for the recipient who has already been infused.

I say we will likely adopt testing when a screening test is available. I say "likely" because there are still these logistical issues about the timing of the collection and whether we are actually narrowing the window sufficiently for it to be meaningful. At the current time we will not recommend a product quarantine procedure or freezing of these products because, again, we feel that the vast majority of the risk comes from blood products and not from the hematopoietic progenitor cell product.

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My summary and conclusion, West Nile virus infections may present significant new risks for HCT recipients. Effective blood donor screening may eliminate the majority of this risk. Effective screening of the HPC donors themselves is problematic and again, to echo the comments of the previous two speakers, in the end it is physicians and their patients who must weigh the risk versus benefit. Thank you very much.

DR. WILSON: Thank you. Is Dr. Harlan here or somebody else from NIAID to present the next talk? We were supposed to have a presentation by Dr. Harlan who was going to give us a similar discussion about the risk of transmission in the pancreatic islet cell setting. Unfortunately, I guess he wasn't able to make it today so we will go on to our next speaker, Dr. Melissa Greenwald, who is a medical officer and also sells tissues and gene therapies.

HCT/P Transmission Issues

DR. GREENWALD: I will be talking today about infectious disease donor screening issues with human cells,

tissues and cellular and tissue-based products, which I will call HCT/P from now on, if you don't mind.

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Specifically, I will be talking about current as well as proposed donor eligibility requirements for infectious disease screening and testing, current industry standards and issues for future development in this area.

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The regulations for current requirements are found at 21 CFR Section 1270, and 1270.21 specifically requires screening for HIV-1 and 2, hepatitis B and hepatitis C. Section 1270.31 has additional requirements that there be procedures that are written, prepared, validated and followed to prevent infectious disease contamination and cross-contamination during tissue processing.

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As proposed in September of 1999, Sections 1271.75, .80 and .85 would require donor screening and testing for what is called relevant communicable diseases and disease agents. There are two parts to the definition for relevant communicable disease. The first part

specifically defines HIV-1 and 2, hepatitis B, hepatitis C, HTLV-1 and 2, transmissible spongiform encephalopathy, syphilis, CMV and gonorrhea all as being relevant communicable diseases.

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The next part of the definition is a little more tricky. It defines relevant communicable diseases to include agents or diseases for which there may be a risk of transmission by an HCT/P because it is potentially transmissible by the HCT/P, and it could be fatal or life-threatening, or it could result in permanent impairment of a body function or permanent damage to a body structure, and for which appropriate donor screening measures have been developed and/or appropriate test for donor specimens has been licensed, approved or cleared for such use by the FDA. Obviously, this has been written to cover things that are coming out that we don't know about right now.

Even under the proposed donor eligibility criteria, West Nile virus would not yet be able to be considered a relevant communicable disease. We don't have appropriate screening measures and there is no FDA approved test. West Nile virus may in the future be considered a

relevant communicable disease when an appropriate screening test is developed.

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So, there are some industry standards for screening that could be helpful. The American Association of Tissue Banks recommends deferral of potential donors with degenerative neurologic disorders of viral or unknown etiology, and the Eye Bank Association of America recommends the deferral of potential donors who have viral encephalitis or encephalitis of an unknown origin, as well as neurologic disease of unestablished diagnosis.

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Both AATB and ABAA recommend that potential donors with septicemia, including viremia, be deferred. The American Society for Reproductive Medicine guidelines recommend that donors should be healthy.

[Laughter]

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Of course, none of this is going to screen out the majority of people who have West Nile virus, as we all have heard today and know. Though West Nile virus is asymptomatic in 80 percent of people who get the disease,

it is still important for ACT/P establishments to pay close attention to the already established screening criteria because those criteria could decrease potential risk of transmission. It would also be prudent to consider the quarantine of inventory and retrieval of unused tissue in cases where an HCT/P establishment becomes aware of a diagnosis of West Nile virus. There will be further work in this area as more information becomes available.

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So, there are concerns for the future, and screening alone will not eliminate donors with asymptomatic disease and tests will be needed in order to significantly reduce the potential risk of transmission of West Nile virus by HCT/Ps.

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Maybe HCT/P donors are cadaveric and it is really problematic to require testing of samples obtained after death with screening tests that have not been validated for this indication. So we, of course, strongly encourage anyone who is developing tests for screening blood donors for West Nile virus to also please consider testing cadaveric blood samples up front, and to seek an indication

for use of cadaveric blood. That is all I have. Thank you.

Discussion

DR. WILSON: At this time I would like to invite all the speakers from this session to come sit up at the front, and we would like to take questions from the audience.

DR. WOLL: Dr. Judith Woll, from Community Blood Center in Dayton. Given the history of problems with cadaveric blood tests, would you encourage the manufacturers to make tests on lymph nodes for tissue donors?

DR. GREENWALD: What was the last part of that question?

DR. WOLL: To develop assays on lymph nodes for cadaveric donors?

DR. GREENWALD: Certainly, if anybody is able to do that.

DR. FREEMAN: I would just say, being familiar with tissue donations through our organ bank, that the majority of tissue donors are not solid organ donors. Routinely, lymph nodes are not taken from tissue only

donors, although they can be. If it is necessary to do that, they can be.

DR. WOLL: If there were a licensed assay for any virus on lymph nodes, I am sure the tissue banks would be thrilled to do it.

DR. FREEMAN: It would be a lot easier for them if they could do it with blood though. I would say that it would be a better way to go.

DR. GREENWALD: We certainly have all the problems of not knowing how much virus you are going to find in nodes as opposed to blood, and stuff like that. So, there would be a whole lot of science that would need to go into that before we could make a decision regulatory-wise about what to do.

DR. GOODMAN: Jesse Goodman, FDA. I just want to sort of put something out there because it is a confusing area, especially since there is so much new knowledge here, but it is very important, as Lyle Petersen said early today, to point out that in these cases that we have studied so far where there is well-documented transmission, whether by transplantation of organs or by blood

transfusion, these patients have all been antibody negative.

So, in terms of thinking about antibody tests for screening, we are either going to have to find a much more sensitive IgM test that turns positive earlier on, or we are going to have to go not to antibody screening but to detection of the virus itself. It is different than the other infectious diseases we normally screen for in organ transplantation. There is a parallel to HIV, except really all we are looking for here is the window period.

Let me just add to that a little. It may be that there are some patients, particularly tissue and organ donors, where antibody testing will also be needed because it could be that you clear virus from the blood but it is still present for some time in organs and tissues. So, it may be that for tissues and organs we need a combination of virus detection and a good IgM antibody test. But ideally, as somebody alluded to, we would want one that doesn't stay positive for a year and exclude donors from this organ donor pool that is so life-saving.

I hope that comment is clear and helps keep this a little bit in perspective, but at least in blood

transfusion we are really dealing with mostly probably the window period and in tissues and organs probably both the window period and perhaps the early IgM positive period. People have said, unlike CMV and hepatitis viruses, we don't know of a chronic carrier state where this would be present in the organ for years and be potentially only detectable by antibody testing.

DR. FREEMAN: If I could just comment to that, I think the most important thing you said was we don't know. We know that blood donors are not viremic when they donate but half of them have CMV in their white blood cells, and we know we can transmit CMV easily from blood donors and also with organs.

In addition, in the West Nile case that I know of, and I have only read the press releases and so forth so I don't have all the data, the donor got transfused with a lot of blood prior to donation. Presumably, the donor was positive from the blood transfusions. We don't know but it could just as easily have been from the blood transfusions infecting the donor that then infected the recipients of the organs. If that is the case, then the virus doesn't

necessarily need to be replicating. The organ donor was just a vector for transmission of the virus.

So, at least from the organ donation standpoint, I still go back to what I said before, any evidence of infection, viral replication or IgM or IgG, indicates the possibility of transmission. That doesn't mean you rule out the organ donor necessarily but I think until we know more about how this virus behaves--is it latent; you know, how does it behave?--I think we have to assume that any positive test, whatever it is, means that you can transmit the virus from the donor to the organ recipient. Now, that may not be true for blood donation, or there may not be any consequence to the recipient of the blood in somebody who is IgG positive but NAT negative. I don't know.

DR. BUSCH: Mike Busch. I think it is about ten years now since David Kestler convened that meeting of closing the windows, and blood bank plasma programs have been screening for nucleic acids for HIV and HTLV for almost five years. I know NHLVI had issued an RFP to fund organ tissue testing platform development for nucleic acids, and in the last few months we are seeing these big public reports of HCV transmission to organ recipients. I

am just surprised we are not hearing anything and I am wondering what is the status of development of platforms. There were some rapid sort of tests that we were seeing five years ago, but what is the status of assay development for nucleic acid testing in your setting?

DR. BURDICK: Nothing that is in use clinically at present. I don't know what companies or institutions may have down the road.

DR. CHAMBERLAND: Mary Chamberland, CDC. I have a question for Dr. Confer. I was wondering if you could expand on your comments that some of the activities that you are thinking of implementing include questioning bone marrow donors about West Nile virus infection. I was just wondering if you could expand on that, what you are planning to do, and have you established criteria for exclusion of such donors?

DR. CONFER: Yes, what we are looking at now is we have a set of screening questions that are very similar to blood donor screening questions. We are not changing those questions but we are going to instruct our donor centers--these donors are typically managed by over 90 donor centers spread around the country--that they should

inquire whether, (a) the person has been diagnosed with West Nile virus infection and, (b) whether the person has experienced any symptoms that could be consistent with West Nile fever, like have they had fever; have they had unexplained gastroenteritis symptoms, nausea, vomiting, eye pain, those types of things. If a donor says yes to those, we will likely try to investigate further.

Now, what that means I don't know right now because there isn't a good way to investigate further besides doing antibody testing and if a donor has antibodies they may be fine. So, that is not completely worked out. We are basically trying to follow the FDA guidance that was put out and say we think that this may to some extent be applicable to the hematopoietic cell donors. That is it.

DR. MOZES: I am a medical adviser to the Association of Organ Procurement Organizations so you will be hearing a little bit more from me tomorrow. But I would like to emphasize the fact that in order to make the organ donor pool safe, and that is one of our goals in providing organs to patients and the other is to help them and save their lives, but we would like to keep it safe. In order

to keep it safe this relationship between the donors and the blood transfusions is very important. Dr. Freeman mentioned this. We do test our donors but then there are those donors that in the course of the resuscitation receive many units of blood and blood products.

At our OPO that I am associated with, and I believe it may be true for all OPOs, we do test our donors on a pre-transfusion sample as well as a post-transfusion sample at any number of transfusions. That has to do also not only with the possibility that the donor may have been infected through the transfusion, but also because of the dilution factor. We may lose the sensitivity of the test after many transfusions. So, we do do that. That is just a comment.

But I would like to emphasize that until we have a safe blood bank screening procedure in place we will not have safe organ donation. Although we do not have currently, and we plead with you to supply us with that rapid turnover specific test to screen our donors on the short time line within which we work, there is currently a test for West Nile virus that can be performed on stored blood. So, I don't know where the regulation stands at

this point but I would like to urge that at least it be applied to blood banking since there is an assay available that can be done within 36-48 hours and that, by itself, would make the organ donor pool safer. I think the one case that has been cited over and over again transmitting West Nile virus disease to four recipients was probably a donor that was infected through the multiple blood transfusions.

DR. CONFER: Well, that is going to keep those eight to ten people in Fort Collins really busy!

[Laughter]

DR. RIOS: Maria Rios. I just want to comment on what he just mentioned about blood being tested negative. I think that for tissues, and maybe you will correct me if I am wrong, sometimes testing cadaveric blood is not enough because if the transfusions were made and the blood tests were negative, maybe the organ is the carrier. So, you have to be careful and start thinking about this in the organ itself instead of just the cadaveric sample.

DR. ST. MARTIN: Dr. Confer mentioned some of the things they are going to institute for the marrow donors in terms of screening tests and asking them to report post

donation symptoms. I know that is not practical for organ donation. Are there any changes that are under consideration for organ donation in terms of informing the recipients about the potential risks of West Nile virus transmission?

DR. BURDICK: It might be worth the group understanding a bit more about how the process works from the recipient's point of view; we talked about donors a lot. When somebody has a disease that warrants a transplant, they go through an extensive process of evaluation, both medical, sociological, etc., but also a process of really informed consent in which a professional sits down with the patient or their family, if the patient isn't able to deal with it because of their illness, and go through the process, and the risks and the issues, and the pros and cons, and the possibilities of not doing a transplant versus doing it in great detail. That happens sometimes well before the transplant in the case of a cadaveric organ and is repeated at the time of transplant for any recipient at the time the routine informed consent is obtained. They know that there is a theoretical small chance of infection with various viruses. That is one

element in the great, long list of uncertainties that a transplant recipient faces, and Rich Freeman alluded to that as well.

So, yes, the patients are already educated about this in many ways. West Nile virus, interestingly just as an aside, has not come up in our patients because they already have some sense of perspective I think on this, and it certainly would be discussed with anyone who wished to. But I think the general caveat or the requirement, if you will, is that the proper informed consent be obtained.

DR. FREEMAN: Just to elaborate on that, and Jim is exactly right. when we first meet these people for the most part we go through an extensive process and the possibility of getting a disease from the donor, not just even infectious disease, malignancies too can be transmitted. That is discussed at length and in detail. You have all the experts here, what should we tell our recipients? What is the risk of getting West Nile virus and dying from it from a cadaveric organ donor? We don't know what that is. I mean, that is one of the reasons why we need to have some screening I think. But we tell them in blanket terms.

I think the organ banks also do a great job of screening these donors for clinical histories under very difficult clinical history situations. You have to remember what the situation is for this donor and how difficult it may be to get the clinical information, but fever and headaches and encephalitis and all those kinds of things that lead to anything that potentially could be medically valuable is there. And, sometimes we are faced with having to tell our recipient that the donor history here isn't exactly great, that there are some factors here that may lead to an increased risk of such-and-such and sometimes it is a malignant risk--a woman who had breast cancer 25 years go for example. Sometimes it is a viral risk--somebody who is in prison, an ex-IV drug abuser but serologically negative now. I mean, all that stuff comes up and you tell the recipient at the time that these things exist but, on the whole, the risk-benefit, at least in this professional's opinion, is to go ahead or not with the transplant in that situation.

DR. CONFER: Yes, there are similar things that happen in the blood and marrow transplant community. I think the big problem is that when you have a recipient who

already has terrible disease and multiple risks associated with the treatment of that disease, and then you throw one more onto the pile, you know, they are going to go anyway. I mean, at that point they are kind of like, "well, what other news do you have?"

So, practically speaking I really think it still is incumbent on us to try to address these risks from the prevention standpoint and figure out ways so that we don't have to talk to them because I don't think the patients can really do a whole lot about it.

DR. BURDICK: I agree with what has been said on both sides but, you know, nothing is 100 percent perfect, at least I can't think of anything right this minute so you are always going to be telling the patients that they may get an infectious disease but what we want to do is minimize it.

DR. GOODMAN: Jesse Goodman again. I just want to second a couple of comments. For many donors who receive multiple blood products and then many organ recipients who often also receive many blood products, by protecting the blood supply you will reduce most of the

likely attributable risk to those patients. So, I agree with that.

Somebody did make the comment that we have a test that we can screen people with for West Nile virus and I just want to reiterate that we really don't. That is an IgM antibody test that seems to stay positive for out to two years and doesn't seem to identify those individuals so far who have transmitted disease by transfusion or transplantation. So, I think while one could think about the role of the current antibody tests in different kinds of donor screening and while it may have a role, we should be a little careful about what we think we might do with those test results right at this moment with so little knowledge about them.

It may, in fact, be that if you have most people with IgM antibody against West Nile virus, it may actually be in an epidemic among the lowest risk populations to spread the virus if, indeed, they at that time have protective antibody. I think part of the reason this is all difficult is a general lack of knowledge right now and, hopefully, as we get more information we will understand the proper role of those tests because I do also think, as

I said before, that there may be people who are blood PCR negative who may have a window during which virus is still present in an organ or elsewhere. Thank you.

DR. BURDICK: Just to mention sort of a conceptual idea on that, it seems to me obvious that there would be a period of time--and somebody, maybe you, showed a graph very nicely and I am probably the one who said we had a test--you know, there is a period of time where you have virus neutralizing antibody in virus and you don't see the antibody and you don't see the virus. I don't think anybody has quite said that and it seems quite logical that would be an issue, and I couldn't agree more that being able to look for the RNA is clearly critical.

DR. NELSON: One question with regard to the last comment, there seems to be some overlap between the presence of the IgM antibodies and PCR. I wonder if there could be infectious virus during part of the time when IgM is present. I wonder if any of the lab people have done any antigen dissociation tests to find out if you dissociate complexes could you in fact show either infectious virus or PCR positivity? If so, how long does that persist? It is not a question for this panel.

DR. FREEMAN: I would like to follow-up on that question too because I would like to know, the people who have looked at tissue and found virus, what has been the serologic status of those patients? Because, again, every virus that we worry about in transplantation has been transmitted from donor to recipient in which there is systemic viremia. That is why I worry about focusing only on the viremic aspect of this for organ transplantation. It may be true for blood donors. Has anybody looked at that? When you find the virus in the tissue is the patient still serologically positive or negative or vice versa? Patient or animal even?

DR. WILSON: I have one more question for the panel to comment on. Some of you did discuss the risk of West Nile virus transmission relative to other viruses that we already know about, but I just wondered if you could do it again specifically addressing if you had an organ donor or a marrow donor that you knew was West Nile virus positive and there was an FDA approved test, how would you deal with that?

DR. BURDICK: Well, if there were viremia I would not use the organ, knowing what I know now, with the caveat

that maybe if we were vaccinating patients that might allow a patient to be safe and also we may have a treatment, even the immune globulin although I have no idea where that stands.

DR. CONFER: In the hematopoietic cell transplantation arena, particularly unrelated donor transplant, we don't use any donors with positive infectious disease marker testing, with the exception of CMV and we use those donors all the time and use preemptive strategies. A West Nile virus positive donor would not be used at the current time.

DR. FREEMAN: I think if we had a viremic donor it would have to be an extremely high risk recipient, somebody who is going to die tomorrow or the next day, and that would be a case where you might do the transplant regardless.

The bigger question to me though is somebody who is IgM or IgG positive who is not viremic, what is the risk of that organ infecting the recipient, and how big of a risk is that relative to what the risk is of the recipient dying without the transplant? That, to me, is the bigger question. I think knowing the windows of positivity of

these tests, if you routinely screen people you are not going to find very many viremic people but you are going to find antibody positive people more frequently. So, what to do with that I don't know. I don't know how we would handle that.

DR. NAKHASI: I think some of you may remember studies done in the 1950's. It was interesting that in the cancer patients West Nile virus was given to them to determine the viremic period. So, I think it depends upon what you are looking for. As you said earlier, risk versus benefit ratio is important.

DR. WILSON: If there are no other questions from the audience, I want to thank all the panel members.

DR. BISWAS: I just want to remind everyone there is no break at this point. Come back--all right, a five-minute break.

[Brief recess]

IV. Industry Perspectives on the Development of WNV Tests for Donor Screening

DR. BISWAS: We will start this session now. This session is called industry perspectives on the development of West Nile virus tests for donor screening.

The manufacturers of the assays will be giving eight talks altogether. This session will be chaired by myself and by Indira Hewlett. I will be introducing the first four speakers and then Indira will be introducing the next four. At the end of this session we will be having a one-hour discussion period so, please, keep your questions until that time. The first talk is called update on National Genetics Institute West Nile virus screening assays, by Any Conrad of National Genetics Institute.

Update on National Genetics Institute

WNV Screening Assays

MR. CONRAD: I am going to go over the NGI West Nile virus NAT tests that we have developed using the same platform as our FDA approved tests.

[Slide]

I am not going to go over the history of West Nile virus since others, far more qualified, have done that ad nauseam.

[Slide]

There is some data here about the rapid conversion of donors from IgM to IgG and the stability of

the IgG, which I think is important and other people have talked about it.

[Slide]

We have developed and validated a quantitative and qualitative assay for West Nile virus, again, using the same platform as our FDA approved assays for HIV and HCV. I am going to give you a brief description of that assay and give you data from serologically positive samples that were sent to our Viramed laboratory for clinical evaluation. These were patients suspected of having West Nile virus. Then we are going to give the preliminary results on our unlinked study where we have begun screening donors.

[Slide]

To optimize the assay we actually evaluated seven different primer pairs that come from all over. They originally came from all over but we settled for 3' UT and an NS primer set, with careful temperature profiles, and we demonstrated that the virus could be actually concentrated ten-fold through centrifugation, which is an important step. I have heard lots of comments on the utility of pooling and I think people forget at CDC that you can take

large volumes of some of the material available to us in the whole blood or in the plasma industry and concentrate the virus, different than what they are getting when they have clinical samples where they may not have as much material.

[Slide]

Basically, we used a positive control liver tissue obtained from the famous snowy owl, somewhere in the Bronx Zoo, then cultured in cells, strained 385-99. We made serial dilutions of 1:10 and also did Poisson analysis, and then worked on some other quantitative mechanisms to determine that the supernatant, as we have it, is about 1 time 10^{10} copies/ml. The sample was then diluted to create several different pools, both a quantitative and a qualitative pool.

[Slide]

To begin validation of the assay we obtained 100 samples that were sent to our clinical laboratory in Minnesota, called Viramed. These were obviously patients in the clinical environment and suspected of being exposed or clinically having West Nile virus. It is important to note that 20 of these samples were just wrong. They had

some other type of fever. They were West Nile virus IgM, IgG PCR negative. Seventeen of the samples were positive for IgG and IgM; 62 of the samples were positive for IgM only; and one sample was positive for IgG only.

[Slide]

Of the 100 clinical samples that we obtained and tested, nine percent of those samples--remember, we were only given 100 mcl of these samples so it wasn't as sensitive as our qualitative assay which uses 2 ml as the inoculate--but nine percent of these samples tested NAT positive. The average viral titer of these samples was approximately 100 copies/ml, with an estimated range of 10-- we can't super-well quantitate down at 10--up to 2000. Two of the samples had viremia of 2000.

So, the impression that has been given that samples which are already immunologically or serologically positive have low viral titers, our data supports that concept. Again, eight of the samples were IgM positive. Only eight of the positives were IgM positive only. Those also had the highest viremia. There was a single NAT sample that was positive for IgM and IgG.

[Slide]

Having this test in hand, we decided to implement West Nile virus testing using the same algorithm as the other assays. In this algorithm we would have access to either pools of 512, 64 or individual samples. So, we could screen and we decided what we would do is look at all the three different levels to see if there was a sensitivity issue in determining positivity. We used this T-can device for pooling large numbers of samples. Currently, National Genetics tests almost a quarter of a million samples a week.

[Slide]

The concept here is that you can generate pools of 512 that have row, layer and columns which contain 64 samples each. The collective 8 by 8 by 8 pool or cube has 512 samples.

[Slide]

The idea is if you test the whole cube and it is negative then you know all its member components are negative to a certain sensitivity. If you test the row, layer and columns they will point to a single positive sample. So, in this pooling algorithm we automatically have the row, layers and columns so any row contains 64

samples. The entire cube of 512 has all the samples in it and you can, of course, test individual samples.

[Slide]

I am going to give you the first week's data. We began doing this a few weeks ago and it takes a while to go down and track down the individual sample. It is important to note that the samples are blinded as to donor but we store the samples in a freezer so we can get some geographic information. We know what centers they come from but we have blinded them to the ID of the donor.

The samples were collected in the late summer of 2002 so due to the 60-day hold you can guess that they were three weeks ago, minus about 60 days, that these samples have been stored. The geographic information was retained and 5366 of the original 7101 donations came from Indiana, Louisiana, Mississippi, Missouri, Ohio and Texas which are traditionally, as you can see from your CDC maps, thought to be unpleasant states for both mosquito bites and West Nile virus. The remainder were from states not associated with significant West Nile virus risk. The reason I bring this up is because some of these numbers are going to be

skewed because the first three weeks we were using cherry-picked, high risk sort of populations.

[Slide]

In week one we obtained one West Nile virus positive NAT sample. It was confirmed positive by all three of the primer sets that we used. It was positive at both the 64 and the 512 pool level, again sort of defeating our original concept where we thought these titers would be low. The titer was quantitated at 196,000 copies/ml. Again importantly, it showed up absolutely in all primer sets in the 512 dilution and then confirmed through the primary pools out to the individual donation. So, the chances of it having been the result of a contamination is extremely low. We would have had to selectively contaminate three different samples, as it were. As of two days ago, we have not received back the serologic data on that individual. We just got the individual and got it quantitated this weekend, as a matter of fact.

[Slide]

Also, with the quantitative test we have performed a series of experiments, that will be described tomorrow, where we looked at inactivation, filtration,

pasteurization and the effect that that has on viral titers. Currently, we have begun screening approximately 10,000 blinded samples per week. They have not all reached resolution but we are going to up that now to 25,000 to 30,000 samples a week, starting next week, in an effort to sort of survey the waning of the epidemic to see if we can get enough numbers to look at these. We will have the units of these samples eventually available, or left over plasma in the case of recovered plasma.

But I can tell you that although we have not resolved them, it is clear that we are detecting West Nile virus positive samples in both pools of 64 and 512. So, something is different than is the conceived sort of notion that comes from the clinical data that we see from CDC. I think that basically sums it up.

The other important thing is that the reason we are seeing this profound difference may have something to do with the mechanism of preparation. Remember, in the whole blood or in the plasma industry we are much more familiar with taking large, large samples of blood and preparing the blood or plasma and preparing the putative virus from these larger samples. So, again, we are using a

minimum of 2 ml except in the clinical samples where we are only provided 100 mcl. Thanks.

DR. BISWAS: That was short and informative. The next speaker is Christina Giachetti, from GenProbe. Her talk is titled West Nile virus transcription mediated amplification, TMA, assay.

WNV Transcription Mediated

Amplification, TMA, Assay

DR. GIACHETTI: I would like to thank you, the organizers, for the opportunity to give this presentation where I will present our program to develop a TMA assay for detection of West Nile Virus.

[Slide.]

The first slide shows which are our West Nile Virus objectives. The objectives are to develop and manufacture a TMA-based assay, for West Nile detection in blood, plasma and organ-donor specimens. This program will initially support the unlinked and then the linked donor-recipients West Nile Virus epidemiological study and IND/BLA submissions.

[Slide.]

I show in the next slide, we structure the program in three phases. Phase I is just to generate research-use-only reagents to support the unlinked studies. These are planned for early next year. Phase II is to generate our reagents to support linked donor-recipient studies. Following that, in Phase II, we are going to file an IND and we will support ongoing West Nile Virus screening under an IND.

Phase III of the program is to confirm the IVD product requirements, refine the formulations to validate product and production processes, to complete the pivotal study and to submit a BLA.

[Slide.]

This slide shows which are our performance goals for Phase I and II; initial sensitivity as a goal is 95 percent detection at 50 copies per ml. We plan to detect all West Nile Virus variants. Specificity will be higher than 99.5 percent. The assay contains an internal control for assay monitoring in each sample. We plan to use the same assay formulations and assay format for organ and blood-donor specimens. The assay formulations will be

applicable to Procleix as well as the fully automated TIGRIS platforms.

[Slide.]

For Phase I and Phase II, the assay will run in the Procleix semiautomatic system which is the same platform that is used at our licensed HIV-HCV assay. Basically, the protocol steps are described here. The first step is pipetting of the specimens using the TECAN, the TECAN pipetted specimens, calibrators, controls and the capture reagent. Then we proceed with sample processing using the target-capture system.

Then we do amplification using transcription-mediated amplification followed by detection with a hybridization protection assay and then the results are read out in a luminometer.

[Slide.]

The next series of slides shows which is our basic technology. For specimen-processing, we use target capture, magnetic microparticle separation. The first step is the viral lysis where the specimen is titrated with heat and with detergents. These release the nucleic acid and then the nucleic acid is captured using capture oligos that

contain a sequence that is complementary to the target RNA and also the capture oligos contain a polyate tail that will bind to tails that are attached to magnetic microparticles.

We apply, then, a magnetic field to be able to separate, to capture our RNA and separate it from the unwanted specimen. Following the isolation of the RNA, we proceed to amplification.

[Slide.]

Amplification is used in transcription-mediated amplification. TMA uses two enzymes, reverse-transcriptase and T7 RNA polymerase. The system works well with RNA or DNA targets and produces an RNA amplicon. Amplification is exponential, very efficient. We have 1 billion-fold amplification in one hour and the reaction is isothermal.

[Slide.]

Following amplification, we do detection. We have a hybridization protection assay that utilizes acridinium ester level probes. The first step is a hybridization where the level probe is bound to the RNA amplicon. Following hybridization, there is a selection step where the acridinium ester attaches to the probe that

binds to an RNA target is protected while the probe that hasn't bound to a target, because the target was not present, is destroyed.

Then, following the hybridization during the detection step, the protected label is detected by chemiluminescence.

[Slide.]

Because our reaction contains an internal control as well as a target, we use the dual kinetic analysis to be able to differentiate the signals within each sample. So what we use is acridinium ester probes with different kinetics of lights off. We use orthofluoro for the internal controls that we call flasher probes with a very fast kinetics of lights off and we use 2 methyl-acridinium-ester labeled glower probes to label our probes against our target West Nile Virus.

And then we use the exponential tail-filled algorithm to be able to discriminate and calculate the signals within each samples that belong to our target or to the internal control.

[Slide.]

As I said, this assay for West Nile is going to be used the same system as we are using for current HIV-HCV assay. Some of the reagents that we are going to be using are going to be common to the HIV-HCV assay or other Procleix assays and these reagents are the enzyme selection and the TMA fluid. For the West Nile Virus assay, we have a specific regent. There are other reagents that contain oligos. And this is a target-capture reagent amplification and probe.

[Slide.]

Regarding assay calibrators and controls, within each assay run will be three replicas of the negative calibrator and three replicates of the positive calibrator. The calibrators in the run are used to calculate the cutoff for internal control as well as for analytes and also is part of the criteria for run validity.

Also, the assay contains an internal control. The internal control is an RNA transcript that is added in each specimen with the target-capture reagent. It is detected with internal-control flasher probe and monitors performance of reagents, operators as well as instrumentation.

Also, each run will include external quality controls, one replica as negative control and one as a positive control.

[Slide.]

My final slide is just to show which is our current assay performance. I should say that we started this program in mid-September. So it is just about six weeks old.

[Slide.]

What we did here is the termination of which is our current analytical sensitivity. We did it using a synthetic RNA transcript that expands our area of amplification. So we prepare the transcript. We purify. We determine the concentrations by absorbance and then we did serial dilutions going from 600 copies per ml down to 0.3 copies per ml and also we included some negative samples.

We tested replicates going from 20 to 30 copies, replicates depending on level, and then we determined which is our percentage detection at each copy level and we have 100 percent detection going down to 20 copies per ml of the

transcript RNA, 79 percent detection at 6 copies per ml and 43 at 2 copies per ml.

We utilized this data to use Probit analysis to determine which is the 95 percent detection and 95 percent confidence intervals. For 95 percent detection, it is 7.6 copies per ml. Our confidence intervals go between 6 to 10 copies per ml.

Similarly, using this analysis, we can calculate 50 percent detection and, in the system, it is 3.5 copies per ml with the confidence interval between 2.5 and 4.5.

[Slide.]

We also tested the CDC viral lysate standard that we provided to us by Dr. Lanciotti. According to the instructions that he provided to us, we made serial dilutions of the RNA. We diluted from 10^{-4} down to 3.2 times 10^{-7}.

Here are our percent detections. We have 100 percent detections at 10^{-5}, 95 percent at 3.2 10^{-6}, 76 percent at 10^{-6} and 38 percent at 3.2 10^{-7}. Again, we used Probit analysis to determine which was the 95 percent detection. In this case, we don't know exactly the concentrations so we expressed at dilution level. We have

95 percent detections at the dilution of 2.7 10^{-6} and 50 percent detections at 4.5 10^{-7} dilution.

[Slide.]

Conclusions at this point are really minimal. It is just that we have demonstrated sensitive detection of West Nile Virus RNA using TMA. Analytical sensitivity is 95 percent detection of the CDC standard, 2.7 times 10^{-6} dilution and 50 percent detection at 4.5 time 10^{-7} and using a synthetic transcript, the detection is 95 percent at 7.6 copies per ml and 50 percent detection at 3.5 copies per ml. [Slide.]

Finally, we have to thank the National Heart, Lung and Blood Institute which has given us a contract to develop this program.

Thank you.

No Denial About West Nile: Update on Roche's West Nile Virus Program

DR. GALLARDA: No denial about West Nile. This actually wasn't my idea for the title but it gained popular support.

[Slide]

I think most of us in this audience would like to be in denial about West Nile. However, I think based upon the recent popular press and discussions that have been going on between industry, FDA and the blood screening community, we view this pretty much as a "must do" kind of project. So, by no denial I mean to say that Roche is committed to going forward to pursue this program.

[Slide]

I have three sections to this talk. The first one are two slides that just talk about critical issues. Then, the second part is related to our understanding of FDA's current thinking for requirements. The third part is our thinking of FDA's current thinking on the requirements.

[Laughter]

Most importantly, I think like with mini-pool NAT testing in the last few years, the partnership between the FDA and the manufacturers and the blood screening community is going to be the key for us to succeed in pulling this off by the middle of next year. In order for us to nail a quality system regulation compliance system that includes hardware, software and integration, the nine-month time line, which is kind of what we are talking about, is pretty

challenging. So, we expect to kind of crunch a lot of things into a very short time. In addition to the development effort, the capital layout and the training and support costs to support a multi-site IND are pretty challenging. The time frame only exacerbates the problem.

[Slide]

We have an interesting opportunity at Roche. We really have a lot of options here for West Nile and with the data that was presented by the public health labs which, by the way, I thought was outstanding, really we are beneficiaries of a lot of the TaqMan observations that have been made by the public health labs.

One of the problems that we are grappling with is that a few weeks ago we had a conference, I think BPAC, where we were talking about going to single unit NAT. So, our reality is that in order to pull of the West Nile program we have to take some of those resources away from the single unit program to devote to executing the NAT program. No problem--no problem! We think that there are some synergies that exist with our options and we will be having discussion with FDA soon on what those options are to really provide, number one, the customer with a platform

that they can use; secondly, a platform that performs as intended.

I highlight this. This is kind of PC talk for opportunities or actually problems. I think based upon the atmosphere of the September 20th meeting that we had with AdvaMed and AABB, there will be a need for a lot of collaboration between the stakeholders to pull this off. So, we really view the success of this being due to the past history of the partnerships we have had and continuing going forward.

[Slide]

Reference points--our understanding of FDA's initial guidance points to consider. A couple of weeks ago at AABB FDA provided initial thoughts regarding the development of donor screening tests. In that, it was encouraged that there be around mid-2003 a nationwide IND, if possible. A focus on donations of whole blood was the emphasis of that presentation. FDA rightly recognized that there are several technology transfer challenges ahead of us related to the urgent time line. From our view, this is not simply a research exercise. It is one thing to put together a research kit that can detect dilutions of

positive samples. It is altogether another thing to put together a system with validated hardware, software, system integration and reagents in the time frame we are talking about.

It was mentioned that the validation would be done in a donor screening environment, i.e., clinical studies to support a BLA, and FDA was very clear this was going to be managed through an IND BLA 510(k) process. This is not an RUO exercise.

[Slide]

In the AABB presentation, Dr. Epstein pointed out the September 20th meeting which we had in which he mentioned, and we all agreed, that there was excellent collaboration between the stakeholders in West Nile. The initial guidance on sensitivity stated 100 copies/ml to reliably detect a sample that was at 1000 copies or genomic equivalence per original sample.

It was also mentioned that this must be in compliance with quality system regulations and, again, that is not just assay stuff; that is hardware, validated software and reagents.

[Slide]

Our thinking of FDA's current thinking--the issues regarding the multi-site IND, in an ideal world all the manufacturers would just like to go to three or four sites, pick up 10,000 samples and file a BLA. I think that day is probably over with NAT. So, our expectation is that there will be a need for whole blood screening for West Nile in the second quarter. This is not a limited IND.

We anticipate lots of discussion with FDA to identify the minimal geographic sites, the strategy of collection and managing data for the submission of the clinical performance. Rather than rely on 13 or more sites to manage that data, that became a very difficult and cumbersome project to manage 13 sites over a two-year IND.

We agree with FDA that we are going to have to come up with some creative solutions regarding the multi-site logistics. Multi-site logistics for us basically means that when we start an IND, and I think the users can attest to this, bears a lot of resemblance to a fully-launched product. Even though it is not a large product, the work to get there is the same work to launch a product. So, we would like to talk to FDA about how we might

strategize so that we can pull it off for the greatest benefit.

[Slide]

FDA talked about the challenges of technology transfer. I mentioned it is not so tough to come with a research assay. However, the rules of engagement to make this safe and efficacious where you have a high positive predictive value and a high negative predictive value, this means we are migrating towards GMP, which is what GMP is all about. We would like to discuss the timing of this relative to the start of the IND. I think there is room for negotiation there.

Rob Myers mentioned earlier this morning that, you know, one preparation of virus could behave completely differently than another. So, one of the issues that FDA rightly recognized was standardization specially of panels that can be considered reference panels or gold standard panels to allow manufacturers to target the development of a system in the way that should be uniform.

FDA discussed validation in a donor screening environment. For us, this is a multi-site clinical trial. We have questions on the scope of the IND. Interestingly,

the role and quality of the serologic referee assays, IgM and IgG which we discussed this morning, it is not clear how we can rely on that serological analysis to help resolve discrepants. However, we think it is an important thing to consider.

The quality of those assays and trusting the call that they provide relative to the interpretation of the NAT result will be important.

We think, as with HCV and HIV, it is a good idea to have alternate NAT provide a confirmatory role in a clinical trial. Although most people in the audience here are interested in claims for screening blood, there are other claims that you might want to entertain from the very beginning. Some of those include what about a confirmatory assay? I think there is an opportunity from the very beginning to strategize how we would tackle that from the beginning.

In a lot of discussions it came up, what about tissues? Tissues are equally important, although for most of our thinking we have historically not considered them from the beginning.

[Slide]

BLAs and 510(k)s are going to be filed for the kit and systems respectively. In addition to the guidance on sensitivity, what are the other critical performance criteria? FDA has a very nice guidance document for development of NAT assays for HIV-1 and HIV-2. West Nile just arrived in the U.S. in 1999 so we don't have as much of a history as we did with HIV, but we look forward to working with the agency on how we can come up with the critical performance criteria that are important to design in our study.

Collaboration among stakeholders, I mentioned the reference materials which would be important. I think critically, you know, we are targeting really the pre-seroconversion window period for West Nile. For that, we have to figure out a way to get pre-seroconversion panels to help us assess the duration of the window period and the level of viremia. I think there will be a talk later by Steve Kleinman on a study to look at prevalence. That might be informative in giving us some of that information.

Customer needs and confirmatory assays as well, the serologic assay--who can do that? So, we are mostly

working in PCR technology. So, we may want to partner with someone to help us with some of the other referee assays.

[Slide]

On the issue of the initial guidance for assay sensitivity, 100 copies/ml to ensure 100 percent detection of a 1000 copies/ml sample was the starting point, and FDA and AABB pointed out that single sample NAT is likely to be needed in settings where virus concentration procedures are impractical.

I think our view is that at the end of the day the system should be designed to interdict successively infectious units irrespective or what those titers are. So, it may be, and we agree, that this is a good starting point. However, the movement towards single sample NAT--I think it is fair to say nobody really knows what is going to be required. The facts are that mini-pool NAT may be sufficient to achieve the desired sensitivity.

[Slide]

I apologize for this slide. Our system is designed around pools of up to 24. The pools will facilitate implementation to the sites in the real world because it accommodates their work flow. If we go towards

a single unit, that would definitely threaten the time line because it creates enormous logistic issues.

[Slide]

So, a single unit NAT scenario, and we talked about this a couple of months ago--for a mandatory move towards single unit NAT we have to address what is the incremental yield of single unit NAT versus mini-pool? What is the impact on the labor resources and availability of those resources in the users environment, and because of the semi-automated systems that are in place right now, for potential documentation errors?

So, the bottom line I think is summarized by this bullet. There need to be for manufacturers objective criteria from the beginning to develop a system towards those criteria. The time line will depend on these predetermined standards. So, we think this is a good starting point, 100 copies/ml sensitivity as analytical sensitivity. Our system's development has to rely on the specification. Alteration later to make it more sensitive could abolish the gains that we made in developing a system for this spec.

[Slide]

The last item that was mentioned, this has to be done with the quality systems requirements. West Nile really represents an opportunity to develop a general rapid response IVD mechanism for the U.S. This is in agreement I think with Dr. Goodman's opening remarks this morning. Outside of the West Nile virus question, Dr. Goodman said how can we develop robust platform technologies with the ability to rapidly respond and alter agents that will be detected using those platforms? That is the bigger question. I think that is one of the directions that we would like to discuss with FDA for how we can implement a mechanism for that rapid develop so that we can solve the West Nile problem and, in addition, any future threats that might come to the U.S.

[Slide]

The bottom line, we are going forward to do this and our resources are really calling upon a multi-national effort to help out with the development of this system. Thank you.

DR. BISWAS: Thank you very much, Jim. You brought up a large number of very salient points and some

of those we can discuss today. Some of the items you brought up I will be sort of talking about tomorrow.

Our next presentation is by Dr. Lynell Grosso, of BioMuriex. Her talk is entitled real-time nucleic acid sequence-based amplification for West Nile virus detection.

Real-Time Nucleic Acid Sequence-Based Amplification for West Nile Virus Detection

MS. GROSSO: Good job on the pronunciation, between the company name and my name it is a challenge.

[Slide]

I would also like to thank the FDA for inviting us to participate in this workshop, and I am going to talk about BioMuriex' real-time nucleic acid sequence-based amplification for West Nile detection. I am going to apologize to Rob Lanciotti; I used a couple of his slides. We talked about it and never really got back to each other so you are going to see some data you have already seen. So, this will be short.

[Slide]

What I would like to cover is some of the CDC assay design, the West Nile virus surveillance data, the

assay evolution and goals for us, and then the proposed NAT platform.

[Slide]

The sequence of events for us has been in 1999 the CDC developed a West Nile virus application using our kit for nucleic acid amplification. In 2000 and 2001 various public health departments throughout the United States implemented West Nile virus testing using the CDC's basic kit application for the surveillance of West Nile virus in mosquito pools and avian tissue. In 2001, a JCM publication by Lanciotti et al. described the real-time NATs using molecular beacons for the rapid detection of West Nile virus.

[Slide]

We have already had a description of what a molecular beacon looks like. This is the actual beacon that was described by the CDC and used in a real-time assay using NASBA amplification. Again, there is a structure that this is actually the part that anneals to the target and this keeps the quencher and the fluorescent dye in close proximity, therefore, not releasing any background

fluorescence, or very little compared to some other technologies.

[Slide]

This is a NASBA reaction that is identical to two other FDA-cleared assays that we have, and what this does is just add molecular beacons in the beginning of the reaction to make the reaction real time. It is very similar to TMA so I won't have to go through the actual amplification process, but it also is exponential in amplification.

[Slide]

This, again, is the structure. It finds the amplified target, anneals and then we get growth of light being produced by the fluorescence once the quencher is far away from the fluorescence of the reporter molecule.

[Slide]

One of the things that is really great about molecular beacons in our technology and other technologies similar to it is that there are several available fluorescent labels which allow for both multiplexing and diversity in the applications. The molecular beacon probes in the proposed system have filters that minimize cross-

talk so that you can actually see the differentiation between two different targets, as well as use internal calibrators.

[Slide]

This is some of the data that was already shown by Rob. It just shows the sensitivity of the assay that has been described compared to the TaqMan. This is our ECL platform, which is an older platform, and this is the new platform using molecular beacons.

[Slide]

There is a fair amount of data on this slide but, hopefully, you can see it. This shows the sensitivity and the specificity of the reactions. The molecular-beacon assay is over here. We already looked at the sensitivity. This is cross-reactivity to related viruses.

[Slide]

This is not West Nile virus, but this is another assay that Rob Lanciotti developed. This is for SLE virus using NASBA as compared to the TaqMan assays, very similar in sensitivity.

[Slide]

This is a comparison of the amplification profiles. You can see that between the two different types of reactions we have an RT step and a TaqMan reaction. The NASBA reactions or, similarly a TMA reaction which occur very quickly.

[Slide]

In conclusion, the West Nile virus surveillance assay that we have going to blood screening, an assay of elution is what we are looking to do when we proceed with the test goals. This is the current CDC West Nile application using our NASBA reaction and we are looking to have at least 100 copies/ml for sensitivity of this assay. We have no cross-reactivity with related viruses, although it appears we need to look at syphilis. We didn't think of that one--who would have thought? Then, of course, we want at least 95-100 percent but clearly, 100 percent is unattainable for new assay goals.

Time to result right now in a positive sample with high viremia or viral load is about 15 minutes. So, our time for amplification and detection is anywhere from 15 to 45 minutes. We would like to maintain that speed. Our isolation--it has already been done with the NucliSens

Boom isolation and with the Qiagen BioRobot, and we want to look at improving our sensitivity with magnetic silica, and also increasing our throughput. That comes down to our run size on our proposed platform. We will be going from our ECL platform which we currently have to our molecular-beacon platform and increase to 96 per run.

[Slide]

This is just a photograph of our analyzer. It is a fluorometer that reads fluorescence and allows us to do the multiplexing and the real-time assays using our NASBA reaction with molecular beacons. That is it.

DR. HEWLETT: Thank you, Lynell, for that very clear presentation on the NASBA technology as it applies to the West Nile virus.

Moving on, I would like to invite Martin Munzer, from Cygene. He will be talking about target enrichment strategy: proposal for improvement of sensitivity and specificity of RT-PCR-based West Nile virus blood assays.

Target Enrichment Strategy: Proposal for Improvement of Sensitivity and Specificity of RT-PCR-Based WNV Blood Assays

MR. MUNZER: Well, thank you very much, Dr. Goodman and FDA, for inviting Cygene to attend this presentation.

[Slide]

We are a very small company so we are prone to make bold statements, and you will see that from our first slide. This is not intended as a claim but a gold standard is something that we are looking to achieve, of course.

[Slide]

Essentially, what we started with at Cygene was a new concept for doing molecular diagnostics, and we recognized early on that for detection of infectious disease and residual disease and bugs like the West Nile virus we are going to need to be able to detect low copy number targets. In order to accomplish that, we pulled the analogy of the haystack processing because essentially the current technologies are very limited with respect to the sample size that they initially evaluate.

[Slide]

Therefore, we tried to find something that would give us the ability to look at the whole haystack, not just a pinch of the hay, and try to protect that target and

eventually degrade the background material, the non-specific nucleic acids, in order to be able to pick up those individual targets. This has been quite a challenge, of course, and we have a partial technology that we would like to propose and present right now.

[Slide]

One of the ways that we found that we could protect these targets was by forming a triplex. So, our contribution to the current West Nile virus detection is by way of target enrichment. This target enrichment strategy is accomplished by using our triplexes.

[Slide]

A number of the considerations that need to be looked at with respect to triplex formation, or stable triplex formation are, number one, we are looking for polypirimidine strand. That is one of the requirements. What we do to form a stable triplex in those regions is we use parallel-stranded anti-parallel hairpin probes. These hairpin probes have been modified by using 8-aminopurines. These base modifications result in a very highly stable triplex structure.

[Slide]

The probes can be configured in a number of different ways. We can have the polarity with the two, five prime ends exposed, or the two, three prime ends exposed with single-stranded template. Then, we also have the ability to do the same thing in a double-stranded template.

[Slide]

The synthesis of these probes would start initially in a very conventional way where we do the purine strand with regular phosphoramadites. We then introduce a linker molecule and then begin doing a synthesis with reverse phosphoramadites. That gives us the ability to produce these anti-parallel strands.

[Slide]

Here we have also an alternative method. Working with reverse phosphoramadites obviously gets pretty expensive and we have a method now of using asymmetric branching units to bind together the probes that allow us now to make these probes a lot more cost efficiently. The top obviously is the DNA and the bottom is the RNA type structure.

[Slide]

To give you an idea of what our modified aminopurines look like, the 8-amino-A, here you see the difference. We add an additional branch to the Hoogsteen binding forces, and this is what gives us a much stronger binding ability, and the same thing with the 8-amino-G.

[Slide]

We are looking at an infected sample, say, of whole blood in this example. But, most likely, we are going to find a lot of advantage using this technique for target enrichment out of tissue samples which are, obviously, going to be a very big challenge, as well as what we heard with respect to the transplant applications. We are going to be looking at DNA haystacks or RNA haystacks and this is where we see an application. So, we need to implement an extraction process that is highly efficient and gives us a lot of the RNA out of the sample, and then introduce our hairpin probe to capture the specific RNA and then go into a detection step, which obviously we can do with RT-PCR or some of the other methods that have been presented here today.

[Slide]

This is what this capture process would look like. In this example we have the viral RNA with our hairpin structure with a biotin-streptavidine conjugate. We, of course, recognize that this is a method that not only is very common in the industry but also brings some background issues along with it because of the non-specific binding. To overcome that, we have addressed this by coming up with a method of synthesizing the hairpins directly onto the magnetic bead, which allows us to saturate the beads with oligos and reduce the non-specific binding.

[Slide]

This is the device that we have kind of settled on to do our magnetic separation. It is a very efficient liquid handling, very simplistic liquid handling robot that does the magnetic separation in the tip of the pipette. This is a very neat way of doing things rather than working in the microtiter plate. It gives you the ability at the end to have just the target RNA of the material left over in the microtiter plate instead of having it mixed with the beads.

This unit is an eight-channel unit and does what we call mid-throughput. We already have a device that will do higher throughputs and also higher volumes, which obviously is going to be necessary for this particular application. It will also help in the pooling of samples so that we can do 10 ml samples, for example, and start with the extraction process which we also have working on magnetic beads, and then move forward to the hairpin capture of the specific targets.

[Slide]

Again, going back to the chemistry, here is the standard adenine and guanidine structure with the Hoogsteen bonds, in the low rectangular shape.

[Slide]

Here you see how the amino modification works and adds an additional Hoogsteen strand. We see that the stability increases quite substantially, approximately 40 percent.

[Slide]

Some of the data that we have generated with this, and this is in the three motif. As you see, the five prime ends are exposed and the three's are attached. We

have stability actually preferentially working in a low pH environment, which is obviously beneficial to working with RNA because the RNAs aren't as active at low pH. With melting temperature of 67 degrees, with the modified amino-G, amino-A adds to the stability of the unmodified probe as well but the 8-amino-G seems to be the method of choice, and 67 degrees gives us a very high melting temperature and, therefore, specificity.

[Slide]

This is the same experiment series done in reverse, exposing the three prime ends and having the two five prime ends butted together. You see that there is similar data that is generated, again, the 8-amino-G being the modified base of choice.

[Slide]

Here we have a comparison of a straight polypirimidine tract which is, of course, an ideal type situation. With this method we can allow for up to two or maybe even three interruptions. Here we are showing one interruption and we are down now to 47 degree melting temperature, which is still stable enough for very good and efficient capture.

[Slide]

To show you how this works, those were DNA examples; this is an RNA. With RNAs, we know that RNA as a center strand in a duplex probe structure forming a triplex gives you the most stable unit. Here we see the melting temperature up at 71 degrees, which is quite excellent, giving you a high degree of specificity.

[Slide]

Here are some data that basically demonstrates that we do have the Hoogsteen bonds taking place in just the probe structure, and this is proven with circular dichroism and nuclear magnetic resonance. Then, of course, we did the work with the triplex and showed with the gel shift and NMR that we have got what we are looking for.

[Slide]

The next question obviously is do you have these pyrimidine tracts available for the West Nile virus, and this is just a preliminary search that gave us a whole host of different target regions. The 66-70 region not only gives us a straight 12 base pyrimidine without any interruptions, but it also is found in about 30 of the different West Nile virus strands. So, this gives us the

ability to use one probe. Obviously, we can also use a multiple of probes attached to different beads. That will give us specificity for different targets. Obviously, this lends itself then to doing multiplexing.

[Slide]

I would like to thank our collaborators, Ramen Eritja at the University of Barcelona, and our group at Cygene, Spain, for having developed these probes and done this work for us, and thank you very much.

DR. HEWLETT: Thank you, Dr. Munzer. That was a very interesting presentation and certainly provides insight into new ways to capture and to enrich your target, which is going to be necessary for West Nile virus assays, given the levels of viremia we are talking about in these donations.

Moving on to the next presentation, the speaker is Dr. Bruce Phelps, from Chiron Corporation, and he will be talking about production of materials in support of West Nile virus assay development.

Production of Materials in Support of

WNV Assay Development

DR. PHELPS: Thank you very much, Indira. I would like to thank the FDA and the organizers of this workshop for giving me the opportunity to present this data to you today. It has been a very interesting discussion. As Jim said earlier, the relationship between the manufacturers and the FDA is critical in making this successful in terms of implementing these assays in such a rapid time frame.

[Slide]

What I would like to do today is to describe some of the activities that we are planning to do at Chiron in support of development of the screening assays, particularly support of the TMA assay that we are developing in conjunction with our partners at GenProbe.

[Slide]

This slide just lists the types of activities that we plan to undertake over the next few months at Chiron. In particular, we plan to acquire culture and characterize West Nile virus isolates from several different sources and provide materials which might be used for controls and calibrators in the context of developing the TMA assay.

We also plan to prepare transcripts of relevant viral sequences of West Nile virus, as well as other members of the Japanese encephalitis complex that might be useful for confirming the assay sensitivities and potentially even looking at multiple detection in these assay.

We also plan to develop supplemental NAT assays for West Nile virus and other related viruses. As Jim said also, there are no confirmatory tests right now for these that are very useful. So, we are looking to develop a sensitive test that might be helpful in confirming the NAT result.

We also plan to express and purify recombinant proteins for use in assay development, and to use these proteins in developing West Nile virus antibody assays, both IgG and IgM.

[Slide]

In terms of the West Nile virus suspensions and our plans on what to do with those, we plan to propagate the West Nile virus strain which is derived from the 1999 New York outbreak in vero cells. We have a BSL-3 facility

at Chiron and we are very adapt at doing cell cultures, as we have in the past for several different viruses.

We then plan to harvest the West Nile virus, estimate titers both by the plaque-forming unit assay as well as TaqMan, and try and get some correlation. We see that there has been some concern and really not a very in-depth understanding of what the relationship is between the plaque-forming units and the gram equivalents per milliliter, and we hope to do some studies to elucidate that correlation.

We would then also plan to inactivate the virus. We have in-house validated protocols for inactivating HCV, which is another flavivirus, and we think that these procedures should be very helpful and very applicable to the use for West Nile virus inactivation. So, we would inactivate that virus and then spike the inactivated virus into human West Nile virus negative serum and, in so doing, construct dilutional panels for QC of the West Nile virus TMA assay development at GenProbe.

I addition, we would evaluate the use of these same materials for potential kit controls that could be

used for the TMA assay. We actually would base these on actual viral isolates.

Finally, we plan to culture and characterize virus from various sources to evaluate strain differences and, hence, the mutation. We heard earlier today that this appears to be a very stable virus and doesn't seem to mutate very much, which is good in terms of the epidemic, but we need to make sure that our assays will detect all of the potential variants that might be arising around the country. So, we will be taking a look at that from multiple samples.

[Slide]

In terms of preparation of viral transcripts, we plan to construct nucleic acid standards that would contain relevant regions of all of the Japanese encephalitis virus complex, West Nile virus, St. Louis encephalitis virus, Dengue virus, as well as other related viruses, and prepare transcripts of these relevant sequences which could then be used to prepare reagents, primers, probes and internal controls for developing other discriminatory NAT assays for these particular viruses as well.

We have a good history at Chiron of developing transcripts that have been used for quantitation in the past. These references are just given in terms of support for that historical ability that we have at Chiron, and we did this for HCV quite some time ago very successfully and plan to do with West Nile and the other viruses.

[Slide]

In terms of developing the supplemental NAT assays, we have developed a procedure and a protocol which utilizes some of the best features really of the TMA assay as well as a TaqMan assay. Utilizing a magnetic bead separation, we use specific oligonucleotides bound to magnetic beads to separate the target nucleic acids and internal control sequences in the capture phase. It is a single tube target isolation. It uses between 05 and 1 ml of plasma or serum, with no centrifugations and a semi-automated wash step. It is a high throughput assay using the TaqMan technology in a 96-well microtiter plate format, and it allows for the simultaneous detection of at least three targets in one assay, if so desired. It is a user-friendly design; requires hands-on time of only about an hour and a total time of about 3 hours and 45 minutes to do

96 samples. It is also quantitative over a wide dynamic range and it has the ability to rapidly evaluate mutations because of the sequencing potential for the amplicons that are produced.

[Slide]

We haven't produced the assay yet for the West Nile virus but we have been using this protocol to develop supplemental assays for several other viruses. The HCV confirmatory assay, which actually was used in support of our submission to the FDA for the TMA assay, uses a slightly different sample preparation. It uses a silica gel-based method for that, but we are able to obtain a very high sensitivity within a half log, which is the requirement for the screening supplemental assay, and use that in terms of support for the BLA submission.

We have also developed in-house an HBV confirmatory assay, again with a very sensitive claim, 45 IU/ml, to support the introduction of our assay and clinical evaluations of that, as well as a parvo and an HLV. So, I just show these by way of showing that we have been able to apply this particular protocol to the detection and quantitation of several other viruses, and

the West Nile and the other viruses of the Japanese encephalitis complex should be no different.

[Slide]

In terms of recombinant protein expression, this is again one of the other major areas of expertise that we have at Chiron, we are planning to develop and express these recombinants in bacteria, E. coli and yeast, and potentially even insect cells. In an interesting discussion this morning, Dr. Wong was indicating that the tertiary structure of at least the envelope proteins may be important in terms of constructing the IgM assays. We also will be looking at some of the other linear structures in other types of antigens to see how they will provide efficacious reagents and antibody detection.

These recombinant antigens will also consist of various types of constructs, both full-length, truncated, chimeric, fusion and non-fusion proteins that could be derived from viral genomic regions that encode for both structural and non-structural proteins.

[Slide]

In terms of development of antibody assays, we plan to look first of all at the Chiron RIBA format, which

many of you are familiar with. We have developed several assays in the RIBA format at Chiron, in particular for HCV and also HIV. We plan to look at a format which could allow us to detect both IgM and IgG in this format for West Nile virus potentially. We may even be able to apply specific antigen bands for St. Louis encephalitis virus, as well as West Nile virus and potentially the other viral components of the Japanese encephalitis complex so we could differentiate in one assay the existence of antibodies to those viruses.

We would evaluate inactivated virus preparations, synthetic peptides and recombinant peptides as the antigen source for these assays, and we will also look at other antigen configurations as well, for example, anti-immunoglobulin specific antibodies in solid phases, and label specific antigens or peptides as conjugates.

In terms of timing for these activities, we plan to have each of these done in terms of the development of the materials and the assays to meet the requirements to have the assays implemented for screening purposes during the next season, in the late spring and early summer of next year. So, it is a very aggressive plan but we hope to

be able to support the development of the TMA assay in this context. Thank you.

DR. HEWLETT: Thank you, Bruce. It is always encouraging to get a nice summary of the kinds of things that a company is doing, and you provided us with a nice summary of the types of reagents you are putting together and the strategy to bring West Nile virus screening into the donor setting.

I would like to move on to the next speaker, George Dawson, from Abbott Laboratories, who will be speaking on an update on Abbott laboratories' strategy for West Nile virus serological testing. Instead of that, I think we will move on and go to the last presentation by Charles Tackney, from Ortho Clinical Diagnostics. The talk is entitled feasibility of an improved immunoassay for West Nile virus.

Feasibility of an Improved Immunoassay for West Nile Virus

DR. TACKNEY: I was going to make a small statement about being last and the privileges one could take about prattling on forever, but now I am disoriented.

[Slide]

Back in September, we were pleased to be invited to the AABB-FDA meeting that we had down here on strategy and discussions around the West Nile epidemic and what we might do with it. In that meeting we had a significant body of individuals there of significant technical and financial resources, along with a lot of our corporate colleagues. It was clear that within that group there probably was no problem that they couldn't solve.

[Laughter]

Actually, I don't mean that flippantly. That is actually true. Listening to Jesse Goodman and Jay Epstein talking about how we perceive this disease and what it meant in terms of our ability to respond to a perceived infectious disease risk to us, it was clear that something had to be done.

If you look up here, you will see that there are really two big areas. The first is the nucleic acid focused area, which involves PCR and related TMA platforms, along with other things that are basically emerging. The immunoassay area, which is more the expertise of Ortho because NAT is not part of our basic core, involves the IgM and the IgG ELISAs, along with the potential of developing

an antigen assay. The antigen assay would go a long way to solving certain problems. The bottom line is that both NAT and serology are required for successful detection, diagnosis and clinical follow-up.

[Slide]

We propose an improved IgM class capture format which involves enhanced sensitivity and specificity. We hope to offer a uniform platform performance and ease of use in interpretation. The West Nile antigen assay detection potential is there. The question is which antigen and what would the sensitivity be. Of course, there always remains the possibility of a novel assay format.

[Slide]

The challenges are clear from what you heard today. We really don't know too much. There is a lack of basic reagents. The present technology is all home-brew, which is sort of reminiscent of where PCR was in the early days. The viremic period is very, very challenging. There is a lack of specificity. And, there is a question of IgM prevalence and its time line. That calls basically for addition of new tools.

[Slide]

What do we need to do this job? We need certainly a better understanding of the patient. There is not a lot of information. We need to understand more about viral epitopes. There is very, very little known there. We have a big armamentarium of knowledge around HCV, BV and IV; we have very, very little there. In our organization, probably most of our expertise resides in understanding that. We need reagents that would enable us to distinguish this virus from its sister viruses. We want something that could be deployed with relative ease and surety of use and interpretation and that has the characteristics that would make it a useful test.

[Slide]

We have a diagnostic format which is basically within this structure that we use for detection of IgM antibodies for several agents, one of which is HIV or HB core as well. That assay involves the use of a very, very high performance monoclonal antibody to human IgM which, in solution, allows us to capture the IgM with great sensitivity and, with solution kinetics, drive it down onto

a streptavidine coated well on an ECI device, which is basically a box.

The development of the reaction is through the use of a viral antigen and a specific conjugate, which in this case would be a monoclonal antibody. The key to using this assay is not necessarily this part because basically all the assays do that with different flavis, but it is the kinetics of the reaction and how specific you can make this react with this to give the necessary information.

[Slide]

In this particular diagnostic assay for HAV, it has pretty typical sample requirements. It is a homogeneous reaction. Again, it uses chemiluminescent readout. It has well established ability, sensitivity, specificity characteristics. It has on-board sample dilution and runs with a time frame something like this. If you look at the MAC ELISA out of CDC, it is basically a three-day test when you really start doing it. So, we already have essentially a prototype platform here that we can exploit.

[Slide]

How do you do that? Well, what you put up here determines the flavi of that assay. In its simplest embodiment you would have lysate or you would have a cloned antigen. Those materials could be labeled in some way, or you might have a peptide, or you might have monoclonal antibodies, lighting them up. So, you would have solution capture of the patient IgM, homogeneous capture onto the solid surface, and then development with one of these reagents.

Now, if you seek to reach out to make this assay more specific, it is going to be determined by what these things are. We have a fairly high confidence level that it would be possible to generate reagents here that would enable us to distinguish the various flavi members within the family.

So, we have the advantage of an existing platform. We have some preliminary work in this area, developing these reagents, and are presently putting this together. One of the things that happened as a result of that meeting in September was that we realized that all of these folks out there, in the state labs, were really on the forefront, and there was an awful lot of knowledge

there that we were able to take advantage of. There have been some very, very generous labs which have given us a lot of material and a lot of expertise and help, and we feel pretty good that we are going to be able to get this together in a fairly short time and get it back into their hands for some early testing.

[Slide]

Thinking of antigen, as an aside because this hasn't come up except as a brief comment, there is no reason why, if one knew what antigen and if the sensitivity were suitable, one could not mimic what we have already done with one of our diagnostic tests for hepatitis C, which is to take samples and disrupt them. In this case this disruption is sufficient in the presence of human antibody to the agent, liberating subparticles, which in this case is core, and detecting them with a new conjugate that we use which gives us a very, very high signal to noise ratio or P to N. If that were West Nile and this antigen were identified and suitable monoclonals were developed, we would basically be able to put this together pretty quickly.

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This is an example of the response of such an assay, our core antigen diagnostic assay. You can see that it tracks quite reliably along with viral RNA. In terms of clinical sensitivity, there is a tremendous amount of data on this assay and it has been very, very successful in this format and monitoring patients under interferon therapy.

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So, the path forward is to take advantage of the platforms that we already have to develop new peptide and protein reagents, which we have begun doing and have had some success in distinguishing reagents that will help us to distinguish the flavis. We have identified some monoclonals from the field and we are developing some additional ones. Of course, that is going to take a little more time to leverage the technology that we already have for antigen detection in the HCV area and, finally, to get these things out into the hands of the folks who actually are seeing the patients and, hopefully, offer some contribution to the solution.

I don't think anyone sees immunoassays necessarily going away, but being really part of a longer picture of total patient management, and that involves

surveillance, diagnosis, treatment and screening. So, thank you.

DR. HEWLETT: Thank you, Dr. Tackney. I think I would like to now call on George Dawson, from Abbott Laboratories. Just to reiterate, it is an update on Abbott laboratories' strategy for West Nile virus serological testing.

Update on Abbott Laboratories' Strategy for WNV Serological Testing

DR. DAWSON: I wanted to go last anyway so this worked in my favor.

[Slide]

Good afternoon. I am George Dawson, from Abbott Laboratories. Thanks to the organizers for inviting me here today, and I am very pleased to be addressing the audience pertaining to Abbott's strategy for West Nile virus.

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I have been in the viral diagnostics business for 25-plus years and one of the things that I really think that I have learned is that the more enlightened you are about the natural history of a given virus infection, the

better you will be able to identify the correct reagents and assays to use.

With West Nile virus we have been seeing a lot of data in the literature and presented here today, but one of the most sorely lacking areas is the specimens from the pre-symptomatic phase of infection. These are exactly the type of samples that are needed to develop blood screening tests. So, there is a real lack here of having the correct specimens to make the right conclusions about the assay formats and their needed sensitivity.

I know we are going to find a lot of these samples over the next few months. There will be blood screening efforts in volunteer blood donors in various places. An alternative is to look at animal models, non-human primates, maybe mice, which may yield some useful information especially pertaining to how early RNA comes up after infection; what the titer is; what the duration of IgM detection is. Any results that are obtained on these natural history studies really depend on the selection and the quality of reagents. We know that as time goes on better and better reagents will be developed and we will likely see, as time goes on, that our current knowledge of

the natural history of West Nile virus will be enhanced by the availability of improved reagents.

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There are three general strategies one could utilize for screening blood. These are nucleic acid tests, IgM tests and antigen tests. Each has their advantages and disadvantages. Nucleic acid testing has a great advantage in that it directly detects virus or viral RNA.

Fifteen years ago, ten years ago it was probably unthinkable to develop a screening test for West Nile virus for screening blood but in the last two or three years we have seen in the United States HCV and HIV RNA tests used very regularly now and, in fact, in many other countries of the world. So, it is not so unthinkable anymore. However, the viral yield for this infection is much lower than what we have been seeing for HCV, HIV or HBV. So, there are some distinctions that need to be made with this virus and perhaps pooling strategies will not be a tenable way to go about making a test that really makes the blood supply safer.

IgM tests represent a second alternative and they have the advantage of utilizing known platforms, probably

being less expensive than nucleic acid tests. We have seen today that RNA and IgM are co-detected on many samples. We have also seen some data this morning indicating that there are differences in IgM levels of detection, that there are some newer tests, and I recall the work from Dr. Wong this morning showing some improved IgM detection tests. How do those improved IgM detection tests perform on samples that were previously RNA positive only? So, there is still a lot to be learned on the value of IgM testing.

Another question is why do some individuals become IgM positive for such a long time? Is the virus continually present and presenting antigenic stimulus to the immune system? Is the virus compartmentalized in different tissues, and is this a continuous stimulus for IgM? In other words, is IgM a surrogate marker for the virus being present in the body not necessarily detectable in the serum?

One potential downside of the IgM test is that very likely you are going to see cross-reactivity with St. Louis encephalitis and Japanese encephalitis and maybe some of the Dengue viruses, but this could also be viewed as a positive, that one would not necessarily want to be

transfused with one of these viruses. So, if IgM is truly detecting at least some of the period of RNA detection, it is not necessarily a disadvantage to have a broad-based detection system.

The disadvantages of IgM tests are that we have seen that many of the samples from pre-seroconversion donors are negative for IgM, and it is very likely that there will continue to be at least a subpopulation of RNA positive samples that will always be IgM positive. These are the natures of most types of infections we see with viruses. There is an eclipse phase; there is a period where there is virus; proteins in RNA detected; then at a later stage antibodies are detected. However, as new agents are developed for IgM detection, we must look at how will this change our picture about what the natural history of West Nile virus infection is?

Another disadvantage of IgM testing is that the IgM response appears to be detectable for many months after infection and if one used IgM screening as a primary test, what would be the strategy for reinstating donors? This could lead probably to a battery of tests that would need to be performed, RNA, IgM, IgG, etc.

Lastly, West Nile virus antigen detection may also become a viable alternative. I don't know if we have the correct monoclonal antibodies present today, or necessarily that the envelope is the correct target for detecting antigenemia but, certainly, antigen detection is a very familiar type of assay and less expensive than some of the alternatives and has the advantage of directly detecting viral proteins. The disadvantage is that most likely you are not going to be able to develop a sensitive enough antigen test to close the window or detect enough of the RNA positive samples.

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Abbott has had a long-standing history in viral diagnostics, in viral screening. We have two general areas, immunoassays and molecular diagnostics. We have developed IgG and IgM class antibody tests, total antibody tests, antigen tests, rapid tests. We have both qualitative and quantitative nucleic acid tests. We have a lot of experience in these areas and any prospective amino assay or molecular-based assay that could be useful for West Nile virus diagnostics or screening would be

compatible with some of the systems we currently have in place that are available worldwide.

The particular group of individuals I have at my disposal for the West Nile virus work are a very experienced group of individuals. I have about a dozen people. Probably the mean experience of each is 20-plus years in viral diagnostics and this team has cell culture capabilities. We propagated HIV, HTLV, HAV and rubella in cell culture. We have had a lot of experience with flaviviruses, though not necessarily this group of flavis. We have a group that discovered the GBV viruses. You remember in the mid-'90's three flavi-like viruses were discovered. Most of those individuals that were involved in that currently are at my disposal for this project.

In addition, we have had a lot of experience in identifying and developing serologic and molecular tests for HCV. We developed antibody tests, antigen tests, nucleic acid tests. We have worked on GBV-C antibody tests, qualitative nucleic acid tests. We have done quite a few studies on the epidemiology and natural history of GB viruses.

Individuals in my group have been developers of research assays and licensed serologic tests, and the molecular biology group has had NAT prototype assay development experience. We frequently do nucleotide sequencing and we have a group that expresses recombinant antigens, both prokaryotic and eukaryotic cell systems.

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We put together a few pretty aggressive goals, and I will need the cooperation from many different individuals, many of whom are in this audience. We want to quickly look at the utility of IgM tests and, to that end, we are establishing collaborations with academic and governmental institutions to procure reagents and specimens needed for assay development.

We are going to look first at anything we can about the value of any of the markers that are at our disposal for understanding the natural history of West Nile virus. We hope to make recommendations, at least internally to our own Abbott management, as to the value of IgM. Do we really think IgM is a viable way to look at blood screening? At this point, we have seen a lot of data this morning which indicates very little overlap between

IgM and RNA. I believe that the overlap will be much bigger than what we have seen so far; that we will see IgM detection on a significant number of RNA positive samples.

By the end of February we would be making motions to develop a prototype IgM assay and to provide at least manufacturing plans for how we would generate the needed reagents and to develop the assay and how to scale-up the various reagents that would be needed for this assay.

We hope to have a relationship with the FDA and CDC and other agencies. We will share our data as they are developed so that we make sure that we are in line with other types of research going on in this area.

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Towards those goals, we do not have at our disposal at the present time a lot of reagents because we have not been working on West Nile virus, but we are looking to establish material transfer agreements with different sites. We are looking at different sources of recombinant protein, different sources of monoclonal antibodies. We already have developed our own primers for nucleic acid testing. We are looking to source specimens

that will help us make our decisions about the utility of IgM testing.

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Our key milestones are to complete the preliminary evaluation of serologic and molecular markers for screening and diagnosing West Nile virus infection by the end of December. We are going to look, as closely as we can, at what is the value of IgM in screening blood donors. We are going to look at decisions to implement plans to generate improved reagents. Do we think we are on the right track but we need to generate some of our own recombinant proteins or other reagents internally that will then make our assay prototype more tenable.

For the first quarter 2003, if we are going ahead with an IgM test we will determine manufacturing plans; meet with the FDA to determine the pathway towards obtaining an IND for testing specimens; complete a prototype assay for IgM antibodies by the end of February, and provide manufacturing plans and time lines as reagents are identified. Additional milestones will be determined based on reagent availability and their evaluation. Thank you very much for your attention.

General Discussion

DR. HEWLETT: Thank you, George. At this point I think I will ask all the presenters to come up to the podium and we will open up the session to questions from the floor. DR. STRAMER: Sue Stramer, American Red Cross. My first question, and I have three, is for Dr. Conrad. Could you expand a little bit on your N equals 1? That is, you were screening I think approximately 5300 unlinked samples and you found one positive. I think you said at 260,000 copies/ml.

MR. CONRAD: You were not paying careful attention, Sue.

[Laughter]

DR. STRAMER: No, I could have been napping.

MR. CONRAD: That is a B- on the quiz! Actually, I only reported data from the samples that had gone all the way through the resolution matrix and we actually found an individual sample. We screened 7100-and something samples and 5300 came from states that had a high prevalence, those geographic regions that we knew. So, we cherry-picked. The one sample I believe had 196,000 copies/ml.

In subsequent weeks we have been screening matrices of 64 and 512 and have found them to be positive for West Nile virus, but we haven't gone and gotten the individual sample. We have to go through a process of requesting and tracking, as you are probably graphically familiar with. So, I wasn't going to present the data on those individuals until we have actually confirmed the individual sample.

DR. STRAMER: So now that we have clarified that, my question is all of your screening then was done in pools, none of it was done individually. So, if you had a low titer viremic sample you have a certain threshold of pool sensitivity and you may have samples below that.

MR. CONRAD: Well, but listen to this, because we actually chose to screen in pools of 64 we screened the primary pools. We went back to the pools of 512 to see if we would have detected it in the pool of 512. So, really 64 was the gross screen. Now, every time we had a pool of 64 positive we did a 4 X 4 X 4 matrix, which jumps it down to pools of 16 to resolve the individual, because we were cheap; we didn't want to test all 64 samples. So, the

smallest we have really looked at was pools of 16 and then confirmation in the individual samples.

DR. STRAMER: Right, but you looked at pools of 16 when your pools of 64 were positive.

MR. CONRAD: So, it is an ever decreasing subset.

DR. STRAMER: Right, so the smallest pool you screened was a pool of 64.

MR. CONRAD: Everything was screened in a pool of 64.

DR. STRAMER: So, in theory there could have been samples with lower copy levels than what you screened at.

MR. CONRAD: More than in theory, it is entirely possible. Even if we screened individual samples, as you saw, when we screened individual samples, 100 individual samples, we only detected viremia in nine of them although all of those patients had come in with an idiopathic fever, saying "I have West Nile virus." So, it is entirely possible that some of those would be positive because we only had 100 mcl aliquots for each of those.

DR. STRAMER: Thank you. My next question is for the manufacturers, that is, for GenProbe and for Roche. You made reference to screening, at least Cristina, you

did, for organ donations and, Jim, you made a comment relative to the importance of getting tissue and organ donations screened. So, I would like to know what the plans are to include not only organ donors but also tissue donors, which are very important not only for West Nile but also for HIV and HCV.

DR. GIACHETTI: Our plan right now is to start testing the samples that we receive from you, and we are planning to start next week. So, this is our current plan right now.

DR. STRAMER: That is for HIV and HCV, which is terrific.

DR. GIACHETTI: The way we see it, the way the system works, once we figure out which are the issues with these type of samples, what we apply for HIV and HCV we will apply for West Nile. Basically, our sample process is very universal and certainly it would apply.

DR. STRAMER: Okay. Jim?

DR. GALLARDA: Yes, we are primarily focusing on whole blood screening, however, if we had to do things over for HCV and HIV if we would want to entertain cadaveric specimens, possibly lymph nodes, I think it is going to

become a dialogue with FDA on how broad the scope of the IND should be. I think it is certainly something that warrants discussion.

DR. STRAMER: This also came up previously but my question is for the same two individuals. We are spending a lot of time and effort developing tests for West Nile virus and the specificity of those tests just for West Nile, so I was wondering what the possibilities are of multiplexing those tests with St. Louis encephalitis and for Dengue so we are not sitting here a year from now having the same meeting.

DR. GIACHETTI: Well, for the version basically we are doing right now is for West Nile virus but certainly we don't see any issues with being able to amplify all of them. The problem is that with the very aggressive time line that we have right now we don't have time to also look at the other viruses, but certainly we can do it. I don't see any problems with that.

DR. GALLARDA: I think from our perspective the decision will be made by the stakeholders. You know, if the general consensus is that we should have broader

coverage, then we will try to shoot for broader coverage. However, Cristina brings up the point about the time line--

DR. GIACHETTI: Exactly.

DR. GALLARDA: --and you know, if we add complexity we threaten the time line by multiplexing. So, it is an open question right now.

DR. GIACHETTI: Yes, if you would like a different time line, we can do it.

DR. STRAMER: Thank you.

DR. HEWLETT: I wanted to pursue that point just a little bit and ask whether the panelists would like to comment on whether that would be feasible, to develop an assay for the different flaviviruses at this point while you are just beginning to develop an assay. You are at the starting stages of product development. How feasible is it, and how much more time consuming would it be? And, is it feasible at all?

DR. GIACHETTI: These viruses are all very conserved so it is very easy to find areas that are conserved to target primers or probes. So, it is not an issue. The problem is that we are six weeks into the program so it would delay us six weeks plus extra time to

test. If you test multiplexing for more than one virus, you have to test sensitivity for all three viruses--all the performance is in triplicate so that is a lot of time.

MS. GROSSO: We already have at least five of the arboviruses--assays that were put together by the CDC. So, we have those primers and capture probes and molecular beacons. We also have a Dengue assay. So, we will certainly be looking at those as well as others. But it certainly would interfere with the time line but multiplexing is a capability for sure.

DR. HEWLETT: Thank you.

DR. KLEINMAN: Steve Kleinman. I have several questions as well. The first one is I understand that this agent has been classified as biological safety level three, which nobody has discussed today. I guess two questions on that. The first one is obvious, why? I don't know if anybody can answer that.

The second one is given that classification, how does that affect the ability to move forward? Does that make us go slower, and what is the practical implication for testing diagnostic samples in blood screening labs? Do we also have to have that same capability of working with

that type of agent? Because I think that doesn't routinely exist.

DR. GALLARDA: It makes no sense.

MR. CONRAD: The reason is because there was a laboratory transmission of it but not from human blood samples, from a high titer animal sample, which then yielded infection to a laboratory worker which instantly kicks it into the B-3 category when there is known transmission to laboratory personnel.

I will just speak momentarily, all of us have B-3 capabilities and it does not seem to be an issue, and I don't think the actual testing will be impacted by that because it is more the generation of the positive controls. As you know, all samples shipped to us, we presume to be negative and it is not until they are determined to be positive that any of the constraints of a positive sample are imposed upon it.

DR. KLEINMAN: What about if we find an initially positive sample and have to do confirmatory testing? Would that be an issue?

MR. CONRAD: Yes, confirmatory tests are considered positive and should be done but, I mean, that is

not going to be a huge number of samples and that problem is mitigated profoundly compared to what the general screening would yield.

DR. GALLARDA: We looked at the web sites for guidance on this and it looks like the Canadians have come up with a good idea, that is, for situations where you are working with cultured virus then that should be a biosafety level three, however, if you are working with serological development, then that goes down to a biosafety level two. I think the practical reality is culturing is one environment, however, developing assays and putting them into the intended use environment is something different that we have to consider.

DR. KLEINMAN: My second question I think is basically to GenProbe and Roche. That is, in planning this, do you think that you will be able to start with the same sample tube and then, based on one purification procedure, extraction procedure for HIV and HCV, then transfer the sample and use the same sample to purify for West Nile?

DR. GIACHETTI: We could do it but it is not the way that the protocol runs. Our protocol is just a single

tube so we do capture in the tube and the same tube is used for amplification. So, it is possible to do it but I don't know if we would like the extra step of aliquoting after the capture and separating the reaction. And, this would pose a problem with the HIV and HCV already licensed assay.

DR. KLEINMAN: So, from your point of view, we need another sample tube for this?

DR. GIACHETTI: Yes. The way we are developing this is completely independent, yes.

DR. PHELPS: I think if it can be done in a pool, however, it is not a problem because we would just take another sample of the pool that is already made.

DR. KLEINMAN: Well, that is what I am asking.

DR. GIACHETTI: Yes, certainly you can use the pool to aliquot.

DR. BISWAS: I would just like to ask the samples that you have been working on, these are plasma samples, serum samples? What sort of samples have they been?

DR. GIACHETTI: Virus in plasma.

DR. GALLARDA: I think the easiest path has been to develop transcripts which are very rapidly developed, move to cultured virus and move to clinical samples, most

importantly, the pre-seroconversion type of sample because that is the kind of target area that we want to go after.

DR. KLEINMAN: My last question is in an area that I don't really understand very well, and that is, is it reliable to use RNA transcripts to determine copy number?

DR. GIACHETTI: Our experience says that for HCV they correlate.

MR. CONRAD: Obviously, it is not optimal, clearly, because it doesn't control for the extraction of the virus but it is certainly not unreasonable. So, you know, there are pluses and minuses. All of us use transcripts as some original sort of idea. We also do experiments, and I am sure everyone does, where you do serial dilutions and Poisson distributions and attempt to correlate what you see in a transcript with what you would find in the whole virus environment. So, the idea is that they are partially okay and we look for better things throughout that is uniform.

DR. KLEINMAN: I guess it is almost as much a question for the FDA as it is for the panel, and that is, since they are going to come up with some sort of

sensitivity standard, I am sure, for these assays the question is what kind of referent does that have to be against? Can it be against an RNA transcript, or does it have to be against a viral culture? So, I guess that is an ongoing discussion.

DR. GALLARDA: Steven, one point of discussion is if there are standards that are development that are applicable across the industry, it should be blind to any particular technology. So, a WHO type of approach with a whole genome is a reasonable idea. Whether WHO is interested in doing West Nile or this should be driven in the U.S. remains to be seen, but a whole virus genome I think is something that should be considered.

DR. BIANCO: Celso Bianco, America's Blood Centers. I don't know if what I have is a question or a challenge for all of you and FDA. Obviously, what we are hearing today and in the last couple of months is the absolute lack of reagents that could be used as standards; that could be used as development. George Dawson actually mentioned, during his presentation, the ability, for instance, to infect non-human primates or other types of animals. We discussed that a little bit this morning and

there is low investment in that area but they have to be larger animals so that you can have enough materials. Why don't you guys all get together, and include FDA, and create a consortium to create some animals for reagents and generate the materials that we will need? Because you are talking about having a test out in the street in nine months.

MR. CONRAD: There is culture available. We can make reagents. We can make high titer panels and dilute--

DR. BIANCO: You can make the culture but what you are going to have are laboratory samples. You are not going to have pre-seroconversion samples or samples of titers, varying titers in a more natural thing than the spiked plasma sample that you are talking about.

MR. CONRAD: Celso, a bird model is an animal but I don't think it is at all analogous to a human being--

DR. BIANCO: Oh, not a bird.

MR. CONRAD: I think that we will find pre-seroconversion panels and we will derive them from the tests that we are doing now. In fact, if you carefully follow in my plasma donors, you will derive far superior actual human experiments where you have liters or 850 ml of

plasma derived from a person going through seroconversion. If you look at the best panels that come from HIV and HCV, which all my consortium co-mates have used, they were derived from those sources. So, I think it is not necessary to move to animal models because I don't think they are as good as what we can get from the plasma panels that we have used.

DR. GALLARDA: I will say, Celso, I think the great equalizer will be the CBER lot release panel!

[Laughter]

DR. HEWLETT: I was just going to say that this is a very important issue and we, at FDA, have recognized the need to put out standards. We are talking to the industry and various people from academia, and so on, trying to collect the relevant reagents. This may either be virus isolates or transcripts. If there is positive plasma that people have, we have asked them to share it with us. In fact, we are setting up a virtual repository and I think Dr. Rios, in FDA, has contacted a number of people to sort of store up this to initiate this effort and to start collecting relevant material.

So, I think over the course of the next couple of months we will be seeing a lot of activity in the form of dialogue and certainly in the form of actual testing because what is needed really is a collaborative study among the various test developers--

[Laughter]

--time out!--to come together and get these types of reagents together.

DR. NAKHASI: All right, I just want to focus our attention back again to what we learned this morning from the test developers and you, as the manufacturers. I want to sort of broaden the discussion and sort of concentrate the discussion. How do you see the tests which we discussed this morning, both serological and nucleic acid-based, and how they translate into high throughput?

Basically several questions, is it feasible in donor screening? Is it feasible in tissue screening? What test is feasible for early on or later on? Also, what are the standards we need? I think we need to focus the discussion and I think I would like to hear from all of you because I guess all of you presented some of the data on what the problems are and whether you can provide the

solutions. So, I think the transfer of technology--the purpose of this whole discussion of the West Nile workshop is to really get the two groups together and see how fast we can get this transfer of technology and at least have a test in a pretty reasonable time. Anybody can start the discussion on that.

DR. GALLARDA: Well, I think the likelihood that NAT is going to play a role seems to be high. We are looking at the infectious window period when the virus is present; whether antibody overlaps or doesn't overlap. That seems like a reasonable first step.

I think Jesse Goodman referred to the broader question this morning, and that is how can we do this quickly and do it in a way that builds an infrastructure so that we can respond quickly, whether it is NAT or serological assays.

The next lead into that is what do we do for the future when we know we are going to single unit testing and now we have an array of viruses for which we would like to have a master multiplex, and that creates a pretty significant challenge. So, I think NAT is a reasonable approach, however, only to look at it in the response to

West Nile virus threat I think is a little short-sighted. We ought to anticipate a bigger picture and build in mechanisms to collaboratively respond accordingly.

DR. NAKHASI: So, to follow-up on that, as Sue said earlier, maybe not even multiplexing because if you pick up the primer sequences which can pick up a broad range and see what virus is there and just, you know, total protection and then go over the specific tests and sort of detect whether it is West Nile, SLE or something like that.

MR. CONRAD: I think it is important to note that we already are testing. I mean, we have screened 10,000 samples. We are screening 10,000 a week and we will do 20,000 by next week. That is a fairly large catch basin and we can up those volumes now. So, that is within a few months of the actual call to do this. We are actually screening in donors now. So, I think that those will then become the data that are used to derive what is the optimal strategy, whether there is overlap between IgM, and all those issues will be resolved through these preliminary studies which are actually ongoing fairly rapidly.

DR. DAWSON: Let me answer for the serology side. For IgM testing, if that route is chosen and that becomes a

viable route, Abbott does have capabilities for blood screening. We have bead-based assays; we have prism-based assays that should be licensed some day, some year, in the future. But we do have in place now EIA assays and we are slated to do that. In addition, we do have platforms and tests for IgM testing and we have the capability to do IgM testing for diagnostics, and we are also looking at what is a potential strategy to reinstate donors that were either RNA positive or IgM positive.

DR. PHELPS: One other point, just to take off a little bit on what was said earlier by Jim, I do agree that I think we need to be looking at newer platforms for implementing assays for blood screening, chip type assays, something like that, that will allow us to expand to new viruses as they come up. It will be much easier to put a separate chip or a separate spot on the chip than it would be to develop a brand-new test.

With respect to West Nile virus, if we can do this in pools, if the concentrations are large enough that the pools that we are currently using can be used to screen, then I think we can implement fairly quickly. If we have to go to individual donation testing, as you all

know, right now the automation is not there and it is going to be very difficult to go to individual donor testing.

DR. STRONG: Strong, Seattle. Back to the question of availability of clinical materials, for Dr. Conrad, you are currently screening plasma donor samples to find specimens. My concern here is that the season has just about run out so you will have to pull this from pre-seasonal samples, back a few months. So, you have frozen samples or something of that sort?

MR. CONRAD: Yes. That is why we pushed so hard to get this done now because we are worried that it will taper off, the amount of clinical samples we can detect. Fortunately, some of the 60-day hold that is in the plasma industry is advantageous for helping find things. There are some mechanisms. Also, because of less urgency that is found in the plasma or the recovered plasma because there is not the immediacy necessary for whole blood, we might be able to get some archived material that will help expand these numbers.

DR. STRONG: Okay. As a follow-up question to the others, assuming that we can't find any more, how are you going to do your test development?

MR. CONRAD: I will share!

[Laughter]

DR. GALLARDA: I think you can get West Nile from ATCC. Right? So, one easy solution to start for a virus sample is to culture the virus and make panels. BBI presented this morning the equivalent of that I think. That is a way to get started. However, to encompass the real-world environment, we just don't have much of a history with West Nile like we do with HIV and HCV, hence, the need for the collaboration between the stakeholders.

DR. BUSCH: Mike Busch. You know, screening these for IgM, these plasma donors, would really allow you to pick up the recent converters and then you have your two months of inventory to look back and characterize viremia on a single sample basis.

MR. CONRAD: That is what we obviously thought we would do if we look at IgM as a hallmark. Unfortunately, it is not that proximal. When we first naively went to do that, we said, okay, is anybody's IgM positive. If you look three donations back, they are going to be viremic. But as you see from some of those 100 samples that we tested, that is not the case because the IgM persists much

longer than one would have intuitively assumed. So, it is okay for a screen, Mike, and I think that is a good idea and we may start doing that to try to push that forward, but it may not be as high yield as we would have wished.

DR. BUSCH: I really wanted to comment on really the need for animal transmission studies. The ramp up has to be very rapid. If you are going essentially from inoculation to 10^5, 10^6 copies/ml in four or five days you are talking about a doubling time of five to ten hours. There is no question that there is going to be viremia that will only be detected by single unit NAT, or even non-detectable by blood screening.

The question is when is that infectious. Right now the CDC, I think probably inappropriately, is ruling out a transfusion case if they are not able to detect a viremic donation with an assay that only has 5000 copy sensitivity. So, I really think, as we are doing for the other viruses, we need to be able to understand infectivity versus viral load, and the only way to really do that is to have a good animal model where you can passage, infect, draw and transfuse. So, I think a chimp is really critical.

MR. CONRAD: The one thing we don't know though for sure is how the whole natural history of this virus developed in symptomatic patients. So, I am not so sure that the four to five days, or any of those things, are true of all people who are infected by the virus because we just don't know that yet. You know better than we all do, with the blips and all that stuff, HIV and HCV surprised us all over the place and this may do the same. There may be a period where the viremia lasts longer than we think but not in symptomatic patients.

DR. GOODMAN: Jesse Goodman. First of all, I would just like to thank all of you and all the speakers so far today, and also to compliment the industry for how, in your various ways, you have gone to work on this and taken this seriously, with Andy Conrad starting to run these large numbers of samples getting what looks like a number of positives where you potentially have large amounts of samples. This could be a tremendous resource.

Also, not everybody here may know but I imagine many of these have been repeat donors on either side of this time point, which provides an extraordinary opportunity, once you have identified it down to an

individual donor level, to look at exactly this natural history and the issue of when IgM comes up. So, I think your plasma resource may be extremely helpful here and we will all really look forward to that information.

I was going to ask a couple of things. One, when I started out this morning I did raise this pan-flavivirus primer issue, and I would just encourage--this is not a regulation comment, it is more of an infectious disease comment from me, but I would really encourage people to think about that, and to think about it at a time when you are choosing your pathways because even though I think ultimately you would like a platform technology that could do, let's say, vaccinia tomorrow or something else, here you have this bizarre opportunity where you have three or four potential blood-borne pathogens that you could conceivably design primers for that would detect all of them.

I think it is worth thinking about when you look at the past epidemiology, such as Lyle Petersen has shown, where it could be that next year this goes away and in three years we have Dengue, or this goes on for five years but in two years we have SLE. So, I am not saying that is

what is needed because obviously we are dealing with an acute problem here, but it may have some advantages if it can be accomplished, and the time it is going to disrupt your development the least is probably early on. One could proceed with the West Nile primers you have and also look at some of these other possibilities and see how they stack up at a very early stage before you do a lot of field testing and validation.

I don't know from this point of view whether Rob Lanciotti or people in academia have characterized at all some of the potential pan-flavivirus primers and can give anybody a head start with ones that look decent, but I just wanted to bring that up.

On a completely different note, I wanted to ask the folks who look at antigen assays, and I guess Charles Tackney was one who mentioned it in terms of licensed antigen assays, just how sensitive these can ever get at their best, you know, and what you are thinking that is.

DR. TACKNEY: Well, first of all, our history with HCV, remember, it was built on many, many years of experience and knowledge, which we don't have with the West Nile. The end-game with the core antigen one might think

is rather trivial. One virus equals X-hundred core. You make a good set of monoclonals and you build a good assay and you have a sensitivity of X, which is in the extremely low picograms, let's say. But when you do the math of what that means in terms of viral copy equivalence, when you divide that back into number of viruses you are still talking about several thousand viruses.

Now, with West Nile we really don't have that experience at all. Is it going to be core antigen; is it going to be one of the non-structural proteins or what-have-you remains to be seen. I am sure Dr. Dawson will say the same thing, that we are all out there trying to find out what that antigen might be, and to be positioned to have the reagents to detect it. So, we have ongoing exploratory work developing monoclonal antibodies to a small subset of regions of some of these antigens to attempt to do just that. How low can you go? Theoretically, hopefully not less than one through a combination of antibody development and readout.

DR. DAWSON: How sensitive can antigen tests get? I think with hepatitis B surface antigen we are probably down to around 2500 DNA copies/milliequivalent. For HCV, I

can't come up with a firm number but it is in the thousands, and the same with HIV. But for West Nile we will be looking at antigen detection. We don't know how far we can take that, can we bring it down to the equivalent of 1000 RNA copies or not, but right now there are not a lot of available reagents. We will look at the envelope monoclonals that are available as the first line, but we will continue to look at antigen detection as a second alternative to nucleic acid testing.

DR. KATZ: Louis Katz, America's Blood Centers. Lest there is any media in the room, I just want to be sure that people understood that what Andy Conrad has at NGI is not at this time available for the whole blood industry, and we deal with turnaround times that require that we release platelets usually within 24 to 36 hours of collection, as opposed to the source plasma industry whose time line is actually months long for release of product. So, very seriously, if anybody believes that we can do this now--it can be done in the source plasma industry perhaps but it is not clear where we are with whole blood donations.

DR. RIOS: Maria Rios. Andrew Conrad, I would like to ask you, it seems that your assay is very sensitive. You are picking up one in 64 or one in 512 so it seems very high and it is very unexpected to everybody here I think. So, how much volume do you test for your sample? How much is the pool, and how much after pooling do you test for?

MR. CONRAD: We concentrate the virus from 2 ml.

DR. RIOS: Yes, but the initial pooling, how much do you use of this sample?

MR. CONRAD: The initial pools use 300 mcl of each individual sample, placed into the pooling tubes. That is the first tube, 300 mcl from each individual is withdrawn from the T-can. It is actually 320 because we leave a little extra volume in there for spitting into the tubes.

DR. RIOS: Another thing that I would like to ask, we have data that the HCV is a flavivirus carrying two positive strand RNA in the same virion particle. I was talking to Dr. Margo Brinton and she said West Nile has only one single copy of the RNA per virion and that would cut down the nucleic acid sensitivity in half. If this is

really a low copy number and we have to increase the sensitivity, how far can you go beyond the 300 mcl?

MR. CONRAD: To my understanding West Nile virus is a single-stranded positive RNA virus. Theoretically, you can take as much as is available. It is not hard to increase the volume. For all those media people here, again, we have large amounts of material so that doesn't seem to be the central issue. It looks like we can refine these and it is certainly just to provide these as reagents. We can increase the sensitivity. And, I might do a pilot where I will test 1000 samples from a high risk area with individual testing to see if there is a big difference to try to get some of those natural history numbers.

DR. BISWAS: I would like to ask Andy and the other panelists who said how sensitive the assays were, how did you come to those numbers?

MR. CONRAD: For us, the same way--RNA transcripts and then serial dilutions and then Poisson distribution, the exact same mechanisms we used for the licensed assays to determine probity analysis after detection of serial dilutions and some idea of its absolute

amount by using different methods, and then serial dilutions, which I think is the same way that most of us have to do it.

DR. KLEINMAN: I have a question for Andy as well. I am not sure if I understood you correctly but I think you said that your screening up till now was unlinked. Is that correct?

MR. CONRAD: It is unlinked as to donor identification. So, what we do is we take the matrices and we preserve all of the members of the matrix, the primary pools and the individual samples. We preserve the samples. They are relabeled so we know where they are, but they are not linked to donor.

DR. KLEINMAN: And that leads to my comment. Unfortunately, if they are unlinked you don't have the opportunity to go back and retrieve previous products from that same donor. Is that correct?

MR. CONRAD: Well, what we can do now is we can apply for an IND, go back and we have to look at donor consents because each of the people who send us plasma have very different donor consent forms. Some of them are incredibly general and say testing for virus. Then the

IRBs have to look at that. But what we are doing is we are rigorously storing all that material--

DR. KLEINMAN: Right, but I think I just want to clarify the point, Andy, because you have a tremendous source for generating the plasma seroconversion panels but, unfortunately, since you have had to do unlinked testing you can't do that up until now. You would have to do it going forward in a linked format, which gets into Mike Strong's comment that we have missed the season.

MR. CONRAD: Yes, it would have been better if we had this up in a linked fashion three months ago. It would have been optimal. It is going to narrow and narrow down and there are some constraints but we are trying to do the best we can--

DR. KLEINMAN: It wasn't meant as a criticism. I know you have to work with constraints. It was just kind of a clarification.

MR. CONRAD: Our friends at the FDA can help us with that.

DR. NAKHASI: Hira Nakhasi again. Part of the discussion is on the validation in the tissue setting. I

heard some of you have done a little bit, but where are we in that area and how far are we in that situation?

The second question is have you given thought to what tests will be suitable for confirmatory tests? Even though I asked earlier, I just wanted to throw that back again so that while you are planning for this testing, have you also planned for the confirmatory tests?

MR. CONRAD: For us, we are trying all different types of tissues and we could use other nucleic acid tests, like TMA, and they could use ours. As far as looking in other tissues, we certainly believe that the extractions are not going to be unique to blood. Obviously, some of our positive control material is derived from liver, from cell culture, from brain. We have even had formalin-fixed brain material where we have been able to extract easily nucleic acids for West Nile virus.

DR. NAKHASI: And RNA extraction, there wouldn't be any problem; it will be the same thing? Did I understand correctly?

MR. CONRAD: It has been our limited experience in the other tissues that we have looked at that the

extractions have worked very, very well in liver and in brain, as well as in whole blood.

DR. NAKHASI: How about kidney?

MR. CONRAD: We haven't tried kidney.

DR. GALLARDA: One question we have is what kind of data would FDA consider to be sufficient to make claim for tissues other than blood.

DR. BISWAS: Well, I think at this stage we don't know right now. You know, it is something that we have to discuss with you. But one thing I did want to say is remember that tissue donors are cadaveric donors and what has been done is that you have to test cadaveric serum samples. I will be saying this again tomorrow, but we do encourage you to test your assays also for cadaveric samples as well.

DR. NAKHASI: The next part of the question was a confirmatory test, what have we done and how are we thinking about that?

DR. GALLARDA: I think under IND, you know, one of the strategies is to use alternate primers to verify that the genome was there, and do a follow-up to confirm that the initial index sample, in fact, was hallmark of

true infection. So, the idea for a confirmatory one might be to cross-license claims between nucleic acid technology for confirmatory. You know, with the other viruses we have confirmatory tests, although these don't exist even for screening for West Nile. So, how that will shake out as a confirmatory option--I heard an option about RIBA to see if you can discriminate West Nile from other flaviviruses.

DR. PHELPS: Right. A couple of things, we had planned on constructing the NAT supplemental test, as I indicated, which would serve as the confirmatory or supplemental test for the TMA assay at least as far as the clinical evaluations are concerned. The RIBA assay could be used as a confirmatory test either for an NAT initial reactive and if it were IgM positive or IgG positive you would get a positive result with RIBA. So, that could confirm an initial NAT result. Or, it could be used as a confirmatory test for another screening assay, such as Chuck or George mentioned in terms of a screening assay for IgM. So, we are looking at confirmatories for both the antibodies and for the NAT.

DR. AKOLKAR: Pradip Akolkar, FDA. What is the effect of immune complex on this virus protection of

nucleic acid test? The virus is complexed. Do you think it is going to affect the protection?

MR. CONRAD: I don't know. The interesting thing is obviously in the samples that we know have antibody in them, they were harder to detect the virus in, but I don't know if that has to do with immune complexes or not. In big samples that we will eventually get we will look at things like that.

DR. GALLARDA: But if you look at seroconversion panels, there are classic control panels for no antibody present, virus present, both present and then antibody alone. Although the quantitation may not be predictive of the influence of the immune complex across seroconversion bleeds, you can see NAT for most of the duration--transition from RNA only to antibody only.

MR. CONRAD: The only difference with West Nile virus is that with HCV seroconversion, although there is a diminishment of viral load, is not as profound as it seems to be in West Nile virus. That is why clearly in the models of HIV and HCV there is a different story there, but with West Nile or viruses that obviously don't have what

seems to be a long chronic component to them, it may be very different and so I am not sure.

DR. GALLARDA: I have a question for FDA. Coming back to the multiplex, if you were to have a pan-flavivirus multiplex assay how would you view discriminatory assays? I mean, you could have a screen but then what do you do?

DR. HEWLETT: Well, you would have to follow the HIV, HCV paradigm, which is to have discriminatory NATs for each of the viruses.

DR. GALLARDA: That would exacerbate the time line issues. Although it is a good idea and it is the right thing to do, having that at the same time would be pretty tough to pull off.

DR. HEWLETT: Yes, I think that what was being proposed was a long-term game plan for how to address this type of scenario, rather than at the moment. Jesse, maybe you want to comment.

DR. GOODMAN: Well, I think we are kind of thinking as we go here and I think we would be interested in your thoughts about that. You know if it is only a screening test and not a diagnostic test and you can show

what the sensitivity and specificity is maybe we could think about do we really need to discriminate--

DR. GALLARDA: Right.

DR. GOODMAN: You certainly don't need to discriminate testing under an IND. So, these are things we can talk about.

DR. PHELPS: There are still donor counseling issues you have to deal with.

DR. HEWLETT: Right. There were two parts to that. One was to validate the test, and the second part was donor counseling, donor management issues. But, you are right, you know, we can do that in a step-wise of phase-wise fashion but in the end, obviously, you would want to know what you are infected with.

DR. LANCIOTTI: I just wanted to make a comment. We have actually published at CDC several consensus assays for flaviviruses, for alpha viruses, for some of the California group Bunyaviruses, and we are working on these now. One of the issues we have come across, we are using a real-time system, using SYBR Green and looking at melting curves, and we have never been able to get to the same level of sensitivity as we have with TaqMan using that

approach. So, I don't know if that is unique to our experience or if that is just going to be kind of a brick wall we are going to hit with the way the technology is. A lot of that is being done or has been done already and, you know, we just can't get to that same level of sensitivity.

DR. GIACHETTI: Yes, in our experience with multiplex, it is possible but optimization of the acid to get the same level of sensitivity takes much longer. Everything takes much longer to be able to have the system working.

DR. LANCIOTTI: Just another issue about sensitivity, I mentioned this earlier, that there are a lot of ways you can increase sensitivity through sample concentration and, obviously, at GenProbe you are capturing RNA and that is obviously a great help in increasing sensitivity. When we talk about copy numbers I think a lot of us are calculating it and there is almost a certain sense of smoke and mirrors in how all that is done. But one interesting thing from our perspective is that we sent you and other people the same standard that we have sent to every state public health lab in the nation, and in one of your slides your sensitivity limit was diluting that

something like two times 10^6 I think. That has basically been the experience of every state lab that has used that same standard that you received, and we have sent that same standard for years now and most of the state public health labs using a TaqMan assay are right at that same level.

DR. GIACHETTI: Yes, we get 95 percent detection at that level.

DR. LANCIOTTI: Right. Again, that has been the experience nationwide with using that standard as well.

DR. RIOS: Maria Rios again. My question is since this encephalitis virus has some very conserved region and belong to the same family, and we are talking about multiplexing, was it ever considered that there would be some region, like non-coding region, a very short fragment of the virus that would be well conserved among at least two or three members of the family, or the most important ones, so that we don't have to multiplex but, instead, use a common primer that would have a highly conserved region? Was that ever looked at?

MR. CONRAD: Yes, there is. If you do blast search of some of the primers that all of us will probably end up using, it is certainly likely but we don't know the

sensitivity because we will have to look at that. But there is certainly cross-reactivity--there can be cross-reactivity. We have looked at lots of primer sets and sometimes we select away from that for resolution but thinking more about this, we may go back and reinvestigate single primer sets that are better over a spectrum of viruses and sacrifice the specificity to West Nile.

DR. RIOS: From what I understand, is it possible to get them all? As they are infections that resolve and are not chronic like HBV, can we just get the blood supply safer by ruling out any of these? In case Dengue comes along, can we just use a set of primers?

MR. CONRAD: I have to say that in our cursory look at it we were certainly not able to get the magic primer pair that would equal the sensitivity we could derive using other primer pairs. We excluded those from our earlier catches because they just weren't as sensitive. So, I don't know but we haven't done as much work as we would have liked in that arena, but certainly our experience matches the CDC's in that it hasn't been quite that easy.

DR. LANCIOTTI: I think maybe I wasn't clear. We have a single set of primers that have enough homology to all the flaviviruses that they amplify all of them in one reaction. There are regions--in fact, our primers are in NS5 and they will amplify any flavivirus but the sensitivity is not the same as TaqMan.

DR. RIOS: Can you use TaqMan?

DR. LANCIOTTI: Well, it is not TaqMan, it is a real-time RT-PCR. Instead of having a probe in there or individual probes, we use SYBR Green.

DR. RIOS: [Not at microphone].

DR. LANCIOTTI: We actually looked at that as well, but you can't just pick probes where you want them. A lot of times you have to go by melting temperature and there are a lot of other issues. But SYBR Green was the best we could come up with where we have two primers that amplify all flaviviruses but, even using that approach, we don't get the same sensitivity as with TaqMan.

MR. FREIBERG: I am Glen Freiberg, from GenProbe. I wanted to follow-up on Dr. Goodman's comment and the general audience participation here in designing products--

[Laughter]

It is a lot of fun. My point is that what we have all been talking about today and what is most important is to get the projects started; to get the INDs started. But what everyone seems to be ignoring is that everyone up at the dais up there, and myself, is also interested in getting the project finished.

If we talk about we can start something under an IND with no discriminatory probes, that really doesn't get us anywhere for finishing the project because if we do an IND next summer and we don't have an assay in place that is licensable, how many years is it going to be and how much treading water do we have to do, even if we have grants, even if we have cost recovery? We have a lot of resources in people and clinical monitors tied up with treading water. And, as an industry we can't tread water year after year like we did with the HIV, HCV system.

So, what we are going to have to do with the last panel tomorrow afternoon is ask a series of questions of FDA, and I am not going to use the old term "out of the box" I am going to use the new term "new paradigm"--

[Laughter]

--what are we going to do different to approach a license quicker? You know, even though we want to get started faster we have to figure out what it is going to take also to finish.

DR. NAKHASI: Very good, Glen. I think I appreciate your comment. You are right. I think what we need is to focus on what the goal is and how quickly we can get to that goal. I think you are right about that. However, we should proceed with this thing and see what other problems there are. This is a general discussion. Again, we are not setting up policies here. This is a scientific discussion and we basically want to see anything which can be done at this time put on the table but, again, the reality is how much can you do and how quickly you can do it.

DR. BISWAS: Does anybody else have anything to say or to ask?

DR. HEWLETT: Thank you very much. Thank you for that very lively participation in the audience. We will meet here again tomorrow at 8:00 a.m. Have a good night's rest and be back bright and early tomorrow. Thank you.

[Whereupon, at 5:30 p.m., the proceedings were recessed, to resume on Tuesday, November 5, 2002 at 8:00 a.m.]

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