



Full Meeting Summary

Puerto Rico Brain Trust for Tropical Disease Research and Prevention

Meeting on Rapid Diagnostic Testing for Zika, Chikungunya, Dengue, Leptospirosis and Influenza

Puerto Rico Trust for Science, Technology and Research

Rio Piedras, Puerto Rico - February 8-10, 2016

The meeting on rapid diagnostic testing for zika, chikungunya, dengue, leptospirosis and influenza, an initiative of the Puerto Rico Brain Trust for Tropical Disease Research and Prevention was held February 8-10, 2016 at the Puerto Rico Trust for Science, Technology and Research. The main focus of this meeting was to discuss how to best develop a rapid diagnostic test to differentiate between Zika, dengue, chikungunya, influenza and leptospirosis.

The Puerto Rico Brain Trust for Tropical Disease Research and Prevention

The Puerto Rico Brain Trust for Tropical Disease Research and Prevention is a research and development organization that brings together scientists from multidisciplinary backgrounds to guide, strategize, connect and provide technical and subject matter support on research initiatives in tropical diseases focusing in areas of rapid diagnostic test design, vaccine development and trials, and vector control all leading to improved patient care and preparedness. The **aim** of the Brain Trust is to accelerate research into translational work that will result in prevention and improved treatment.

Mission

The mission of the Puerto Rico Brain Trust for Tropical Disease Research and Prevention is to promote research and development in area of tropical diseases that will accelerate their prevention and control.

Vision

The vision of the Brain Trust is a world free of tropical diseases.

The Roles of the Brain Trust are to:

- conduct timely scientific forums for advancement of tropical disease research and prevention in Puerto Rico,
- connect experts from multidisciplinary backgrounds to collaboratively advance science, promote preparedness, and improve patient care in the area of tropical disease research and prevention,
- strategize and advise key decision makers in matters of science, technology and research related to tropical disease research and prevention, and
- contribute to the technological renaissance of Puerto Rico by promoting Puerto Rico as a hub for tropical disease research and prevention initiatives.

II. Background

A small group of scientists in Puerto Rico developed the concept of the Brain Trust. Subsequently, a small grant was prepared in December of 2014 to the Puerto Rico Trust for Science, Technology and Research to hold an initial meeting to address the need for rapid diagnostic testing following a Chikungunya epidemic that occurred in Puerto Rico in the spring of 2014. Our initial meeting on the topic of Rapid Testing for Zika, Chikungunya, Dengue, Leptospirosis and Influenza held on February 7, 8 and 9, 2016 was the birth of the Puerto Rico Brain Trust for Tropical Disease Research and Prevention.

Our three specific objectives for the initial meeting were:

- A) To compare the available tests by characteristic for example, diagnostic accuracy, biological sample needed, preparation of biological sample, cost, rapidness, ease of use, technology employed to create test, timing of test with regard to disease procedure, requirement of lab equipment and use of reader device.
- B) To compare available rapid tests for chikungunya, dengue, zika, leptospirosis and influenza with that of the existing gold standard for each disease with regard to diagnostic accuracy.
- C) To use this information to determine the best way to create a rapid and effective diagnostic tool to test for zika, dengue, chikungunya, leptospirosis and influenza.

In an ideal world, the test we are hoping to develop would meet the following criteria (mostly adopted from the World Health Organization's characteristics of an ideal test): affordable, sensitive, specific, user-friendly, rapid and robust in different climates, equipment free and affordable (possibly at different price points), delivered or available to those who need it with a short turn-around time to effectively treat the patient and control the spread of the disease.

Between 25-30 participants were carefully selected and recruited overtime. A complete list of participants can be found in the appendix of this report along with their bio sketches. Experts from each disease being discussed (zika, dengue, chikungunya, influenza and leptospirosis) were invited. An expert in influenza was invited due to vast experience the influenza group has in detection, treatment, vaccine development and ultimately control and prevention of the annual influenza disease. Experts in the areas of test design and development from the private sector both local and US based were invited along with test developers from the academic world with expertise in areas of immunobiology. Representatives from the Trust for Science, Technology and Research, our funding agency attended. A Pharmaceutical Systems Specialist and the Deputy Director of Viral Disease both from Walter Reed Army Institute of Research participated in the meeting. A contractor from Defense Advanced Research Projects Agency (DARPA) with interest in mosquito abatement attended along with Centers for Disease Control and Prevention Dengue Branch Chief, Manager and the State Epidemiologist.

The recent arrival of the Zika virus to the United States in December of 2015 has made diagnostic testing for these aforementioned diseases an extremely high priority because it is believed to cause congenital microcephaly in unborn fetuses (BMJ article neural imaging study). It is hypothesized that congenital microcephaly is just one of the most extreme results of a possible spectrum of conditions that attack the brain and central nervous system of unborn babies that are now also theorized to be caused by Zika, but additional epidemiological research will be needed to better understand this. Additionally, Zika is also believed to cause Guillain-Barre Syndrome in adults which is a rare illness where one's own immune system damages the nerve

cells causing varying degrees of paralysis. We do know that the Zika virus is transmitted via blood, semen, and body fluids. Measures have been taken in Puerto Rico to protect our blood supply to protect from further spread of the disease.

Meeting preparation materials included(appendix): detailed descriptions of tests available for each disease along with a summary of the disease and our objectives for rapid test creation. A **test table** was created that describes diagnostic testing platforms typically used for zika, dengue, chikungunya, influenza and leptospirosis (test tables can be found in the appendix). **Key articles** on rapid test development and **bulletins** about current Zika epidemic as it was progressing through Brazil, Puerto Rico and the United States were included. A **list of research questions** was developed as meeting materials were prepared to get participants to focus their thoughts and better understand what outcomes we were hoping to achieve.

The meeting agenda can be found in the appendix of this report. Day one of the meeting consisted of mainly presentations by experts in each of the disease areas, presentation by the state epidemiologist, the CDC Dengue Branch Manager and academic test developers. A more detailed summary of these presentations can be found in the full meeting summary. The second day of the meeting focused more on how to actualize a new diagnostic test learning from the experience of inventors in this area. One of the main points was not to re-invent the wheel, not to underestimate meeting the needs of the clients and the great importance of packaging.

Based on the recommendations that evolved from our first meeting on diagnostic testing, future work of the Puerto Rico Brain Trust for Tropical Disease Research and Prevention can be categorized into the following three core areas: **Core 1: Research and Development of Rapid Diagnostic Testing**, **Core 2: Comprehensive Vector Control Strategy**, and **Core 3: Capacity Development and Preparedness**:

Research and Development of Rapid Diagnostic Testing and Vaccines for Infectious Diseases

- 1) Need to urgently create a diagnostic test for Zika virus that can clearly define and differentiate it from dengue virus and take advantage of research funding being allocated by government agencies and foundations to address and contain the unfolding Zika epidemic.
- 2) Promote public-private partnerships that can enhance the opportunities to move forward with additional testing platforms that will increase testing capacity, reduce turn-around time, by validating existing tests and move to obtain FDA approval.
- 3) Reach out to countries that have tests that could be suitable for expedited FDA approval.
- 4) Improve the marketability of a rapid test. There is a need to educate health care providers, health insurance companies, and the public about the importance of early and rapid diagnostic testing.
- 5) Partner with Zika vaccine developer at the University of Georgia to explore the testing and manufacture the vaccine in Puerto Rico.

Comprehensive Vector Control Strategy

- 6) Create a comprehensive Aedes Aegypti Vector Control Strategy for Puerto Rico by holding a subsequent meeting to explore the combined approaches of traditional vector control strategies with use of Sterile Insect Technology (SIT) to eliminate the Aedes Aegypti mosquito vector.

Capacity Development and Preparedness

- 7) Develop a Biorepository that will address the need for biological samples required to validate testing in the development pipeline and those seeking FDA approval. This may be an activity that may also require a public-private partnership.
- 8) Strengthen the Brain Trust's collaboration with the CDC Dengue Branch in Puerto Rico to enhance lab capacity and support rapid test and vaccine development.
- 9) Working to streamline the FDA process for use of sterile insect technologies, diagnostic test and vaccine approval when there are outbreaks that require immediate action like Zika and Ebola.
- 10) Work with the Governor's Administration to create an administrative order for diagnostic tests to be covered by medical insurance so that price is not a barrier.
- 11) Facilitate efficient communication and collaboration between mutually beneficial private and public (local and federal) partnerships to enhance our ability to prepare to an infectious disease crisis.

Results

There have been several tangible outcomes as a direct result of our initial meeting on Rapid Diagnostic Testing for Zika, dengue, chikungunya, influenza and leptospirosis. One main key result is that this meeting brought together key people in government together with biotechnology experts and this combination has resulted in real traction.

1) First, the immediate planning of our second follow-up workshop, which is also an initiative of the Puerto Rico Trust for Science, Technology and Research, on the **"Creation of an Integrated Strategy for Aedes Vector Control and Elimination in Puerto Rico"** which will take place on May 23, 24, and 25 of 2016. Eliminating or greatly reducing the Aedes vector from Puerto Rico will eliminate or greatly reduce Zika, chikungunya and dengue all viruses transmitted by this vector.

2) Second, the Brain Trust for Tropical Disease Research and Prevention through the support of the Puerto Rico Trust for Science, Technology and Research has secured funding for Dr. Ignacio Pino and CDi Laboratories rapid diagnostic development project titled, **"Use of Proteome arrays for screening and diagnosis of Zika, dengue and chikungunya."** Dr. Pino's work will be supported by the purchasing of two microarray scanners with autoloaders which are essential for aim three of his proposal to generate a prototype array in a 4 x 16 arrangement that is 384 well format compatible using the serodiagnostic antigen set identified in aim 2.

3) Thirdly, Dr. Jorge Munoz and his colleagues at **CDC Dengue Branch have created a rapid trioplex assay to distinguish Zika, dengue and chikungunya.** This test is being replicated and put into use by state and local health departments around the United States.

4) Fourth, The **US Army Advance Development Agency (USAMMDA) will be selecting the Puerto Rico Trust for Science, Technology and Research's Consortium for Clinical Trials as a site to conduct a multi-center prospective clinical trial for the Next Generation Diagnostic System (NGDS), a multiplex system currently being developed by BioFire Defense** and the Army for the diagnosis of dengue, Zika, chikungunya and various other pathogens in the blood of patients.

5) A visit from Dr. Ted Ross about collaboration for possible Zika vaccine development and sample collections.

6) The Puerto Rico Trust for Science, Technology and Research has agreed to **continue to support the Puerto Rico Brain Trust for Tropical Disease Research and Prevention for two years** as an initiative until we can secure partial to full support from outside funds.

7) A future initiative to bring together collaborating partners (CDC, Galveston National Laboratory, and University of Georgia) for the **creation of a biorepository in Puerto Rico** for the storage and use of blood, serum, plasma, core placental blood and tissue samples. This would be important to enhance research and development in Puerto Rico, store samples of rare tropical diseases, and help to enhance preparedness for potential bio-terrorism.

Second Follow-up Meeting to Create an Integrated Strategy for Aedes Vector Control and Elimination in Puerto Rico - May 23-25, 2016

The Puerto Rico Brain Trust for Tropical Disease Research and Prevention held an initial meeting on the importance of the diagnostic testing for zika, dengue, chikungunya, leptospirosis and influenza to improve patient care and prevent and contain epidemics. Following this meeting, key participants suggested holding a follow-up meeting to address the Aedes mosquito vector and the diseases it carries (Zika, chikungunya and dengue). The Brain Trust proposes to host a meeting to determine whether or not we can create a comprehensive integrated pest management using a variety of interventions to eliminate the Aedes mosquito vector. Participants will be charged with developing an integrated strategy using interventions that are feasible for the Puerto Rican context. We will be utilizing a planning matrix tool to create an integrated intervention strategy with a timeline (short-term, mid-term and long-term implementation), an understanding of how interventions will be implemented, and an analysis of the barriers and challenges associated with the proposed strategy.

Participants will carefully weigh the effectiveness of a group of diverse methods of Aedes vector control. These methods include source reduction, the use of Sterile Insect Methods (SIT) (traditional irradiation-based SIT and innovative methods such as Genetically Engineered Mosquitoes SIT and Wolbachia SIT), use of Autocidal Gravid Ovitrap (AGO traps), use of pesticides and larvacides, bed nets, screens and other low-tech methods for vector control of the Aedes mosquito species. The proposed workshop will bring together a diverse group of scientists from multiple disciplines to develop the best integrated approach using all the available tools in the toolbox. The primary goal of this meeting would be to create a playbook for the integration of vector control and elimination strategies that could be implemented in a public-private partnership.

The main objectives of this meeting would be:

- 1) to utilize this diverse group of participants to create an integrated pest management strategy utilizing a combination of Aedes vector control interventions;
- 2) to create an intervention matrix showing combined options of the best integrated pest management strategy along a timeline of short, medium and long term implementation. Followed by an analysis of how these strategies will be implemented and an identification of the barriers for each proposed intervention;
- 3) to produce a feasible draft blueprint of how a combined vector control and elimination strategy could be implemented in public-private partnership with an understanding of the barriers and challenges associated with the proposed interventions;

- 4) to have a panel of key meeting participants summarize what they think the best approaches to an integrated Aedes vector control and elimination strategy would be based upon the meeting process.

This meeting would involve key participants from the Centers for Disease Control and Prevention, the Puerto Rico Department of Health, The Puerto Rico State Epidemiologist, the US Department of Agriculture, key genetic entomologists and ecologists, representatives from the International Atomic Energy Agency (irradiated mosquitos), Representatives from Walter Reed, DARPA and BARDA, representatives of companies like Oxitec (genetically engineered mosquito interventions) and Mosquito Mate (Wolbachia mosquito interventions) worldwide, Spring Star, Senecio (mosquito release) and experts in mosquito abatement and trapping.

A full summary and presentations from the meeting on Rapid Diagnostic Testing for Zika, dengue, chikungunya, influenza and leptospirosis follows.

Rapid Diagnostic Testing for Zika, Dengue, Chikungunya, Influenza and Leptospirosis Meeting Summary

The meeting agenda was strategically organized to have the meeting sponsor Lucy Crespo, representing the Puerto Rico Trust for Science, Technology and Research welcome participants 2), Dr. Jose Lasalde welcomed participants on behalf of the University of Puerto Rico and provided comments regarding our local research environment. Dr. Jose Cordero gave the official meeting charge to the attendees emphasizing our meeting objectives with regard to considerations in diagnostic testing for tropical diseases. Our state epidemiologist, Brenda Rivera provided an update of Arboviral disease in Puerto Rico, with most up to date counts for confirmed Zika infections, microcephaly and Guillian barre syndrome counts. This information was key to participants' understanding of the current local situation of simultaneous endemic dengue virus, influenza epidemic, along with recent memory of the chikungunya outbreak from 2014-1015 and our current unfolding Zika epidemic in 2016.

Our agenda then shifted to have experts from each disease present on diagnostic challenges with regard to their area of expertise. Dr. Elizabeth Hunsperger presented a comprehensive talk on Dengue Diagnostics, Dr. Jorge Munoz, current Chief of the Surveillance and Research Lab of the CDC presented on the challenge of Zika and Chikungunya diagnosis in dengue endemic regions. Dr. Scott Weaver, Director of the Institute for Human Infection and Immunity at the University of Texas Medical Branch and Scientific Director at the Galveston National Laboratory presented a comprehensive talk on epidemiology of and diagnostics issues pertaining to the Chikungunya virus. Renee Galloway, the CDC expert on Leptospirosis presented an overview of the state of diagnostic testing. Dr. Ian Lipkin and his team presented a multiplex assay for detection and quantitation of Zika and Ebola viruses and the Vir Cap Seq – VERT, A tool for viral surveillance and discovery. Following Dr. Lipkin, Dr. Lee Gehrke presented how a Paperfluidic diagnostic device could be used to detect and distinguish arbovirus infections. Dr. Ignacio Pino, from CDi Laboratories, a local device manufacturer, and his partner Dr. Xiaowu Liang from Antigen Discovery, Inc. presented how they could identify biomarkers for infectious diseases like zika and dengue. They demonstrated that Puerto Rico has the capacity to become a true hub for tropical disease testing, trials and product development.

Day two began with a brief summary from day one's meeting and a joint presentation from Dr. Francis Mandy, Vice President at LeukoMetrics in Ottawa, Canada and Dr. Abe Schwartz, President of the Center for Quantitative Cytometry. Dr. Ivan Lugo presented on the unique opportunities for research and development on tropical diseases that Puerto Rico can offer. Dr. Jose Cordero led a closing discussion among participants about how we can best organize ourselves to act quickly to develop a diagnostic test for zika, dengue, chikungunya, influenza and leptospirosis.

Presentations

Welcome Remarks and Presentation

Dr. Jose Lasalde Dominicci, PhD

Vice President for Research and Technology

University of Puerto Rico

Dr. Lasalde's brief presentation focused on a brief description of the zika virus situation and how it's presence in Puerto Rico is changing the focus of this meeting by creating a true sense of urgency and a funding stream for increased research on expanding our knowledge base on the topic. Dr. Lasalde emphasized how Puerto

Rico is a natural laboratory for the study of these existing and emerging tropical infectious diseases. We have the presence of the Aedes vector, all strains of DENV, CHIKV and now ZIKAV, an ethnically diverse population, experience in conducting clinical trials for vaccine research and have the most pharmaceutical industry per square mile on the planet. Institutions in Puerto Rico receive federal funding from agencies like NIH, NSF and CDC. Our medical school at the University of Puerto Rico is solid, the Trust for Science, Technology and Research is a developing asset to promote and support this type of research. In addition, Puerto Rico offers attractive tax credits for businesses that chose to invest in Puerto Rico. This is an urgent issue, we need to take effective action and collaborate across disciplines to achieve success in creating a point of care rapid diagnostic test and work to identify vaccine development strategies.

Update on Arboviral Diseases in Puerto Rico

Dr. Brenda Rivera, DVM, MPH

State Epidemiologist

Puerto Rico Department of Health

Dr. Brenda Rivera began by presenting Puerto Rico's current numbers for zika virus confirmed infection. The first confirmed case was on December 30, 2015, but retrospective testing of more than 200 negative DEN and CHIK samples show that the first locally acquired case of zika was found the week of November 23, 2015. Highest numbers of locally acquired zika are found on the north eastern coast and in municipalities around metro area of San Juan.

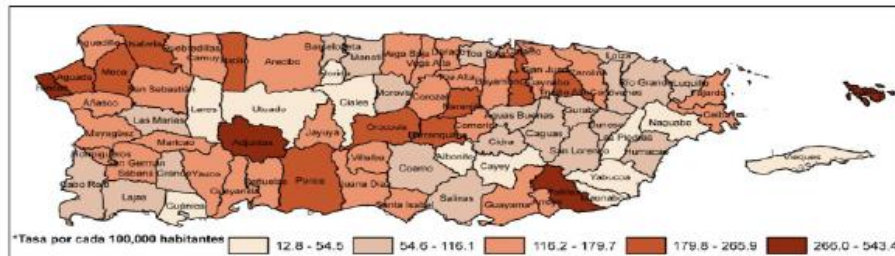
Dr. Rivera presented all of the epidemiological data for each of the diseases of relevance to our meeting. First focusing on zika, showing cases confirmed by week, the distribution of cases on the island, case profile, cases confirmed for zika, chikungunya, and by week for 2015-2016 and by age group, the distribution of chikungunya, dengue, leptospirosis and influenza. Confirmed number of leptospirosis cases by month from 2011-2016, with July being the peak month with over 35 cases. Leptospirosis cases by sex and age group with greatest number of cases being among males ages 25-65+. Males ages 50-64 with more than 80 reported cases since 2011.

Influenza now of great concern with 7,914 cases reported in season of 2015-2016 already 5 deaths and 579 hospitalizations. She estimates about 2000 cases of influenza per week. The virus is spreading very quickly throughout the entire island. Possible reasons for high rates of influenza in our population is the low rates of vaccination. Reasons for increased complications and hospitalizations due to flu are pulmonary (26%), cardiovascular (9.5%), metabolic (8.8%), obesity (15.9%), pregnancy (5.4%), ICU (4%), and mechanical ventilation (3.3%). It was estimated that about 20% of those hospitalized with complications from the flu were not vaccinated.

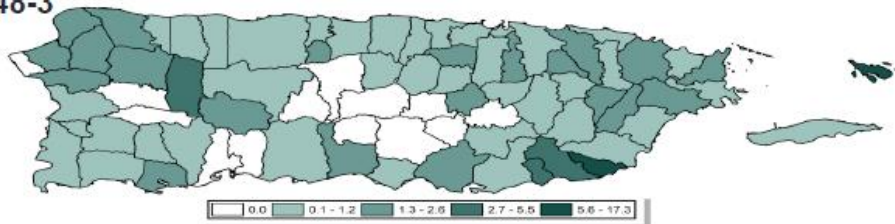
A map of the rates of influenza by municipality of residence in Puerto Rico was compared with a map of rates for arboviruses and the municipalities with the highest rates for both correlating. Indicating that where one lives is an important variable in their likelihood to become infected or not regardless if it is flu or an Arbo Virus. See slide of Comparison rates maps influenza and ARBOV below:

Comparisson rates maps Influenza y ArboV

Mapa de tasas de influenza* por municipio, semanas 1 – 4



Tasas de casos sospechosos de ArboV* por cada 10,000 residentes, semanas 48-3



Dr. Rivera commented several times on how the climate, specifically draught or unusually heavy rain affects the Arbo Virus population and intern the rates of dengue, chikungunya and zika infections. If it is dry, like Puerto Rico's recent draught due to el Niño, the vector population is reduced and number of humans infected with the viruses is also less.

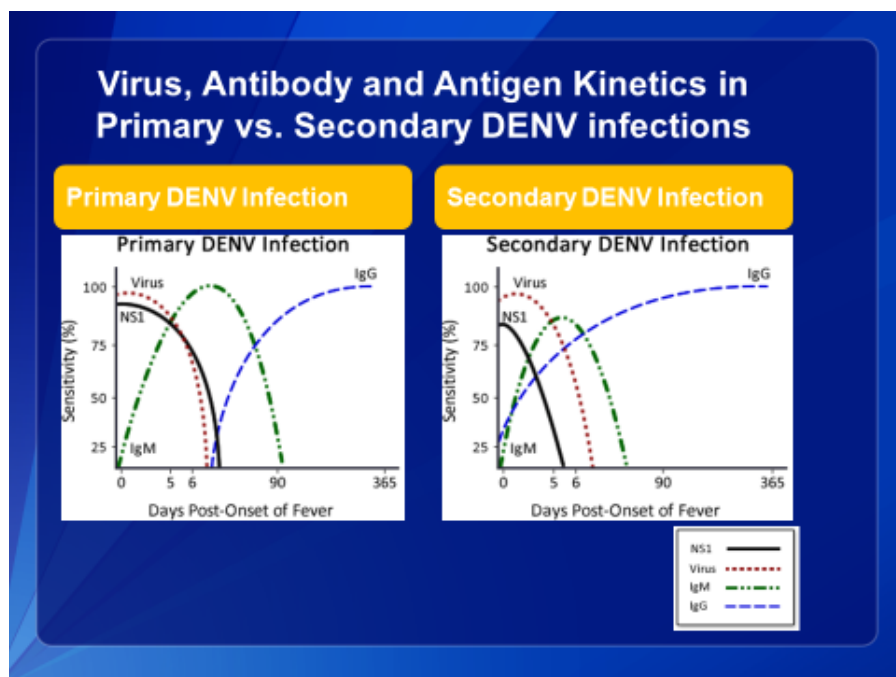
Dr. Rivera hypothesizes that an epidemic for Dengue is nearing because they have noted a shift in serotype among cases reported. In the past they have primarily seen Dengue I and IV, but recently there has been a sharp increase in the number of DEN II cases. DEN II has not circulated in our population for many years, therefore there will be many susceptible individuals with no immunity. Emphasized strongly, was that underreporting and probable misdiagnosis of all of these diseases occurs frequently, especially difficult to diagnose is leptospirosis. The state health department is currently monitoring the companion animals for leptospirosis infection to better understand health of human population. Dr. Rivera feels that physicians have a difficult time diagnosing because all of these diseases have very similar symptoms and partly due to the fact that the physicians do not take time to verify the public health reports that are publicized every Thursday describing epidemiology of disease in Puerto Rico. It is essential for physicians to understand what is happening in the community to provide effective patient care and management of illness.

Dengue Diagnostics and RDT tests for Dengue

Dr. Elizabeth Hunsperger, Ph.D.

Former Activity Chief

Dr. Elizabeth Hunsperger covered in great detail the dengue virus, transmission and diagnosis. She followed this by describing current techniques used for clinical diagnosis and surveillance followed by a summary of commercial assays currently available. Dengue virus requires a diagnostic tool that takes into account differing genotypes, differing serotypes and differing co-circulating flaviviruses. It is endemic in Puerto Rico, so it is estimated that between 80-90% of people will have dengue virus antibodies present in their blood. Dengue, like zika, is from the Flaviviridae family and the genus Flavivirus. It is single stranded, enveloped, positive sensed RNA virus with four serotypes. It is transmitted to humans by *Aedes aegypti* mosquitoes. The dengue virus, antibody and antigen kinetics in primary and secondary infections is shown in this slide.



Typically, the secondary infection for an individual results in the severe dengue which can lead to hemorrhagic fever and death. It would be useful to create a test that could discern whether it is an individual's second infection with dengue virus in order for patient care providers to understand the level of seriousness of the infection, especially when dealing with medically fragile patients. The 2009 WHO Guidelines revised the criteria for Dengue Case Classification and create two categories based upon level of severity. The non-severe group contains dengue without warning signs or severe dengue (with warning signs). Warning signs are: abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement, and increasing haematocrit with decreasing platelets. The severe group is defined by the leakage of plasma, severe bleeding or severe organ impairment. Risk factors that can lead to a more likely case of severe dengue are: secondary or greater infection (four possible infections), infants born to seropositive mothers, elderly, a short interval of 5 years or less between dengue infections, infection with DEN2 strain, other co-morbidities like asthma, sickle cell, and diabetes.

Dengue is now endemic in all regions of the tropics and subtropics through the world. The WHO has a conservative estimate of 3.6 billion people living in endemic areas, with 500 million symptomatic DENV infections occurring each year. Resulting in roughly 20 million annual cases of dengue hemorrhagic fever and 21,000 deaths. Rates of dengue fever infection has been steadily doubling with each decade, with increasing rates since the 1980's with rate of 16.4/100,000 pop, the 1990's with a rate of 35.9/100,000 pop and the 2000's with a rate of 80.1/100,000 pop. Epidemics tend to be cyclical and happen in an endemic region when a new strain is introduced to which the current population does not already have immunity.

There are different needs for differing diagnostic tools in general for tropical infectious diseases and more specifically for dengue virus. Diagnostic tools are needed for 1) patient management to confirm and clinically diagnose, for 2) surveillance in order to monitor trends and give early warning of outbreaks, for 3) outbreak investigations in order to rapidly identify and implement appropriate prevention or control strategies, for 4) monitoring of effectiveness of drugs, vaccines, and vector control interventions and 5) for risk assessment in order to identify patients at risk for severe disease. Below is a slide that shows the types of diagnostic tests available for dengue virus with an indication for what the test is measuring.

Types of Available Diagnostic Tests				
Tests	Virus ID	Genome detection	Serological	Antigen
Isolation in C6/36 or mosquito	X			
RT-PCR	X	X		
Nucleic Acid Amplification Tests (NAAT)	X	X		
Sequencing	X	X		
Immunofluoresence	X			
IgM			X	
IgG			X	
Neutralization			X	
Microsphere assay			X	
NS1				X

Many possible infections on the list of differential diagnosis of dengue virus depending upon where patients live and their recent travel history. They are: malaria, chikungunya, leptospirosis, influenza, West Nile Virus, Measles, Rubella, Malaria, Typhoid fever (Salmonella typhi), Enterovirus, Meningococemia, Rickettsial infections, Bacterial sepsis and other viral hemorrhagic fevers, Burkholderia, Scarlet Fever, HIV Seroconversion Illness, Erythema infectiosum, Roseola infantum, Epstein-Barr virus and Scrub Typhus.

Dr. Hunsperger then presented on virus detection by real time RT-PCR and NS1. She mentioned the utility of the rRt-PCR test during acute dengue illness because 75% of patients in Puerto Rico go to the doctor within five days of symptom onset, they have high viremias and a late IgM response with detectable viral RNA

levels, highly specific and a positive result is a confirmed DENV infection. NS1 was discussed as diagnostic tool as compared to IgM.

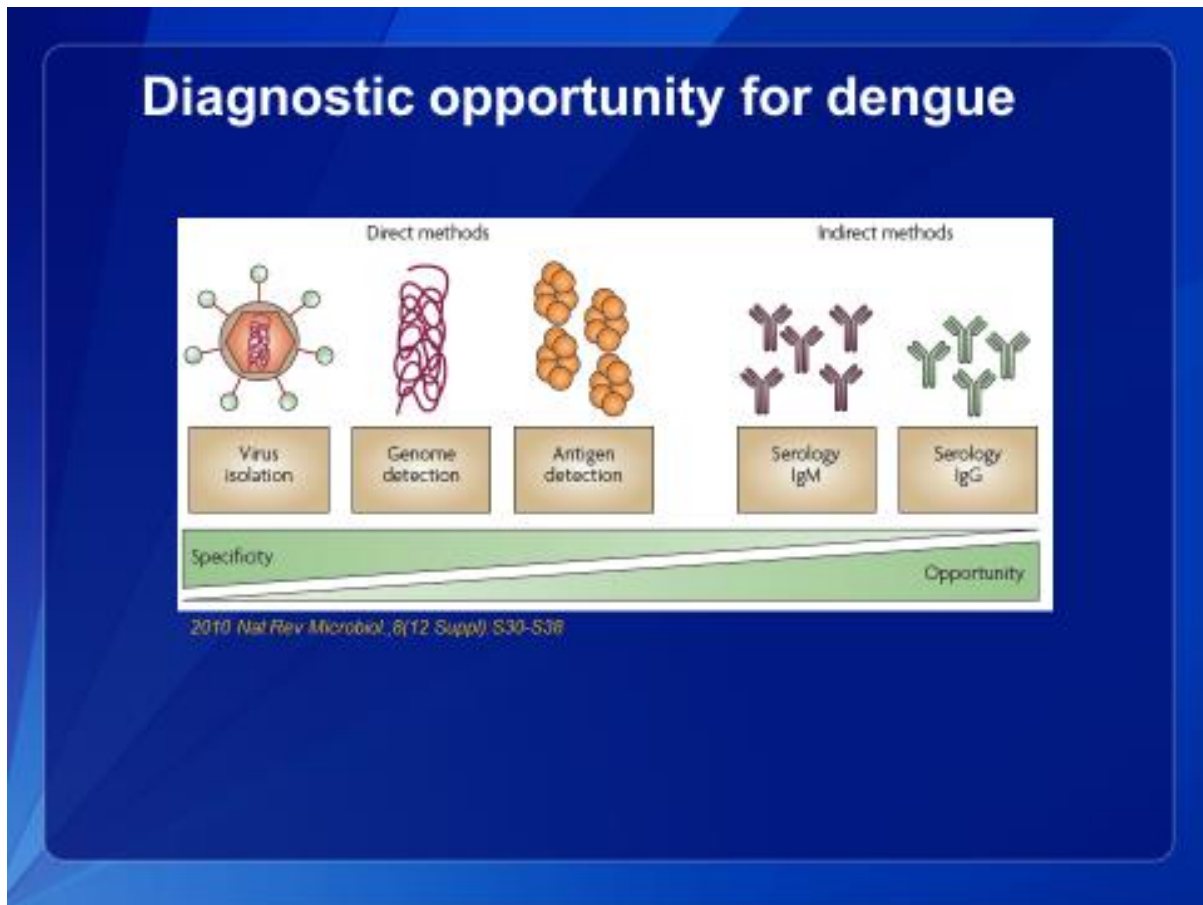
The InBios Dengue Virus Detect IgM capture ELISA test was presented as the only FDA approved Dengue IgM detection ELISA assay on the market. It is based upon similar principle as the CDC MAC ELISA. The test is pre-coated with Goat anti-human IgM, patient's serum is added at temperature of 37 degrees for one hour, DENV VLP Agn and Normal Control Agn are then added for one hour, followed by addition of HRP anti-Flavivirus McAb at 37 degrees for one hour and finally substrate for colorimetric reaction is added. Below is a slide with the IgM Commercial ELISAs for DENGUE as evaluated by WHO/TDR.

IgM Commercial ELISAs for Dengue Evaluated by WHO/TDR					
	Focus IgM Capture Dx Select	Omega Panthozyme Dengue M	Omega Panthozyme Dengue M Capture	Panbio Dengue IgM Capture ELISA	Standard Diagnostic Dengue IgM Capture ELISA
Detection	IgM Capture	IgM Indirect	IgM Capture	IgM Capture	IgM Capture
Antigen	DENV1-4	DENV-2	DENV1-4	Recombinant DENV1-4	DENV 1-4
Sample Volume	10ul	10ul	20ul	10ul	10ul
Storage Requirements	2-8°C	2-8°C	2-8°C	2-8°C	2-8°C
Sensitivity	96.8	61.5	62.3	99	97.6
Specificity	79.9	84.6	97.8	84.4	86.6

Hunsperger et al., EID 2009 15(3):436-440

Dr. Hunsperger then presented various samples of rapid diagnostic tests (RDT). First, she showed how the lateral flow assay test works. Then we looked at the interpretation of results for the lateral flow assay for NS1 test and compared it to the interpretation for a lateral flow test for IgM and IgG. The NS1 test provides clear results on whether or not the patient has a current dengue virus infection. It is easy to interpret because it is either positive, negative (not dengue) or invalid. The interpretation of the IgM/IgG Test is a bit trickier with IgG positive indicating there was a recent dengue virus infection within the last three months, IgG and IgM positive indicate a recent dengue virus infection within the past three months, IgG positive indicates a past dengue virus infection with no evidence of recent infection. A negative result indicates that the individual does not have dengue virus and an invalid test is invalid. According to recent studies on Dengue Duo performance, from Wang et al 2010; Blacksell et al, 2011; Andries et al 2012; Pal et al, 2014; Gan et al, 2014; Carter et al, 2015 tests have varying degrees of sensitivity and specificity ranging from sensitivity of 48.5-81.6 and specificity of 97.5-100. Dr. Hunsperger then presented tables of how the NS1 RDT (BioRad, Panbio, Standard Diagnostics and CTK) results vary in acute phase versus in convalescent phase indicating much higher sensitivity in acute phase. The same tests were also compared for primary versus secondary infections from DENV showing substantially greater sensitivity for primary DENV infections. When looking at sensitivity across the serotypes for these tests, DEN 1 across the board has a much higher sensitivity (95-98%) with DEN2 the

lowest sensitivity (50-60%). Reader to reader variation across labs for tests was shown and finally across reactivity for the RDT's for NS1 and IgM was shown. A long list of commercially available tests was presented by manufacturer, country of origin and type of test. Finally, Dr. Hunsperger reminded us there is a great opportunity for diagnostic development for DENV with closing slide below.



The Challenge of Zika and Chikungunya Diagnosis in Dengue Endemic Regions

Jorge L. Munoz Jordan, PhD

Chief, Surveillance and Research Lab

Centers for Disease Control and Prevention

Dengue Branch

Zika virus is a single stranded RNA virus in the Genus *Flavivirus*, Family *Flaviviridae*. It is closely related to dengue, yellow fever, Japanese encephalitis, and West Nile viruses. Zika virus is transmitted to humans primarily by *Aedes* (*Stegomyia*) species mosquitoes. Dr. Munoz presented a map showing that as of January 23, 2016 21 countries in world had reported Zika virus activity.

Clinical Features: Zika Virus Compared to Dengue and Chikungunya

Features	Zika	Dengue	Chikungunya
Fever	++	+++	+++
Rash	+++	+	++
Conjunctivitis	++	-	-
Arthralgia	++	+	+++
Myalgia	+	++	+
Headache	+	++	++
Hemorrhage	-	++	-
Shock	-	+	-

This table shows the clinical features of Zika virus disease compared with dengue and chikungunya. The number of plus signs indicates the frequency at which symptoms were reported. In comparison to dengue and chikungunya, Zika is more likely to cause a rash and fever is common but not invariably present. Cases are also more likely to present with conjunctivitis. Arthralgia, though common, is not as prominent as in chikungunya. Zika virus disease is not characterized by hemorrhage or shock. The differential diagnosis for Zika virus disease includes other arboviruses including dengue and chikungunya; bacterial infections including leptospirosis, rickettsia, and Group A streptococcal infection; malaria; and non-arthropod borne viruses such as parvo, rubella, measles, adeno, and enteroviruses.

Diagnostic Testing for Zika Virus

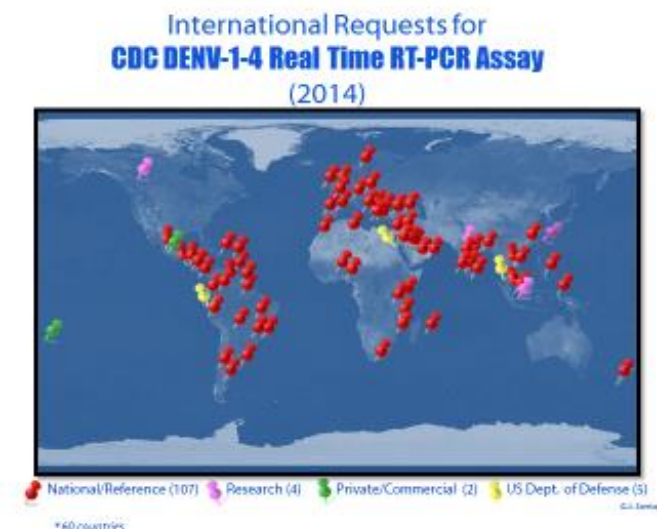
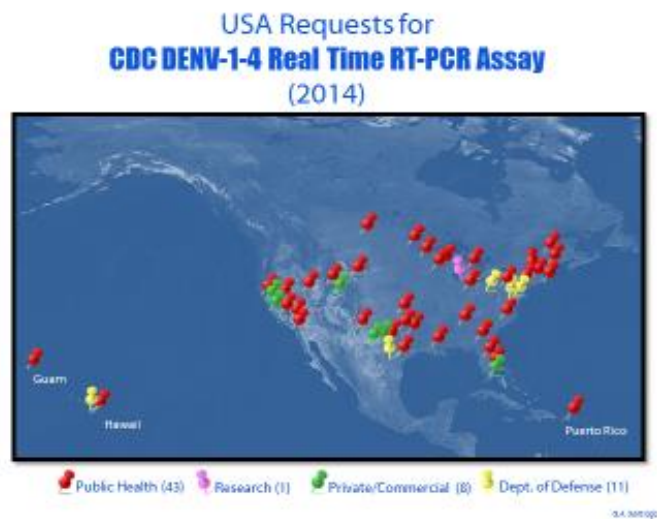
- Reverse transcriptase-polymerase chain reaction (RT-PCR) for viral RNA in serum collected ≤ 7 days after illness onset
- Serology for IgM antibodies in serum collected ≥ 4 days after illness onset
 - Run in combination with dengue IgM serology
 - An option for asymptomatic pregnant women in endemic areas
- Plaque reduction neutralization test (PRNT) for ≥ 4 -fold rise in virus-specific neutralizing antibodies in paired sera

There are number of diagnostic tests for Zika virus infection, including: RT-PCR to detect viral RNA in serum collected within 7 days of illness onset, serology to detect IgM and confirmatory neutralizing antibodies in serum collected at least four days after onset. Plaque reduction neutralization titers (or PRNTs) can be used to detect a four-fold or greater rise in virus-specific quantitative antibody titers on paired sera, and immunohistochemical staining for viral antigens or PCR on fixed tissues. There is high level of cross reactivity with other flaviviruses. The complexity of

testing and interpretation of results is that Zika virus serology can be positive due to antibodies against related flaviviruses such as dengue and yellow fever viruses. Neutralizing antibody testing may discriminate between cross-reacting antibodies in primary flavivirus infections, but it can be difficult to distinguish the infecting virus in people previously infected with or vaccinated against a related flavivirus.

A true measure of the Zika outbreak and its implications will be impossible until better diagnostic tools are developed. Scientists all over the world are rushing to develop a better serology test that will distinguish Zika antibodies from other viruses in the same family that have a similar structure. The CDC is moving towards improving lab capacity in areas where arbo virus infections are frequent and/or endemic. New developments in testing are: the implementation of RT-PCR in Puerto Rico (CDC and PRDH), a Triplex test which allows for the simultaneous detection of DENV, CHIKV and ZIKV and will be adapted to technology already present in Puerto Rico. The implementation of a Zika IgM ELISA test by the CDC Dengue Branch and the Puerto Rico Department of Health as of March 2016.

An example of the precedence set by DENV detection has been made with the FDA approved CDC DENV 1-4 RT-PCR Assay. Kits and manuals are made available by the Centers for Disease Control and Prevention, the Ancillary Reagents are purchased from Invitrogen in pre-qualified lots and the equipment a ABI 7500 Dx from Roche and Qiagen (present in most labs). The maps on the slides below show domestic and international requests for the CDC DENV-1-4 REAL TIME RT-PCR ASSAY.



Dr. Munoz presented the before and after ZIKAV RT-PCR testing options. Prior to ZIKA, serum was collected between 0-6 days of infection and the Dengue PCR test detected and subtyped 80% of Dengue cases and the Chikungunya PCR test detected 85% of cases. Post Zika Virus, the serum is tested between 0-6 days (more precise between 0-4 days) and does a pan dengue PCR test (no subtyping), a Chikungunya PCR test, and a Zika PCR test with a similar performance as seen in the single plex test.

Dr. Munoz presented tests under evaluation (as of February 2016) in three categories: RT-PCR, ELISA and RAPID TESTS. They are as follows:

RT-PCR

Altona diagnostics Zika RT-PCR Assay (approved in Europe)

ELISA

EUROIMMUN IgG/IgM ELISA

BIOCAN combo Dengue and Zika IgG and IgM (approved in Brazil)

RAPID TESTS

BIOCAN IgM/IgG Antibody Rapid Test

MIT Rapid Test

DENV Subtyping

DENV/CHIK/ZIKA

Chikungunya

Scott Weaver, M.S., Ph.D.

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The name “Chikungunya” is derived from a Makonde word meaning “to become contorted,” describing the hunched posture of persons with severe, debilitating, frequently chronic arthritis that often lasts for years. There were several million cases during near-pandemic proportions since 2005, the attack rates approach 50% in many regions, there is high apparent: unapparent ratio (unlike dengue). There are few fatal cases. Fatalities occur mainly in the elderly, during perinatal infections, and among persons with underlying medical conditions. The CHIKV infection can cause chronic arthralgia in up to 60% of patients as long as 2-3 years post infection. The DALY (disability adjusted life years) estimates can exceed 2/3 of the total population morbidity during outbreaks. Cases are greatly underestimated due to the overlap in clinical signs and symptoms with dengue and other tropical infectious diseases.

Dr. Weaver presents the difficulty of differential diagnosis of febrile illnesses especially in dengue endemic areas. This underscores the need for a sensitive, specific, rapid diagnostic tool that can differentiate between zika, dengue, chikungunya, influenza, and leptospirosis among others. Chikungunya is an old world virus that has emerged repeatedly, probably for centuries, from its enzootic cycle in Africa into an urban human-mosquito cycle in Asia, Europe and the Americas. It is spread to the human population via the *A. aegypti* and *A. albopictus* mosquito vector. *A. Aegypti* originated in sub-Saharan Africa and spread throughout the tropics centuries ago. The *A. albopictus* originated in Asia and spread to the Americas, Africa and Europe beginning in 1985.

Urban Chikungunya Virus Vectors

A. aegypti



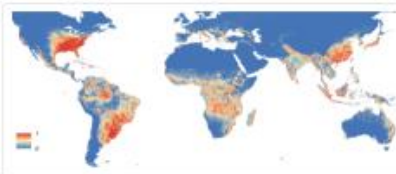
Originated in sub-Saharan Africa, spread throughout the tropics centuries ago



A. albopictus



Originated in Asia, spread to the Americas, Africa and Europe beginning in 1985



Kraemer, M.U., et al., 2015. The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. *Elife* 4.

The ***Aedes aegypti*** are tropical and subtropical, feed almost exclusively on humans and take multiple blood meals within a gonotrophic cycle. They use artificial water containers near houses as larval habitats. The adult females are found mostly inside homes and tend to feed during daytime hours. They are moderately susceptible to the chikungunya virus. The *Aedes aegypti* are a successful vector in the spread of chikungunya to humans due to their behavior. The ***Aedes albopictus*** prefers the tropics and temperate regions and tends to feed opportunistically. They take only a single blood meal within a gonotrophic cycle. They use artificial and natural larval habitats. They prefer humans and eating and resting inside human dwellings (anthrophily and endopophily). *Albopictus* tend to feed during the daytime and they have a moderate to high susceptibility to the chikungunya virus.

Since 2013, there have been about 2 million suspected cases in the Americas alone, with 3,000 imported cases in the United States mainland. Eleven cases were locally acquired in Florida. There are very limited diagnostic capabilities in the regions that have been affected. Only 2% of all cases have been diagnosed by a laboratory test. Attempts at mosquito control have failed in slowing the spread of the illness. Originally, CHIKV existed in Africa, mostly in sylvatic cycles involving arboreal mosquitoes and non-human primates. In 2004, a CHIK epidemic began in East Africa that resulted in its emergence both in the from obscurity. Initially, epidemics were observed in coastal Kenya, followed by its spread to Comoros, La Reunion and other islands in the Indian Ocean during 2005. Many tourists returning to Europe from vacations in this region got Chikungunya and this began to raise global awareness of the epidemic. In late 2005, epidemics occurred in India, followed by exportation via infected travelers to all regions of the world including the USA. Autochthonous transmission of new epidemic strains of chikungunya virus later happened in Southeast Asia and Europe.

Dr. Weaver then presented information about *Aedes albopictus* adaptive mutations. The 2005-2015 epidemic was extensive, due to the sequential, **convergent and step-wise adaptation via 5 natural *A. albopictus*-adaptive envelope glycoprotein gene mutations**, with little or no effect on infection of *A. aegypti* or murine models of human infection. An ECSA strain introduced into Brazil in 2014 from Angola appears to also have this potential. The artificial substitution of additional residues in the acid-sensitive region of the E2 protein with glutamine, as well as the combination of 2 natural glutamine substitutions, **further enhance infection of *A. albopictus***, predicting additional vector-adaptive evolution. Epistatic interactions have prevented adaptation by the Asian CHIKV lineage for *A. albopictus* transmission via either E1 and E2

substitutions, and should limit the ability of the initial CHIKV strains in the Americas to adapt for efficient transmission by this vector. These and studies of the CHIKV 3'UTR suggest that founder effects and stochastic events have dramatic influences on CHIKV evolution and emergence. The potential for establishment of an enzootic CHIKV cycle in the Americas is unpredictable.

A. albopictus-adaptive mutations

Lineage	Year of first appearance	Protein	Substitution	Fitness for <i>A. albopictus</i> infection	Fitness for <i>A. aegypti</i> infection
IOL	2005	E1	A226V	40-fold increase	Slight decrease
IOL SL1	2007	E2	K252Q	8-fold increase	No effect
IOL SL2 (partial)	2008	E2	K233E	6-fold increase	No effect
IOL SL3B	2008	E2/E3	R198Q/S18F (synergistic)	16-fold increase	No effect
IOL SL4	2009	E2	L210Q	5-fold increase	No effect
Asian	Never	E1	A226V	No effect	Epistatic interactions prevent penetrance of these mutations in Asian/American strains
Asian	Never	E2	K252Q	Decrease	
Asian	Never	E2	K233E	Decrease	
Asian	Never	E2/E3	R198Q/S18F (synergistic)	Decrease	
Asian	Never	E2	L210Q	Decrease	

Could Zika virus emergence be mediated by vector-adaptive evolution?

A. albopictus-adaptive mutations in the acid sensitive region of E2

E3; E2; E1 proteins

Natural, second-step adaptive substitutions of glutamine in acid-sensitive 210-252 E2 region

Hypothesis: Gln or Glu substitutions in the E2 acid-sensitive region enhance E1-226V by regulating low pH-induced E2-E1 heterodimer disassociation for efficient CHIKV endosomal entry in *A. albopictus* midgut cells.

Support: E2. substitutions are not in the receptor-binding domain, and artificial Gln substitutions in the acid sensitive region also enhance *A. albopictus* infectivity (Tsetsarkin KA, et al. Nat Commun. 2014;5:4084. Epub 2014/06/17). After demonstrating how these *A. albopictus* adaptive mutations have greatly enhanced the fitness for the *albopictus* mosquito to become infected with the CHIKV, Dr. Weaver then posed the question, « Could Zika virus emergence have been mediated by a similar vector-adaptive evolution ? »

Controlling the Spread of Chikungunya

Dr. Weaver pointed out that there are basically four main prospects for controlling the spread of Chikungunya.

1) **Reduction of contact** between people and mosquito vectors. 2) Reduction of human amplification **using antivirals**. 3) Control of transmission by **reducing populations of mosquito vectors**. 4) Prevention of human infection **using vaccines**. Dr. Weaver reminds us that just one traveler can initiate a new urban epidemic easily.

Vaccine development for Chikungunya

The good news:

- Single serotype, no evidence of reinfection or immune enhancement
- Well established correlates of protection for alphaviruses (neutralizing antibodies)
- Ease of genetic manipulation for rational attenuation

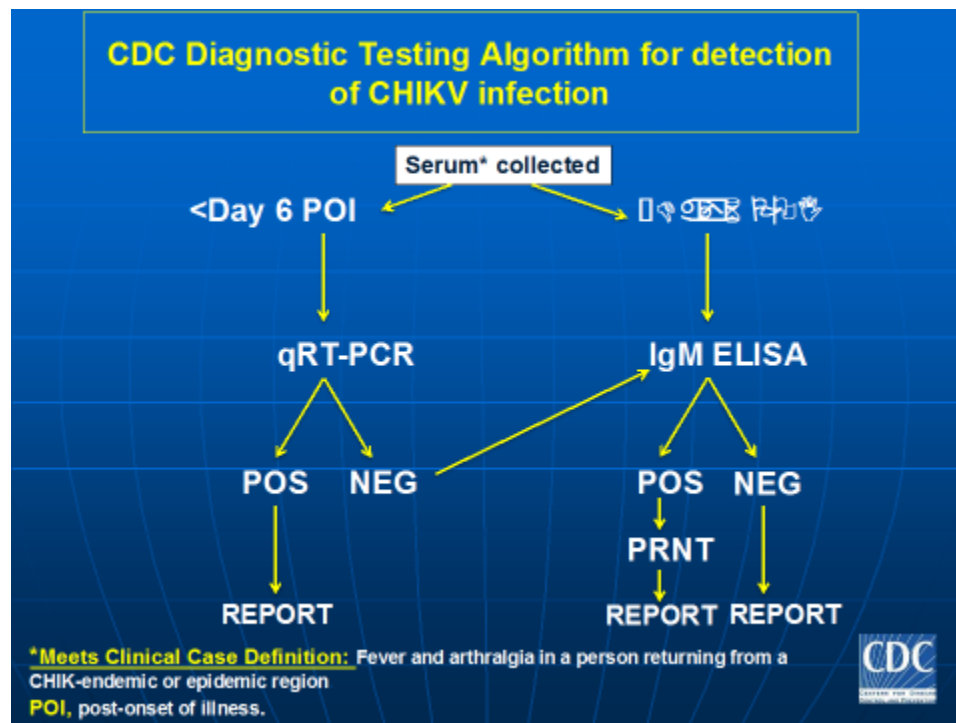
The bad news:

- Wild-type rodents are poor models for human disease
- Unpredictable incidence of disease for human efficacy trials
- FDA animal rule has not yet produced a licensed vaccine

Currently, the following vaccine efforts are in the **preclinical phase**: Bharat Biotech (VLP Vaccine), Inovio (DNA Vaccine), Yale Profectus (UTMB: VSV-Vectored live attenuated), Indian Immunologicals (Inactivated Strain), Indian Immunologicals (Walter Reed Strain), Arbovax (Recombinant LAIV), and Takeda and UTMB (recombinant live attenuated). Only one effort is in **Phase I**: NIAID (National Inst of Allergy and Infectious Disease) VLP Vaccine manufactured in CHO cells, Merk option. **Phase II** level of vaccine development has only been attempted by the U.S. Army, 181/clone 25 live attenuated vaccine based upon 2-point attenuating mutations.

Chikungunya Diagnostics

Great need to improve our diagnostic capability for Chikungunya, because of difficulty in diagnosis by symptoms alone. Serum is collected if patient meets the clinical case definition for Chikungunya which is fever and arthralgia in an individual returning from a CHIK-endemic or epidemic region. Serum is collected for diagnostic testing if has been less than 6 days post infection. Current testing algorithm by the Centers for Disease Control and Prevention for the detection of the chikungunya virus looks as follows:



Eilat virus, is a unique alphavirus with a host range restricted to insects due to their RNA replication. This virus (ELIV) is used as a diagnostic antigen platform for detection of CHIKV. The rationale is as follows: chimeras can be grown to high titers in cell cultures in a biosafety level 1 setting, there is no need to concentrate or inactivate the virus, there is a significant reduction in risk and cost of production and Eliat is antigenically identical to its pathogenic counterparts. Eliat/CHIK Chimera is used as a diagnostic antigen and is less expensive than other options. Below is a slide showing detail of chimera design.

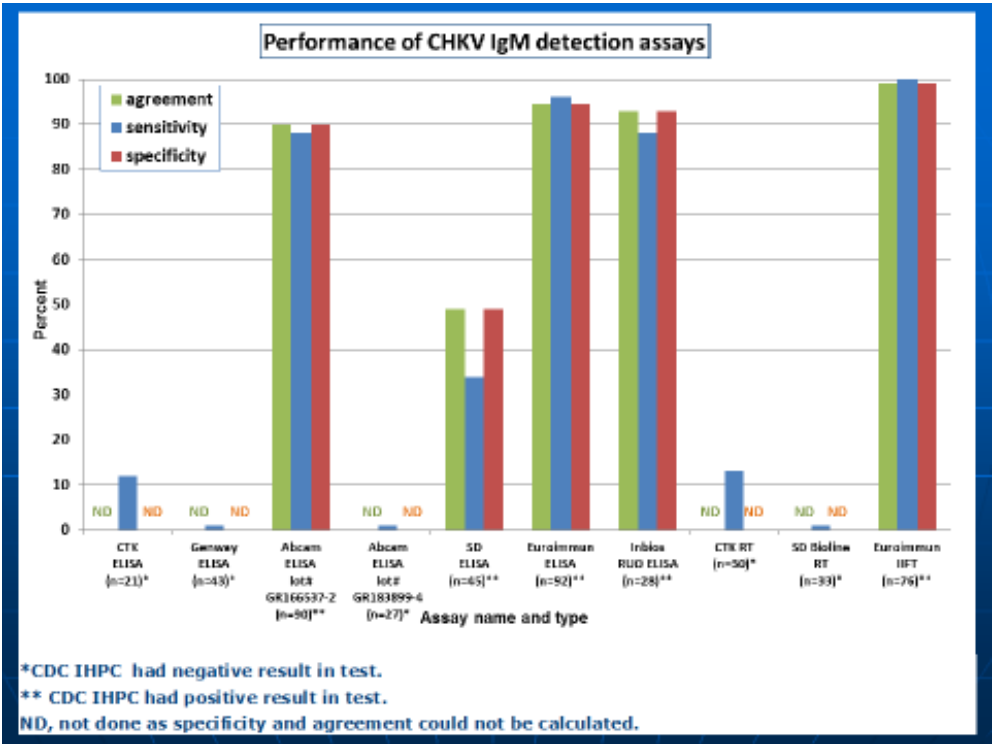
EILV/CHIKV Chimera Design

- Generate chimeras containing structural genes of pathogenic alphaviruses



- Source of structural protein genes
 - Human isolate of CHIKV from Caribbean outbreak (Virgin Islands, kindly provided by the CDC)
- Source of nonstructural protein genes
 - EILV infectious clone

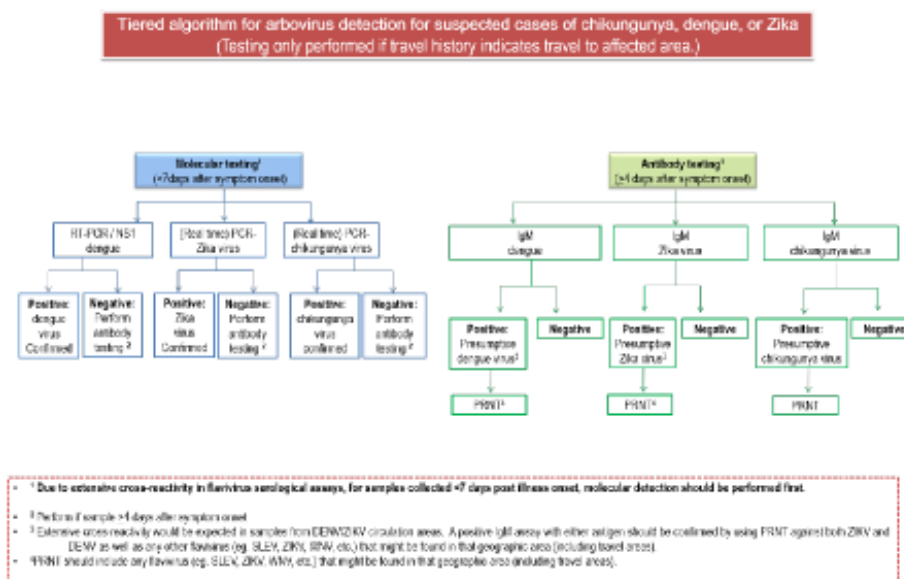
Dr. Weaver continues to compare performances of various CHIKV IgM detection assays by sensitivity, specificity and level of agreement. Strongest performing test was Euroimmun IIFT reaching almost 100% in all categories. Dr. Weaver noted that diagnostic kits are highly variable in terms of quality.



In summary, the Centers for Disease Control and Prevention provide the following summary of existing diagnostic tools.

Summary CDC evaluations of CHIKV IgM detection assays		
Manufacturer	Assay format	Performance /concordance
Plate ELISA		
CTK Biotech	RecombiLISA CHIK IgM Test	Low /ND
Genway (NovaTec)	CHIKV IgM μ -capture ELISA	Low /0%
Abcam (NovaTec)	Anti-CHIKV IgM human ELISA kit	Inconsistent quality/0%-95%
SD Diagnostics	CHIKa IgM ELISA	Low /<50%
Euroimmun	Anti-CHIKV ELISA (IgM)	High/>90%
Inbios	CHIKij Detect MAC-ELISA	High/>90% (research format)
Rapid test		
CTK Biotech	On-site CHIK IgM Combo Rapid test	Low/ND
SD Diagnostics	SD BIOLINE Chikungunya IgM	Low/ND
IFA		
Euroimmun	Anti-CHIKV IIFT (IgM)	High/98%

Dr. Weaver closes his presentation with a testing algorithm for both molecular detection and antibody testing for chikungunya, zika, and dengue as recommended by CDC. Due to extensive cross reactivity of flaviviruses in serological assays for samples collected greater than 7 days post illness onset, molecular detection should be done first. Extensive cross reactivity is expected in samples from DENV/ZIKAV circulation areas. A positive IgM assay should always be confirmed by using a PRNT against both ZIKAV and DENV as well as other flaviviruses that might be present in the area or in area/s of recent travel.



Influenza

Dr. Stephen Lindstrom, Ph.D.

Team Lead, Diagnostics Development Team

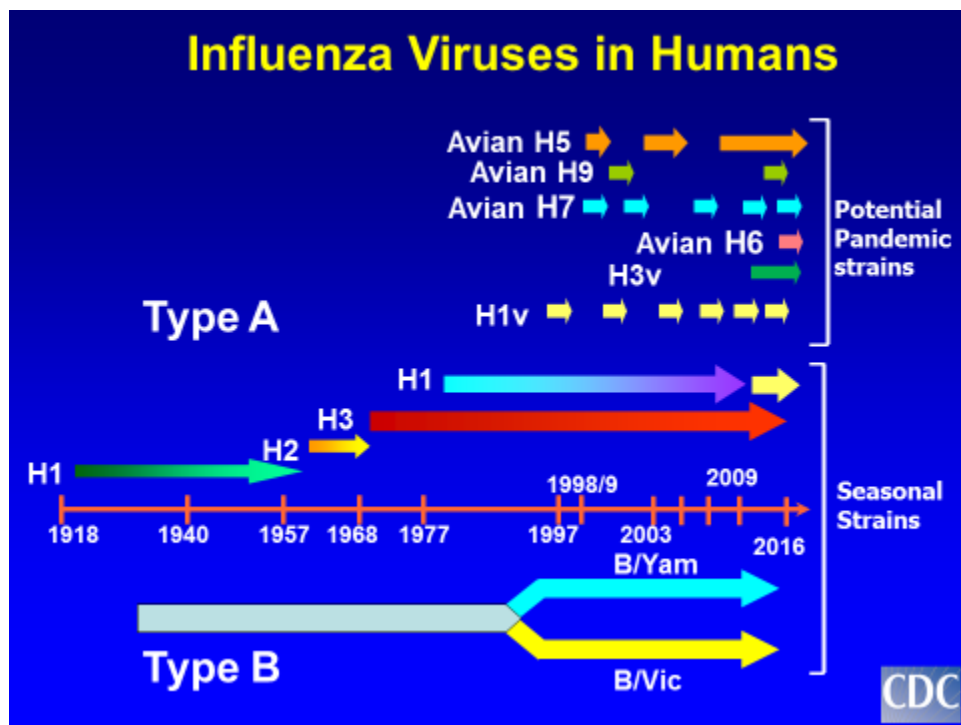
Virus Surveillance and Diagnosis Branch

Influenza Division, NCIRD

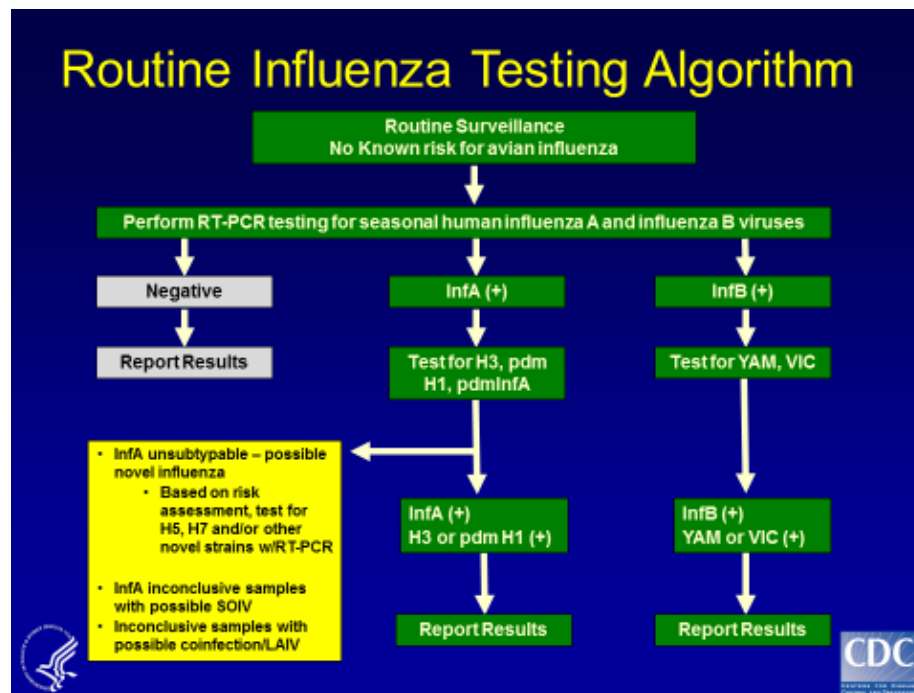
Centers for Disease Control and Prevention

Dr. Lindstrom was invited to this meeting to demonstrate all of the progress that has been made in the area of influenza diagnostics, vaccine development, surveillance and constant monitoring to predict next type of influenza outbreak. The CDC's influenza vaccination program is extensive and prevents many illnesses and deaths in the United States annually. Influenza is a difficult virus to monitor due to genetic drift and shift. It changes more frequently than the flaviviruses discussed earlier.

There are three types of influenza A, B and C. A and B are major human pathogens with negative-sense segmented RNA genome and 10 major proteins. The two major surface proteins of the A and B influenza viruses are Hemagglutinin (HA) and Neuraminidase (NA). Influenza A subtypes (H1-H16 and N1-N9) have many animal reservoirs: poultry, humans, pigs, horses, aquatic mammals, cats and dogs. Aquatic birds are reservoirs for all HA and NA subtypes. Bats are a reservoir for H17N10 subtype.



The CDC Flu rRT-PCR Assays for humans differentiate on the following three levels. First there is an assay to type the flu (flu A or B). Second, Human FluA Subtyping will distinguish between following: Human H1, Human H3, pdmInfA and pdmH1. Human FluB Genotyping will distinguish the following: InfB, YAM, VIC, and RP. Below is the CDC's routing testing algorithm for influenza.

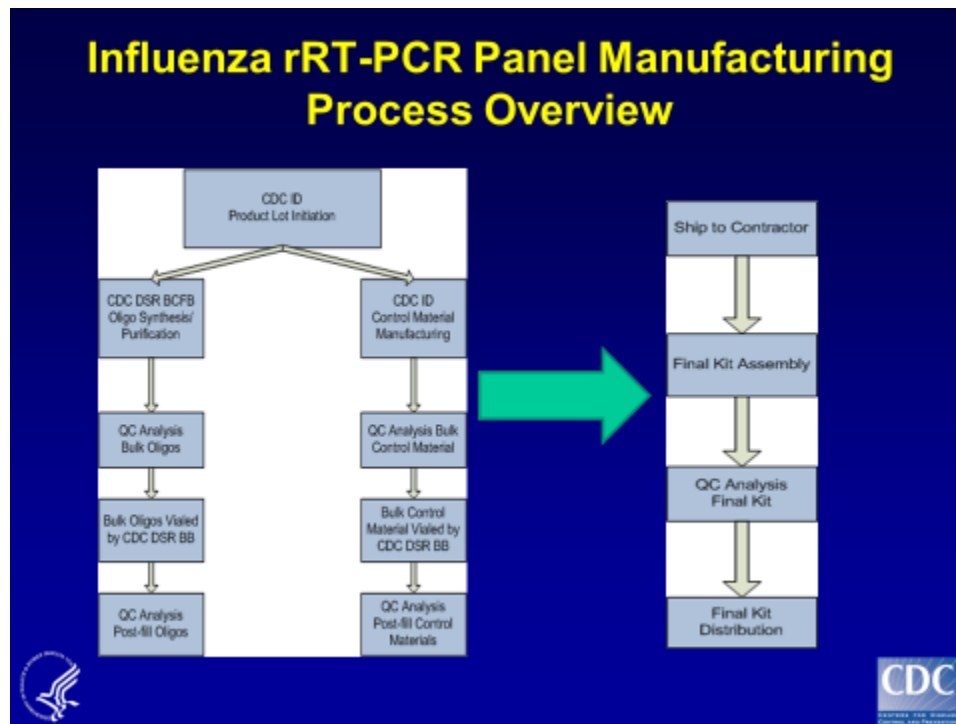


Dr. Lindstrom then presented global surveillance data and number of positive samples sent from public health departments in the U.S. for 2015-2016. There was a substantial increase in numbers of positive cases received from public health departments around the world from only 50 per week (week 42 of 2015) to almost 400 cases in week 2 of 2016. Influenza A H1N1 made up the majority of positive cases detected. The CDC's role in real time surveillance and monitoring of influenza is important to predict what subtypes will be important to include in the annual influenza vaccines. The CDC develops many types of diagnostic tests for the influenza virus. Many of these tests need to be updated frequently due to changing nature of influenza virus. This slide shows when tests have received their FDA clearance, type of panel or kit used and a description of the purpose of the test. The turn-around time for test development, FDA clearance and use of tests by public health departments and others is an established process for influenza virus.

FDA clearances & EUAs

SDG number	FDA Clearance Date	Panel/Kit	Description of purpose
K151446	December 1, 2015	CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel, Influenza A/H5 Subtyping Kit (VER 3)	- Update assays to ensure universal detection of HPAI Avian lineage A/H5N1 viruses, including A/H5Nx (clade 2.3.4.4) - Qualify alternative commercial manufacturer of critical reagent (probe) to include ZEN quenched probe.
K132586	September 23, 2013	CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel, Influenza B genotyping assays	Addition of a new assays within the panel for the determination of the genetic lineage of human influenza B virus as B/Victoria or B/Yamagata lineage.
K130551	May 22, 2013	CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel	Qualify an alternative commercial manufacturer of critical reagent - enzyme mastermix, the "QuantaBio Sciences qScript One-Step qRT-PCR Kit"
EUA	April 22, 2013	CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel, Influenza A/H7 (Eurasian Lineage) Assay	- Detection of Eurasian Avian Influenza A/H7 for use with CDC Flu RT-PCR Dx Panel. - Authorization received under new authority
K123965	January, 2013	CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel	- modification of results interpretation to allow reporting of presumptive positive for H3Nx influenza A virus detection
K111567	August 23, 2011	CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel	- Merge CDC Flu Dx panel with CDC Influenza 2009 A/H1N1 pdm09 Real-time RT-PCR Panel into a single kit
K101554	June 22, 2010	CDC Influenza 2009 A/H1N1 pdm09 Real-time RT-PCR Panel	- Detection and characterization of Influenza A/H1N1 pdm09 with CDC rRT-PCR Flu Dx Panel - Update assays from EUA with H1N1 pdm09 - Clearance of additional commercial nucleic acid extraction chemistries
EUA	April 29, 2009	Swine Influenza Virus Real-Time RT-PCR Detection Panel (rRT-PCR, Swine Flu Panel)	- Detection and characterization of Influenza A/H1N1 pdm09 with CDC rRT-PCR Flu Dx Panel
K896479	September 30, 2008	CDC Human Influenza Virus Real-Time RT-PCR Detection and Characterization Panel	Intended use/capability: - Detection of Influenza A/B - Characterization of human Influenza A (H1N3) - Characterization of avian Influenza A (H5N1) Clearance of ABI7500 test instrument

The CDC provides the following in house tests: live attenuated influenza vaccine (LAIV) – CLIA, swine origin A/H3v – pre EUA, swine origin A/H1v, N. American avian A/H7, Asian avian A/H9, canine/equine A/H3N8 and canine A/H3N2. The CDC also provides reagent manufacturing and distribution support for public health laboratories around the country. The panel manufacturing process is shown in the following slide:



The CDC rRT-PCR distributes two types of panels the IVD kits and the RUO kits. IVD kits are distributed to 97 domestic public health labs in all 50 states including Puerto Rico and 19 domestic and 6 international Department of Defense labs. The RUO Kits are distributed to 147 international sites of which 71 are National Influenza Centers (NIC). The Reagent ordering and technical support are outsourced and done via internet. This allows laboratories access to multiple procedures and methods depending on their specific need (equipment, chemistry, etc.). It allows for coordinate communication with qualified public health liaisons and provides timely notification of assay updates.

An example of critical commercial reagents equipment is: 1) nucleic acid extraction, 2) options for enzyme master mix 3) manufacturing options for oligonucleotide primers/probes and 4) rRTPCR instrumentation. Qualified Nucleic Acid Extraction is done by manual RNA extraction. The Qiagen QUamp Viral RNA Mini kit. Automated RNA/TNA extraction can be done by: Roche Magnapure Compact and LC, the Qiagen Qiacube and the BioMerieux Easy Mag. Extraction platforms under evaluation are the Qiagen EZ1 advanced XL and the Roche LC96. Enzyme chemistry options are: Invitrogen super Script III, Platinum One-Step Quantitative and the RT-PCR System. Probe chemistry options are ZEN dual quenched probes - Integrated DNA technologies and the Black Hole Quencher (BHQ1) – Biosearch Technologies.

Real-time PCR Platforms have FDA requirements for distribution. All users and analysts must be trained to perform and interpret results from the assay procedure by the CDC or a designated trainer. The test must be performed on “ABI 7500fast DX” instrument. Two types of FDA cleared real-time PCR Platforms under evaluation are: LifeTechnologies QuantStudioDx and the Qiagen Rotorgene Q MDx

Types of Commercially Available Diagnostic Influenza Tests

Rapid Influenza Diagnostic Tests (RIDT's)

RIDT's are advantageous because they produce rapid results in 30 minutes or less, they are simple (CLIA waived) and they allow for on-site testing which is very useful for clinical settings and outbreak investigations. Limitations of RIDT's are their high false negative rates (sensitivity only between 50-70%), specificity rates tend to be higher at between 90-95%. Other limitations are: some tests do not determine type of influenza, cannot subtype (H3, H1, H5) and they tend to be reactive against non-human influenza. Antigen detection is one type of RIDT commonly used to detect influenza virus. A common format is a lateral flow ImmunoAssay. Dr. Lindstrom displays a nasal swab test that uses a reagent and results are shown on a test strip after only 10 minutes. Typical RIDT Indicator formats are: Alere AB, Alere BinaxNOW AB, Meridian_TRU FLU, BD-Directigen EZFluAB and Remel – Xpect ID. Two examples of RIDT Readers are BD Veritor Influenza A and B (digital immunoassay, automated results, no user interpretation) and the Quidel Sofia Influenza A and B (digital immunoassay, automated results, no user interpretation and wireless reporting).

FDA Proposed Reclassification of Rapid Diagnostic Tests

FDA is proposing a reclassification of RIDT's. The FDA believes that general controls are insufficient to reasonably assure safety and effectiveness of RIDTs. They propose to reclassify RIDTs from class I to class II. The addition of special controls would mitigate the known risks associated with the use of Class I RIDTs and enable the FDA to enforce higher performance criteria. Criteria could include monitoring of annual reactivity testing and analytical performance validation by the manufacturers. These actions would promote the development and manufacturing of new and improved diagnostics for influenza that will better meet the needs of patients, physicians, and public health.

As part of this reclassification process, CDC proposes an Influenza Virus Panel. This panel is intended to provide diagnostic test manufacturers with characterized influenza viruses. It will assist in the internal evaluation testing for the purpose of generation of analytical performance data to be submitted to the FDA Administration. The FDA refers to the RIDT manufacturers to the CDC to request panels. The CDC human influenza virus panel consists of ten highly characterized human influenza virus isolates for the following five viruses: A(H1N1), A(H3N2), A(H1N1) pdm09, B (Victoria's lineage) and B (Yamagata lineage). Results of the annual analytical reactivity testing will be made publicly available.

Reclassification is currently pending. Viruses can be made available to companies on a voluntary basis and manufacturers are encouraged to report test results to the FDA. They may include performance data in 510(K) submission and in product package insert.

Commercial Molecular Diagnostic Tests



In recent years, a number of commercial assays based on nucleic acid detection have been developed and cleared by CE and/or the FDA for human diagnostic testing. These commercial molecular tests are increasingly being used in clinical and public health settings. Some tests detect influenza as well as other respiratory viruses. There are a variety of testing formats and designs available based on rRT-PCR as well as other nucleic acid detection methods. These tests are usually based upon nucleic acid amplification (NAAT), they are available in a single-plex or multiplex assay format and utilize real-time vs. end point detection.

Advantages are that molecular assays are more sensitive and specific than a rapid antigen test. They can detect additional non-influenza respiratory pathogens and some tests determine seasonal influenza A virus subtypes (A(H1N1) pdm09, seasonal A (H1N1), or seasonal A (H3N2). They may even identify a novel influenza virus strain or type as “unsubtypable.”

There are limitations of the commercial molecular diagnostic tests. Some assays will detect influenza A or B viruses but will not determine the A virus subtype. Some tests provide some but not all influenza A subtypes. Flu A tests are not intended for universal detection of all influenza A virus subtypes – detection of a novel influenza is often unknown. Commercial assays only FDA cleared for nasopharyngeal specimens. Dr. Lindstrom closed his talk with a table of commercial molecular diagnostic tests for influenza.

Commercial Molecular Dx Tests

Product (Manufacturer)	Influenza Virus Type (subtype) detected	Other Respiratory viruses detected	Acceptable Specimens*	Test Time/Complexity*
CDC Hanes Influenza Virus Real-Time RT-PCR Diagnostic Panel [†]	A and B (H1, H3, 2009 H1, H5N1 (A/Sydney lineage)) (B-Vietnam/1194/05, B-Victoria lineage)	None	NP swabs, nasal swabs, nasal aspirates, nasal washes, oral NP throat swabs, bronchoalveolar lavages, tracheal aspirates, bronchial washes, and viral culture	~4 hr/High
Cepheid Xpert Flu Assay	A and B (2009 H1)	None	NP swabs, nasal aspirates, and nasal washes	1.5 hr/Moderate
eSensor [®] Respiratory Viral Panel (RVP) (GenMark Diagnostics)	A and B (H1, H3, 2009 H1)	Yes - 9	NP swabs	~8 hr/High
FluView [®] Respiratory Panel (BioFire)	A and B (H1, H3, 2009 H1)	Yes - 9	NP swabs	1.5 hr/Moderate
ReaPLEX-IG Flu	A and B (H1, H3, 2009 H1)	None	NP swabs	~8 hr/High
IQium List Influenza A/B Assay	A and B	None	NP swabs	0.5 hr/Moderate
Prodesse PROFLU [™] +	A and B	RSV	NP swabs	~45 min/High
Prodesse ProFAST [™] +	A (H1, H3, 2009 H1)	None	NP swabs	~45 min/High
Quidel Molecular Influenza A/B Assay	A and B	None	NP swabs and nasal swabs	~4 hr/High
Qiagen Axiom Influenza A/B Rotavirus RT-PCR kit	A and B	None	NP swabs	~4 hr/High
Simplex [™] Flu A/B & RSV (Focus Diagnostics, IM)	A and B	RSV	NP swabs	~45 min/High
Simplex [™] Flu A/B & RSV Direct (Focus Diagnostics, IM)	A and B	RSV	NP swabs	~25 min/Moderate
Simplex [™] Influenza & H1N1 (2009) (Focus Diagnostics, IM)	A (2009 H1)	None	NP swabs, nasal swabs, and NP aspirates	~4 hr/High
Venue [®] Respiratory Virus Nucleic Acid Test (Ringsberg, Inc)	A and B	RSV A, RSV B	NP swabs	3.5 hr/Moderate
Venue [®] Respiratory Virus Plus Nucleic Acid Test (RVT) (Ringsberg, Inc)	A and B (H1, H3, 2009 H1)	RSV A, RSV B	NP swabs	3.5 hr/Moderate
eTAG [®] Respiratory Viral Panel (RVP) (Luminex)	A and B (H1, H3)	Yes - 8	NP swabs	~8 hr/High
eTAG [®] Respiratory Viral Panel Fast (RVP FAST) (Luminex)	A and B (H1, H3)	Yes - 4	NP swabs	~6 hr/High

Leptospirosis

Renee Hallaway, MPH

National Center for Zoonotic, Vector-borne and Enteric Diseases

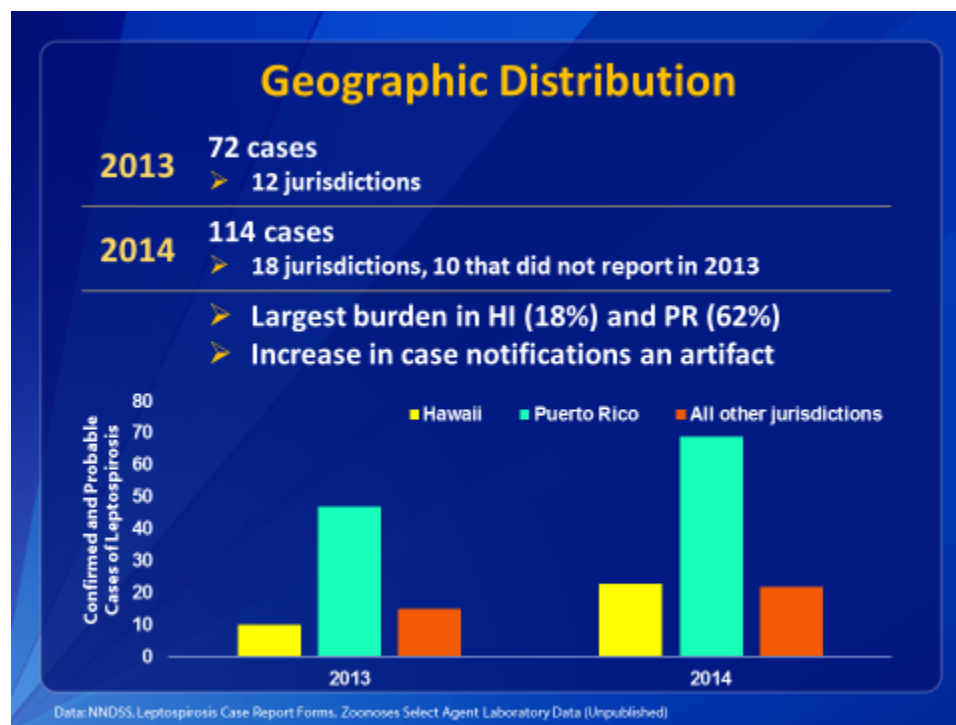
Centers for Disease Control and Prevention

Ms. Hallaway presented an update on Leptospirosis rapid diagnostic testing in Puerto Rico. Leptospirosis is the most widespread zoonosis in the world and has a wide variety of mammal carriers. It is most common in tropical areas of poor sanitation and seasonal flooding. Outbreaks can be associated with water sporting events. For example, in 2000 an Eco Challenge in Borneo after heavy rains from Hurricane Wilma resulted 29 people hospitalized, in 1998 the Springfield Triathlon resulted in 21 hospitalized, 7 underwent a lumbar puncture and 1 a kidney biopsy, 2 others a cholecystectomy.

Leptospirosis causes severe illness in 10% of humans. Up to 25% mortality rate with classical severe leptospirosis (kidney and liver failure). There is up to a 60% chance of mortality related to severe pulmonary hemorrhagic syndrome (SPHS). Early symptoms are non-specific and often misdiagnosed as a viral infection or

dengue fever. Early antibiotic treatment is extremely important. Most common clinical signs and symptoms of Leptospirosis are fever, headache, and myalgia. Other possible symptoms could be: conjunctival suffusion, jaundice, general malaise, stiff neck, chills, abdominal pain, joint pain, anorexia, nausea, vomiting, diarrhea, oliguria, hemorrhage, skin rash, photophobia, cough, cardiac arrhythmia, hypotension, mental confusion, psychoses, and delirium (WHO). Leptospirosis is dangerous because it is difficult to correctly diagnose in a dengue endemic region. Differential diagnoses could include: influenza, dengue and dengue hemorrhagic fever, hantavirus infection, yellow fever and other viral hemorrhagic fevers, Rickettsiosis, Zika virus, Borreliosis, Brucellosis, malaria, pyelonephritis, aseptic meningitis, chemical poisoning, food poisoning, typhoid fever and other enteric fevers, viral hepatitis, pyrexia of unknown origin, primary HIV seroconversion, legionnaire's disease, toxoplasmosis, infectious mononucleosis, and pharyngitis (WHO).

The highest incidence of Leptospirosis is in Puerto Rico in all of the United States and its territories. The highest number of fatalities due to Leptospirosis is also in Puerto Rico. The Geographic distribution for 2013 and 2014 is shown below.



In the 1980's an animal survey was done to assess levels of Leptospirosis in animal population in Puerto Rico (Farrington NP, Sulzer KR. Canine leptospirosis in Puerto Rico. Int J Zoonoses 1982 Jun;9(1):45-50.) The found 63% of dogs had antibodies to Leptospira spp., 42% of monkeys and 75% of mongooses. Mongooses had especially high titers of 12,800.

Leptospirosis diagnostic testing falls into three basic categories: conventional, early diagnosis and rapid diagnosis. Conventional tests include culture (generally insensitive, slow and unreliable) and serology (more than 4 days post onset) by MAT, ELISA and IHA. Early diagnosis is a PCR test that is performed on acute specimens (performed 4 days post onset). Last category is the rapid diagnostic test also serology (4 days post onset) by lateral flow or latex agglutination. Microscopic Agglutination Test (MAT) is the CDC's gold standard and has special requirements: a panel of live antigens and corresponding antisera and a darkfield microscope.

This is rarely performed except in a handful of reference laboratories worldwide and some veterinary laboratories. The MAT requires paired samples or a convalescent.

Ms. Galloway presents the advantages and disadvantages of three tests 1) ImmunoDOT IgM – dipstick ELISA, 2) Leptorapide – latex agglutination and 3) the Test-it – Lateral flow for Leptospirosis. The **ImmunoDOT IgM** is easy to perform, serum or whole blood can be used, registered with the FDA and has an in-country manufacturer. Disadvantages are the cost of \$14.00 per test, requires an expensive heat block and defines “rapid” as 1.5 hours. The **Leptorapid – latex agglutination test** is easy to perform, registered with the FDA, has a long expiration time and only costs \$1.50 per test. The disadvantages are that it is difficult to read with a high number of inconclusive results, uses serum only, shipping costs are expensive and there are insufficient positive controls. The **Test-it IgM – lateral flow test** is advantageous because it is easy to perform and read, has a long expiration period, costs only \$2.00 per test, the lancet is supplied, can use either whole blood or serum and they have a canine test. The disadvantages are the high shipping costs and that it is not FDA registered. In conclusion, rapid tests for leptospirosis are available and easy to use. They may however miss patients who present acutely. Work needs to be done to get the lateral flow tests approved by the FDA.

A Multiplex Assay for Detection and Quantification of Chikungunya, Dengue, Zika and West Nile Viruses and VirCap Seq – VERT – A Tool for Viral Surveillance and Discovery

Ian Lipkin, MD

John Snow Professor

CII Zika Virus Team

Thomas Briese, PhD

Rafal Tokarz, PhD

Nischay Mishra, PhD

Center for Infection and Immunity

Mailman School of Public Health

Columbia University

Dr. Lipkin and his team presented the “virome capture sequencing platform for vertebrate viruses” or the VirCapSeq – VERT, a new tool for viral surveillance and discovery. It is a new method for identifying a virus behind an infection using polymerase chain reaction (PCR) and amplifying of bits of DNA into a large enough sample size. It can simultaneously analyze 21 samples in less than 48 hours at just \$200 a sample. This technique was developed by first compiling a database of 1,000 vertebrate viruses, then synthesizing genetic probes to match 2 million strains of viruses. The probe binds to a matching virus when they come into contact. Magnetic beads are added and a chemical linker binds the beads to the genetic probes and the viruses they have identified. A magnet pulls the probes to the tubes walls. After all probe bead combos are washed, researchers genetically sequence the viruses reducing the likelihood of false-positives.

This approach is capable of multiplexing up to forty including influenza viruses. It is a cost effective approach because there is no need to use cultures to amplify. VERT can find every virus in a given drop of saliva, tissue or spinal fluid with near-perfect accuracy.

Dr. Gehrke opened his presentation by stating the main motivational reasons for a need for a diagnostic test. First, arboviruses and hemorrhagic fever cause serious human diseases. Rapid testing is needed for pathogen tracking and triage patients. Additionally, rapid diagnostics can be delivered and used quickly to address disease outbreaks. Finally, rapid tests are valuable to assess seroconversion in vaccine trials.

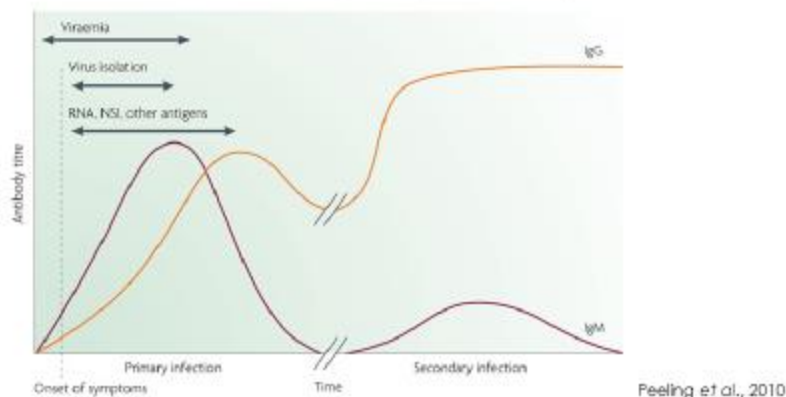
Included in current approaches for Arbovirus diagnostics are laboratory diagnosis using serum, blood, urine, saliva, cerebrospinal fluid and tissue for immunohistochemistry. Important diagnostic clues are the patients' clinical features and recent travel history. Other possible diagnostic methods are: PCR, virus culture and immunological approaches like the ELISA and rapid Paperfluidic tests. Patients will benefit from a wide variety of diagnostic reagents to benefit their health. Finally, rapid diagnostics have the benefit of a fast readout that can triage patients and track viruses.

The goal should be to develop a simple, rapid diagnostic device that will screen for multiple pathogens to focus appropriate medical care on patients with the greatest need. Such a test should be simple with no special training chemicals, reagents or training required for use and is able to perform without a power source. Rapid should be defined as 30 minutes or less. The best scenario would be a multiplexed device that can screen up to eight pathogen markers simultaneously. Important would be to partner with local innovators to develop and potentially manufacture the tests.

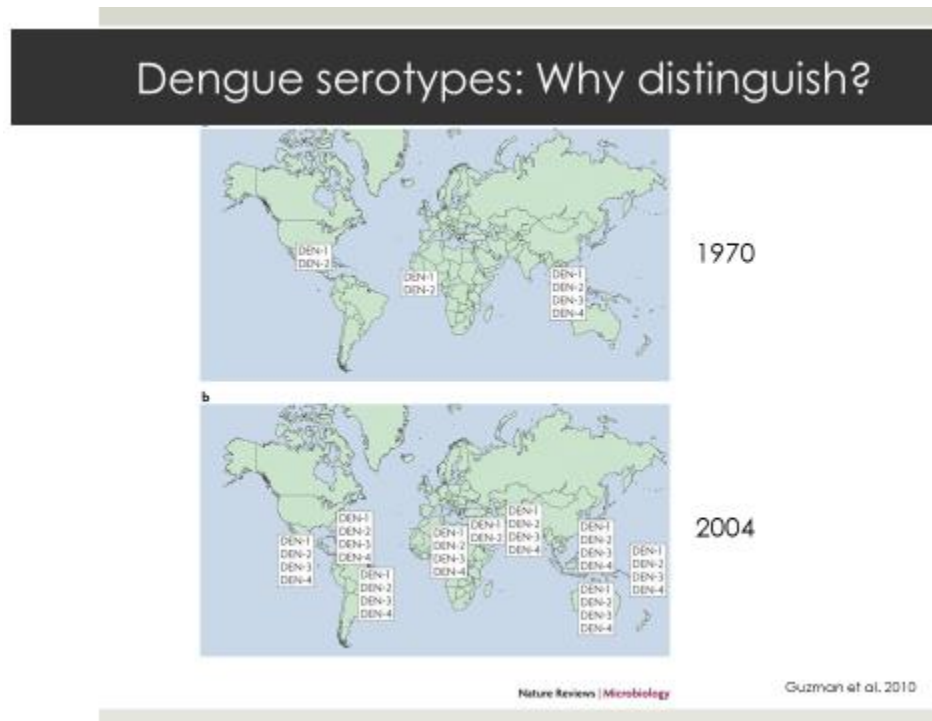
Considering dengue fever, for example we can look for dengue NS1 which is a protein secreted and is an early marker of infection.

Dengue NS1 Protein Antigen Test

Dengue NS1 protein is a secreted early marker of infection



It is important to test for the serotype when considering dengue fever because we know that a population in an endemic region that is hit with a serotype that has not circulated for a block of years, will be more likely to experience hemorrhagic fever and more severe illness. Dr. Gehrke showed this slide comparing two maps from 1970 and 2004 to demonstrate why there is a need to distinguish.



Serotype specific dengue detection is possible and can be achieved with patterned antibody pair recognition (PAPR). The approach would be lateral flow chromatography which uses pairs of eight antibodies on four paper strips. The sample reactivity will generate a unique pattern that will identify viral serotypes with a sensitivity and specificity in the range of 80-100%. The test format can be performed in a single lane or multiplexed for eight markers or viruses. MIT uses monoclonal antibodies generated and characterized in their own lab. They produce and purify their own antibodies and laser cut their own papers. The test housings are 3D printed or injection molded and they are in the process of building a low-cost data recording system.

Dr. Gehrke presents several slides showing serotype specific dengue NS1 detection with each serotype containing four vertical strips and a series of paired positive indicators for each of the four serotypes. For example, DEN1 pairs 1/5 and 3/7, DEN2 pairs all positive, DEN3 pairs 3 and 7 positive and DEN4 pairs 3/7 and 4/8 as positive. These tests are confirmed with purified NS1 proteins and experience no crossover from yellow fever virus NS1.

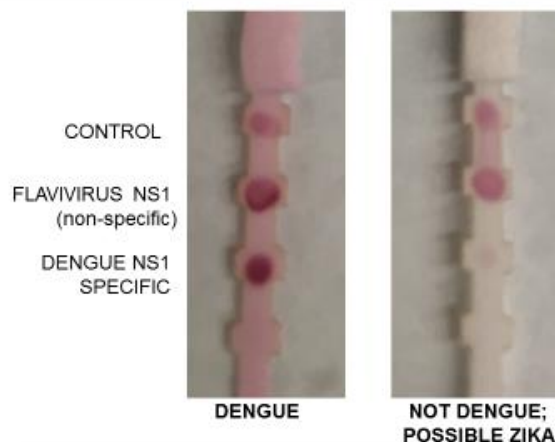
sensitivity/specificity

<u>serotype</u>	<u>sensitivity</u>	<u>specificity</u>	<u>n</u>
D1	75%	100%	4
D2	82%	100%	7
D3	83%	100%	6
D4	100%	100%	4

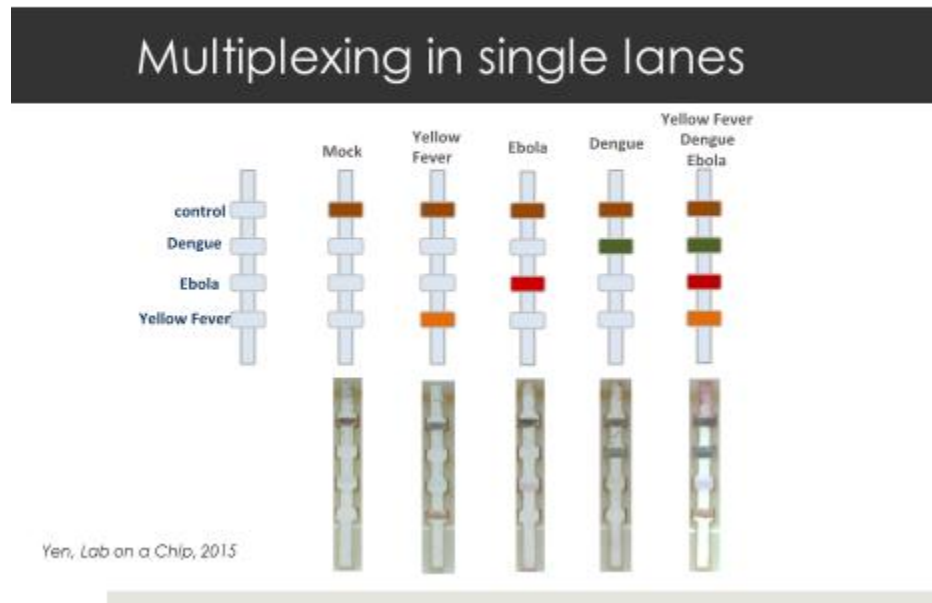
A chikungunya antibody screen is also in development for the detection of baculovirus CHIKV E1 and E2. Using dengue and chikungunya as examples of how to think about Zika virus diagnostics, Dr. Gehrke provides the following recommendations: development of new antibody reagents is needed, antibodies will need to be characterized and tested to define pairs for Zika detection, and in the short term we at least need a test that can rule out dengue in a Zika context.

Dr. Gehrke shows how a rapid diagnostic test for Zika could be achieved using eight vertical strips and an example of a “dengue rule out test for Zika” could be designed using a control, flavivirus non-specific NS1 and a dengue NS1 specific.

Dengue “rule out” test for Zika



Other possible detection strategies could be use of Gold nanostars (Nstars) and multicolored silver nanoprisms. These are being worked on now by Dr. Gehrke and his collaborators. The multiplex lateral flow pathogen detection using multi colored silver nanoplates permits multiplexed analysis in a single channel making it easier to facilitate and manufacture (Chun-Wan Yen, Helena de Puig, et al 2015).



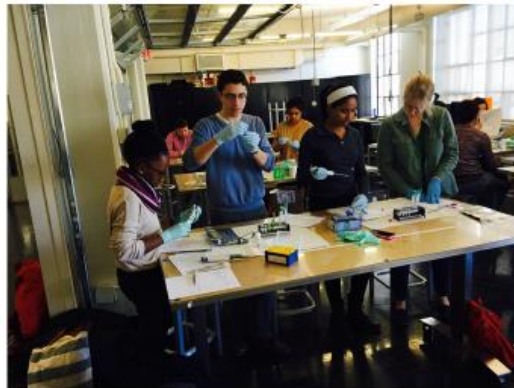
In conclusion, rapid diagnostics are needed for pathogen tracking, patient triage and seroconversion monitoring. A combination of approaches (lateral flow, ELISA, and PCR) are the most ideal for patient care because each has its unique advantages and disadvantages. Detection and distinguishing the four dengue serotypes is possible using multiple antibody pairs that generate a unique reactivity pattern. We are certain that this same approach will work for Zika. Digital fabrication, coupled with local innovation and manufacturing offer important alternatives for delivering healthcare.

Dr. Gehrke, closed his presentation with may photos of his team and his students and emphasized a collaborative will to partner with local researchers and businesses.

Diagnostics Kit Assembly



Education and Training



Puerto Rico, A hub for Tropical Disease Testing, Trials, and Product Development, Opportunities and Challenges

Ignacio Pino, DVM

President and Founder of CDi Laboratories, Inc.

Mayaguez, Puerto Rico

Dr. Xiaowu Liang, Ph.D.

President and CEO

Antigen Discovery, Inc.

Dr. Pino and Dr. Liang co-presented and opened their talk with facts about CDi and ADi which are among the first companies to leverage high through-put proteomics for antibody biomarker discovery. They

use a novel platform which accelerates relevant antigen and biomarker discoveries in weeks-time versus years. They represent the largest collection of expressible ORF clones, comprised of over 80,000 and growing biomarker candidates for human and infectious agents. They have a solid portfolio and a broad pipeline to support their continued and sustained growth. They have created a world class business by developing and commercializing innovative proteome technology which has resulted in rapid economic development. They utilize revenue from grants and contracts to conduct their research and development, they utilized a co-development strategy, a fee for service, and product sales to create their biosciences products and services and they use their revenue from partnerships, out-licensing and royalties to support the manufacturing and licensing of diagnostic products for infectious and autoimmune diseases.

Many proteomes are already available and others can be generated in only weeks. Below is a listing available proteomes.

Many Proteomes Already Available, Others Can Be Generated In Weeks

Total Number of proteins

Retroviruses HIV 1&2 (5 clades) 83

Papilloma HPV viruses (29 types) 232

Orthopoxviruses Vaccinia, Variola, Monkeypox 260

Herpes Viruses HSV

1&2,

VZV,

EBV

300

Flaviviruses WNV,

Dengue4,

YF,

SLE,

JE,

chikungunya 90

Alphaviruses

Bacteria *Brucella melitensis* 3,194

Chlamydia trachomatis 911

Chlamydia muridarum 911

Mycobacterium tuberculosis 4,100

Francisella tularensis 1,933

Coxiella burnetii 2,065

Borrelia burgdorferi 1,600

Borrelia garinii 1,200

Borrelia afzelii 1,000

Burkholderia pseudomallei 5,728

Leptospira interrogans 3,658

Salmonella enterica typhi 4,318

Orientia tsutsugamushi 1,400

Rickettsia rickettsii 900

Bartonella henselae 1,400

Staphylococcus

aureus

(MRSA) 2,628

Streptococcus

pneumoniae 2,200

Parasites *Plasmodium falciparum* 5,643

Plasmodium vivax 5,300

Schistosoma mansoni 9,000

Babesia microti 5,000

Toxoplasma gondii 12,000

Necator

americanus

12,000

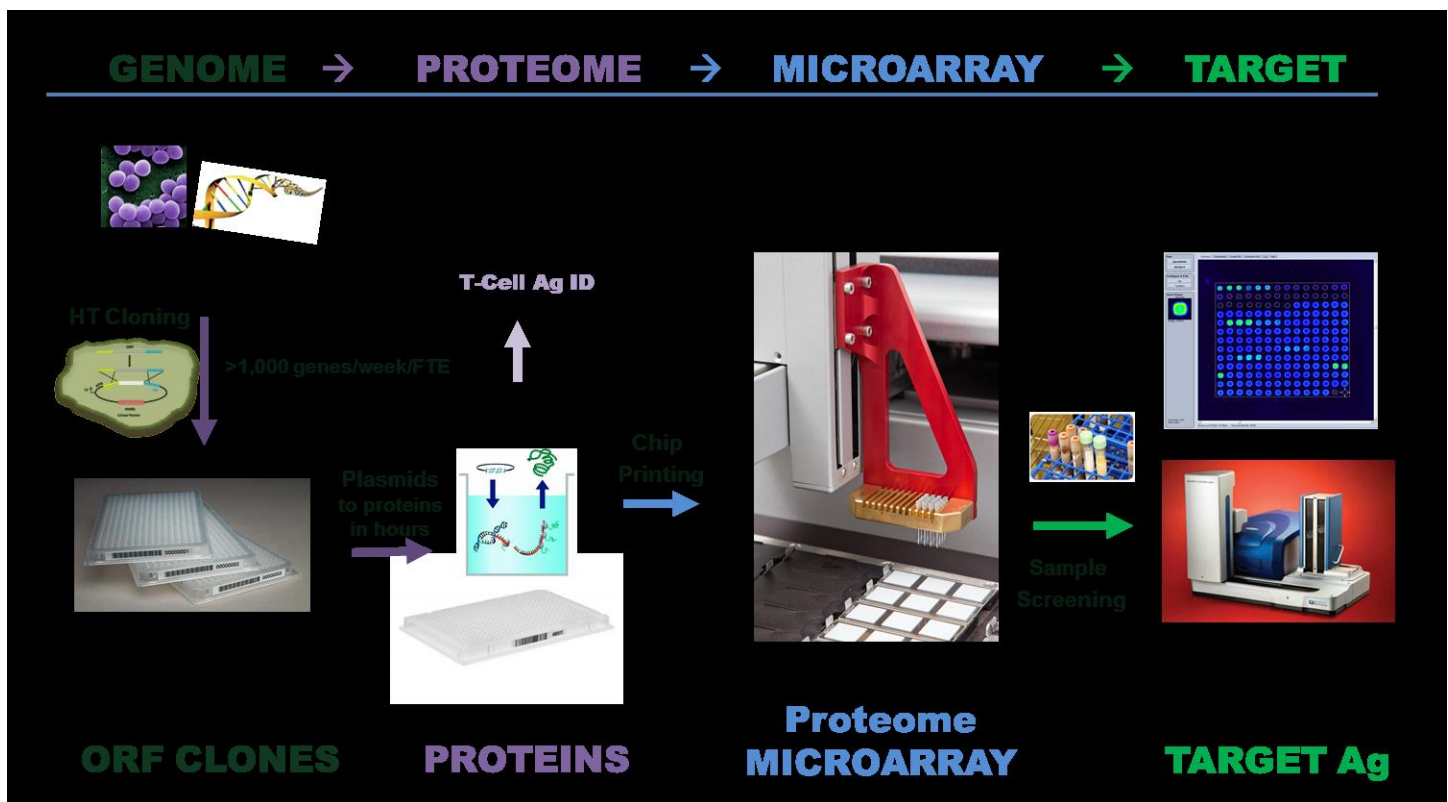
Trypanosoma cruzi 15,099

Trypanosoma brucei 8,529

Human Unique genes 22,000

TOTAL ~135,000

The goal of CDi and ADi Labs is to eliminate the bottleneck that can occur when translating genomics and proteomics into therapeutics, vaccines and diagnostics. Below is a graphic display of their disruptive platforms and how process moves from genome – proteome – microarray – target.



One of the Disruptive platforms is the Fast-Mab Monoclonal Antibody Production which contains libraries of monoclonal antibodies with unparalleled specificity in just eight weeks. It is currently generating MABs to 35 new human proteins every month and it rapidly generates controls and additional reagents for kits. There have been 1,251 MABs to 595 TF's generated to date. The proposed workflow process is as follows: 1) screen multiple patient and control serum samples on Discovery array, 2) Comparison of Ab profiles from both groups and identify candidate Ag markers, 3) fabrication of focused arrays with candidates, 4) testing of a large cohort against focused arrays to validate Ag biomarkers and finally, 5) development of multi-organism diagnostics and vaccines.

CDi has synergistic collaborations with a variety of partners in Puerto Rico. They collaborate with ADi in the development and manufacturing of cutting edge discovery tools and the analysis of proteome arrays and bioinformatics. There is a larger partnership among the University of Puerto Rico, PSM, FIJD and others in the collection of clinical samples and clinical data for diseases under study. The manufacturing and characterization of reagents for diagnostic kits is done in partnership with the University of Puerto Rico which leverages capabilities of the HIV project. Finally, development and manufacturing of final diagnostic devices is done with CDi, ADi and other collaborators.

Technology highlights have been the complete proteome coverage which results in the greatest chance of success by interrogating the entire proteome for targets. The speed and throughput which has become much more efficient than other conventional approaches, which only a small amount of samples needed. The IP Generating Machine which has helped to identify novel biomarkers and antigens and identified their use in therapeutic, vaccine and diagnostic applications. The broad applications in target discovery and validation, product development and clinical trials for diagnostics, therapeutics and vaccines have been fruitful.

Several real world examples of the application of this technology were presented. A diagnostic tool for Malaria using 8,500 P falciparum proteins, was created showing the illumination for proteins detected in the

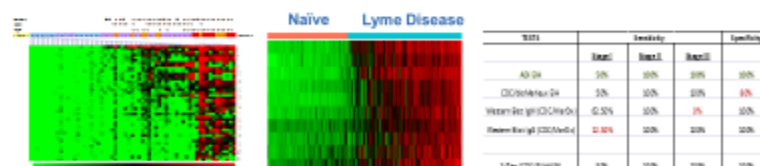
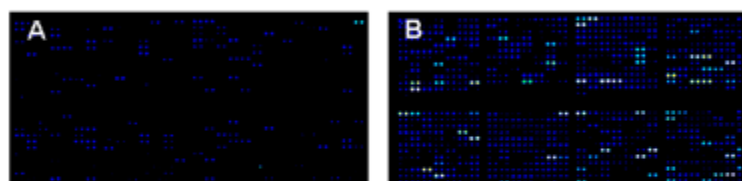
blood of a child naturally exposed from Papua New Guinea compared with that of a domestic naïve adult. A heat map was presented showing the cross-sectional profile of 109 infected Ghanaian children compared to the 12 naïve controls. Subsequently, two antibody kinetic patterns are distinguished in a post experimental challenge. One showing the rapid onset and rapid decay and the second showing a slower onset and slower decay. Lyme disease is also presented using the same proteome array and heat mapping process.

Example: Lyme Disease

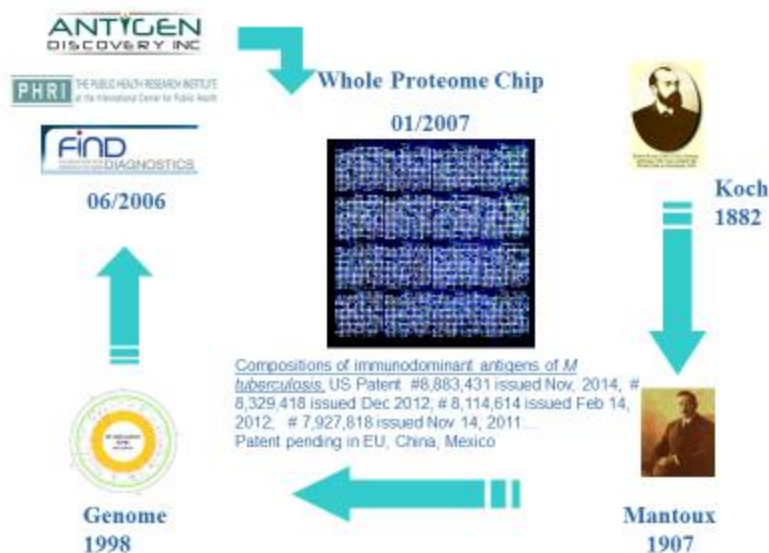
B. burgdorferi proteome array (1,500 proteins)

Naïve Control

Lyme Disease



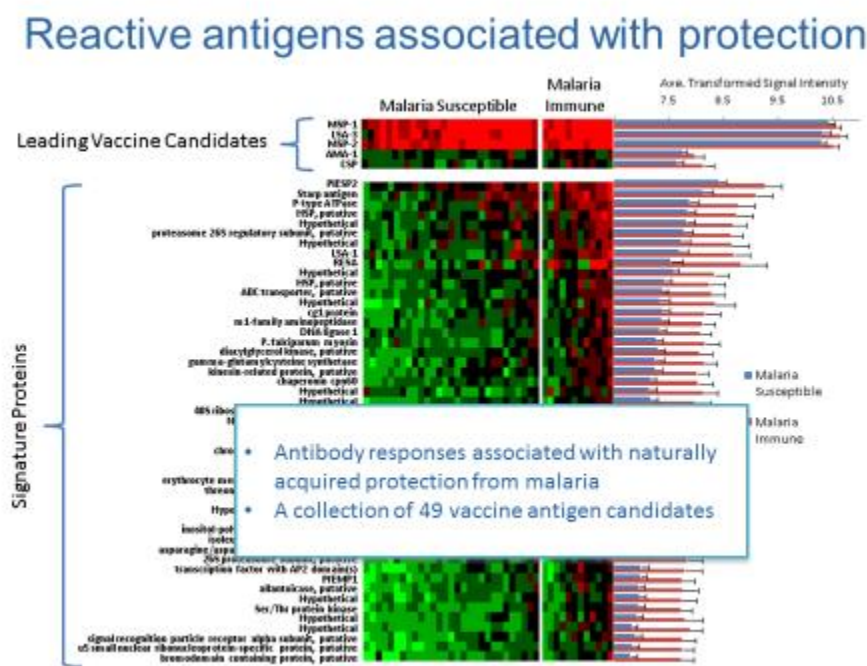
Application: TB Antigen Discovery



This technology has also been used to detect HIV incidence in sera and saliva in work done for the consortium for the evaluation and performance of HIV Incidence Assays. CDi and ADi then present how this

technology can be applied to diagnose multi-pathogens using an example which examines serodiagnosis of malaria and tularemia.

Vaccine Antigen Discovery can also be achieved using this technological approach. Malaria risk decreases with age. Children in Kambila, Mali begin to show protection by ages 8-10. Analysis of 150 protein antigens show an age and seasonal dependent effect on serology in Mali. There are more antibodies in adults than children and there are more antibodies after the malaria season than before. An analysis of signature proteins and how they compare to leading vaccine candidates demonstrate how reactive antigens are associated with protection. Antibody responses are associated with naturally acquired protection from malaria.



Finally, the major challenges to progress in this area are identifying funding and the coordination of serum samples for all diseases with comprehensive clinical information. It is possible to build a protein microarray for a Flavivirus panel. Currently on the chip are dengue 1-4, West Nile virus, yellow fever, TBEV (JEV and SLEV) and CHIKV. It is a matter of adding ZIKAV (3 structural proteins and 7 non-structural proteins), the peptides encoding ED3 of various flaviviruses and the envelope proteins expressed using CDI's novel HSV-based membrane protein expression system. The end product will be a miniaturized table-top hand held system that is powered and controlled via a battery or laptop computer.

Challenges and Opportunities in Developing Tests for Clinical Use

Frank Mandy, Ph.D.

Vice President at Leukometrics

Canada

Dr. Frank Mandy begins by presenting the main challenge – chikungunya, dengue, influenza, leptospirosis and Zika are all infectious diseases that impact on health, life-style and global economics including tourism in Puerto Rico. This presents us with a great opportunity to manufacture kids in Puerto Rico

to detect infectious disease. Specifically, strategies that combine protected innovative elements integrated with 21st century solutions. We can introduce superior, affordable point of care assays that are multiplexed kits that are easy to use and produce rapid results. It is important to have daily external quality management to support users including remote locations. There are many examples of portable instrumentation in 2016.

PORTABLE INSTRUMENTATION IN 2016

Instruments work with disposable cartridges: size of a chewing gum



From left to right DI's: **Pima**, **Avalune**, **FACSPresto**, **ChipCare** & **MBio**



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DIGITAL IMAGERS

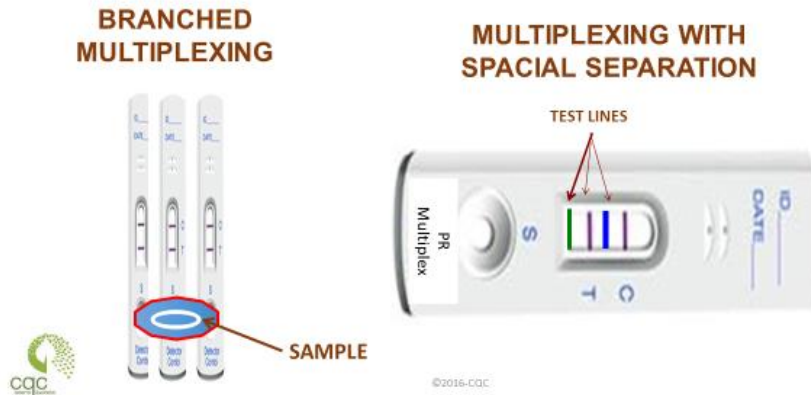


Dr. Mandy offers historic milestones for consideration:

In the 1960's RIA introduced sensitivity to clinical assays. In the 1970's ELISA offered safer and more cost effective technology, in the 1980's commercial Mab technology added specificity and lateral-flow based immunoassay added simplicity. In the 1990's, digital signal processing was introduced along with lasers and personal computers. In the 2000's dedicated FC for multiplexed applications was developed and in 2010 hybrid instrumentation (flow and image together) is being utilized.

Dr. Mandy shows us that there are two possible options for multiple pathogen detection with lateral flow – branched multiplexing and multiplexing with special separation.

TWO POSSIBLE OPTIONS FOR MULTIPLE PATHOGEN DETECTION WITH LATERAL FLOW



The best examples of exceptional unification of multiple technologies has been the Apple I-pod and the I-pad. We need to keep in mind that sometimes it is “repackaging for the consumer” that makes all the difference. Our goal is: innovation, development, product formatting of biomedical technologies to support health care services to monitor vaccination campaigns in rural regions or perform routine diagnostics. We need to bring externally managed quality point of care diagnostic kits to remote health centers. There are innovative applications of noble metal nano-particles for multiplexed lateral flow assays operating with a single strip. There are also several proposals on how to link these devices to smart phones (Zheng et al. Nature Biotech 2005).

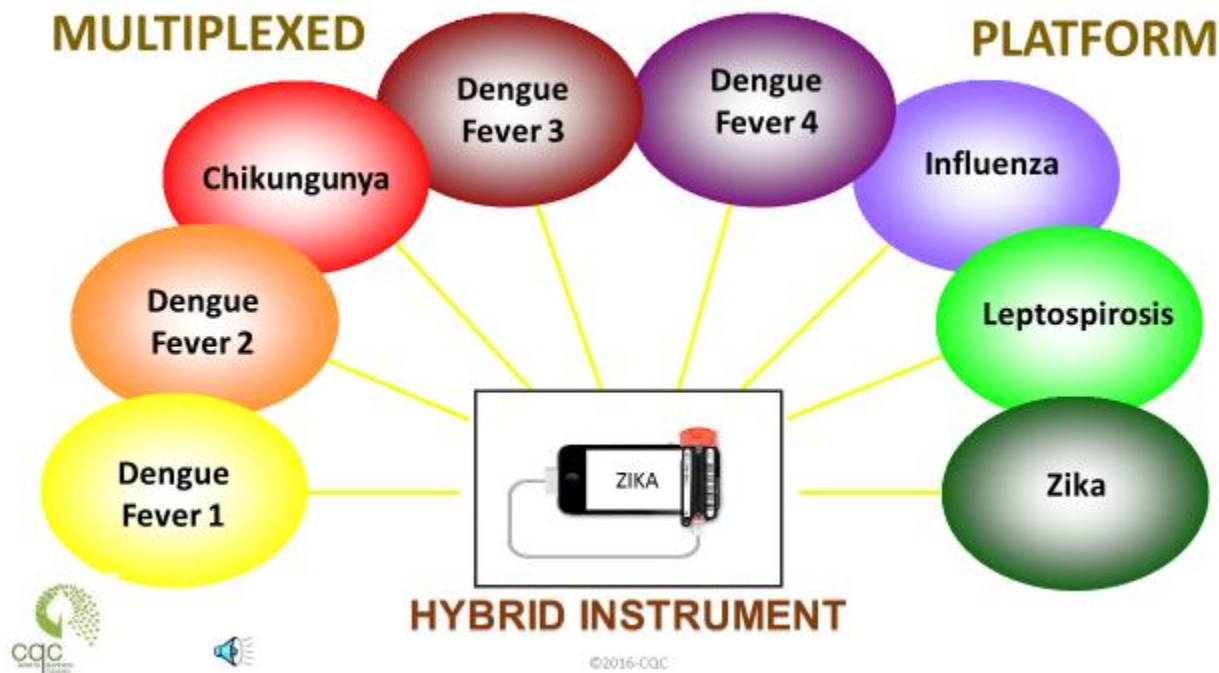
Past examples of synergistic solutions with significant global impact in clinical labs have been flow cytometry (laser, Mab's, PC's) and the digital image analyzer (Digital Signaling Processor, LED/Laser and low cost Charged Coupled Device). Dr. Mandy then presents the approach of flow cytometry signal intensity binning and its variations of specific and non-specific binding. He provides us with a real world example of a multiplexed assay for Rubella Ab's.

Dr. Mandy offers Microflow, (manufactured in Canada) as a robust medical tool that is portable, shoe box size, and battery operated. It uses fiber optics in a uFC configuration is alignment free and has a compact light source. It uses sheathless fluidics and is easy to use and provides rapid analysis. Cartridges load easily and results are available in only 10 minutes. It is being tested now on board the international space station by Canadian Astronaut, Commander Chris Hadfield, CSA.

Asserting that our main objective of this meeting is to use innovation to “re-package immunoassays” to adjust to global socio-economics environment and assemble the ultimate diagnostic bundle. As shown in the slide below. It is suggested that when considering making test available worldwide that we consider the inappropriate import/export traffic in ideas and between the rich and poor countries. Nigel Crip offers the following explanation: rich countries import trained health workers and export their ideas and ideologies about health to poorer countries. Crisp suggests that we reverse the order, poor countries should export their ideas and rich countries should export their healthcare workers (Turning the world upside down, the search for global health in the 21st century, 2010). The socio-economic aspects of re-packaging technologies is an important consideration in the 21st century when interdependence is the way forward with health care management. Sound global perspective must be a critical and constant consideration. Social and economic capacity effect the way we react to global outbreaks. Consider the US and EU response to the Ebola outbreak. Dallas and Dakar both had a single Ebola infected individual identified by local health professional, but with

two very different outcomes. The one patient was successfully isolated in Dakar, West Africa. In Dallas, USA, multiple infectious resulted from a single case of Ebola.

A twenty first century product must include at least these four elements: International IP protection, integrated biometrics linking patent-test-results, assay time reduction by 50% vs ELISA and daily universal external quality control and management. In conclusion, Dr. Mandy reminds us that the opportunity is here to leap ahead by a decade with affordable rapid diagnostics for infectious diseases. It is possible now to implement rapid, cost effective and reliable multiplexed testing. The innovative path offers enhanced based quality management. It will deliver improved health care, including composite vaccination status surveillance which will be essential to combat the ever increasing impact of climate change. He charges our group to move forward and hopes that by 2022 the Point of Care Multiplexed Diagnostics will be a universal thanks to a Puerto Rican initiative.



Dr. Abe Schwartz
President
Center for Quantitative Cytometry
Puerto Rico

Dr. Abe Schwartz closes the meeting by offering some sound practical advice on the topic of Intellectual Property. "If you want to keep a secret, do not tell anyone. Just because you think you are paranoid, does not mean they are not after you." When working with Patent Lawyers and Agents, remember they are not typical lawyers. They have specialized vocabulary and regulations. Perform your own initial prior art search. Write the body of the patent yourself and have the lawyer write the claims. Make sure you read the claims so that they accurately describe your invention.

In this world, there are filing priorities. The new law is that the first party to file wins the invention rights. In order to maintain your global rights, do NOT: publish a paper (including on the internet), give a presentation anywhere, discuss the proprietary information in public, and certainly so not discuss the information with other colleagues. It is always best to consult a lawyer or an agent when in doubt. Dr. Schwartz goes over some patent trivia with the group. He emphasizes that 97% of all patents never make any money (<http://www.allbusiness.com/97-percent-of-all-patents-never-make-any-money-15258080-1.html>). A patent is a license to sue. Inventors do not usually own their patents, the assignee does. The patent examiner cannot be called as a witness. Patents are the most expensive type of publication. We are encouraged to consult David L. Gulley, PhD, RTTP, CLP, the Director of the Technology Transfer Office at the Puerto Rico Trust for Science, Technology and Research at dgulley@prsciencetrust.org for more information.

Main Discussion Points

Next Steps

Appendices

- I. Agenda
- II. Participants
- III. Test tables and Reviews
- IV. Other meeting materials
- V. Bio sketches of participants

Puerto Rico Brain Trust for Tropical Disease Research and Prevention
Rapid Diagnostic Testing for Chikungunya, Dengue Fever, Influenza, Leptospirosis and Zika

February 8 – February 10, 2016
Puerto Rico Trust for Science, Technology and Research

AGENDA

Tuesday, February 9, 2016
Challenges of Rapid Testing and Future Trends

Morning Session
Conference Room 2nd Floor
Puerto Rico Trust for Science, Technology and Research

7:30 AM **Hotel Pick-up**

8:00 AM **Breakfast**

8:15 AM **Welcome**

Eng. Lucy Crespo, CEO
Puerto Rico Trust for Science, Technology and Research

Dr. José A. Lasalde Dominicci, Ph.D.
Vice President for Research and Technology
University of Puerto Rico

8:30 AM **Meeting Objectives and Introductions**
Considerations in Diagnostic Testing for Tropical Diseases

Dr. José F. Cordero, MD, MPH
Meeting Chairman & Project Investigator
Patel Distinguished Professor of Public Health
Chair, Department of Epidemiology & Biostatistics
University of Georgia

8:45 AM **Update on Arboviral Diseases in Puerto Rico**

Brenda Rivera Garcia DVM, MPH
State Epidemiologist
Puerto Rico Department of Health

9:00 AM **Dengue Diagnostics**

Elizabeth Hunsperger, PhD
Activity Chief
Centers for Disease Control and Prevention - Dengue Branch
Immunodiagnostic Development and Research Lab
CDC Division of Vector Borne Diseases

9:45 AM **The Challenge of Zika and Chikungunya Diagnosis in Dengue Endemic Regions**

Jorge L. Munoz-Jordan, PhD
Chief, Surveillance and Research Lab
Centers for Disease Control and Prevention
Dengue Branch

10:30 AM Morning Break

10:45 AM Chikungunya
Scott Weaver, M.S., Ph.D.
John Sealy Distinguished University Chair in Human Infections & Immunity
Director, Institute for Human Infections & Immunity
Scientific Director, Galveston National Laboratory
University of Texas Medical Branch, Galveston, USA

11:15 AM Influenza
Dr. Stephen Lindstrom, Ph.D.
Team Lead, Diagnostics Development Team
Virus Surveillance and Diagnosis Branch
Influenza Division, NCIRD
Centers for Disease Control and Prevention

12:00 PM Leptospirosis
Renée Halloway, MPH
National Center for Zoonotic, Vector-Borne and Enteric Diseases
Centers for Disease Control and Prevention

12:45 PM Lunch Break

Challenges and Opportunities in Diagnostic Testing Development

Afternoon Session

Conference Room 2nd Floor

Puerto Rico Trust for Science, Technology and Research

1:45 PM A Multiplex Assay for Detection and Quantitation of Chikungunya, Dengue, Zika and West Nile Viruses and Vir Cap Seq – VERT – A Tool for Viral Surveillance and Discovery
Ian Lipkin, M.D.
John Snow Professor
Center for Infection and Immunity
Mailman School of Public Health
Columbia University

2:30 PM Rapid Paperfluidic Diagnostic Devices to Detect and Distinguish Arbovirus Infections
Lee Gehrke, Ph.D.
Herman von Helmholtz Professor of Health Sciences and Technology
MIT Institute for Medical Engineering and Science
Professor of Microbiology and Immunobiology

Professor of Health Sciences and Technology

3:15 PM Afternoon Break

3:30 PM Puerto Rico, A hub for Tropical Disease Testing, Trials, and Product Development, Opportunities and Challenges

Ignacio Pino, DVM

President and Founder of CDi Laboratories, Inc.

Mayaguez, Puerto Rico

Dr. Xiaowu Liang, Ph.D.

President and CEO

Antigen Discovery Inc.

4:30 PM Discussion

Dr. José F. Cordero, MD, MPH

5:00 PM Adjourn

5:15 PM Transportation to Hotel

Day 3

February 10, 2016

Recommendations for Tropical Disease Rapid Test Development Future Steps for Creating a Hub in Puerto Rico

Morning Session

Puerto Rico Trust for Science, Research and Technology

Conference Room 2nd Floor

7:30 AM Hotel Pick-up

8:00 AM Breakfast

8:30 AM Welcome and Objectives

Dr. José F. Cordero

9:00 AM Challenges and Opportunities in Developing Test for Clinical Use

Dr. Frank Mandy - Canada

Vice President at LeukoMetrics

Ottawa, Canada

Dr. Abe Schwartz - Puerto Rico

President

Center for Quantitative Cytometry

10:00 AM Opportunities for Rapid Testing Development in Puerto Rico

Group discussion

11:00 AM Challenges and Feasibility Issues for Rapid Testing Development in Puerto Rico

Group discussion

12:00 PM

Meeting Closure

Dr. José F. Cordero, MD, MPH

12:30 PM

Lunch

1:45 PM

Adjourn

2:00 PM

Transportation to Hotel or Airport

Meeting Participants
Rapid Diagnostic Testing for Zika, Chikungunya, Dengue, Influenza and Leptospirosis
February 8-10, 2016
Puerto Rico

	Name and title	Physical address and Email address	Confirmed & Role
1.	Dr. Jose Cordero, MD, MPH Patel Distinguished Professor of Public Health Chair, Department of Epidemiology & Biostatistics PI Brain Trust for Tropical Disease Research	706-583-8202 jcordero@uga.edu B.S. Miller Hall Room 105 Athens, GA 30602	Confirmed Host
2.	Dr. Scott Weaver, M.S., Ph.D. Professor Departments of Pathology and Microbiology & Immunology Director Institute for Human Infections and Immunity Director, Galveston National Laboratory	University of Texas Medical Branch 6,200D Galveston National Laboratory 301 University Blvd. Galveston, Texas 77555-0610 409-266-6500 409-457-7970 sweaver@utmb.edu	Confirmed Presenter on diagnostic tests and chikungunya
3.	Dr. W. Ian Lipkin, MD John Snow Professor of Public Health Center for Infection and Immunity Mailman School of Public Health Columbia University	Center for Infection and Immunity 72 West 168 th Street Room 1703a New York, NY USA 10032 Wil2001@columbia.edu 212-342-9033 212-342-9044 Ejk2162@cumc.columbia.edu Ellie Kahn Administrative Coordinator www.cii.columbia.edu	Yes
3.	Dr. José Lasalde Dominicci, Ph.D. Adjunct Professor Structural and Molecular Biology Laboratory Sciences JGD Bldg. Lab 114-115	Structural and Molecular Biology Laboratory Sciences JGD Bldg. Lab 114-115 PO Box 23360 University of Puerto Rico-Medical Sciences Campus Rio Piedras Medical Center Américo Miranda Ave. San Juan, PR 00931-3360 jlalalde@gmail.com fax 787-753-3852	Confirmed Welcome remarks
4.	Dr. Ignacio Pino, DVM President and Founder of CDi Laboratories, Inc. Bioprocessing Technology Development Center	CDi Laboratories, Inc. Bioprocess Development and Training Complex Guanajibo Research and Innovation Park 4005 Street B, Road 114 km 1.3 Mayaguez, PR 00682 787-806-4100 Fax 787-806-4006	Confirmed Presenter on industry in Puerto Rico

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		Ignacio.pino@cdi-lab.com	
5.	Dr. Emma Fernandez-Repollet, Ph.D. Principal Investigator Center for Collaborative Research in Health Disparities (NIMHD-RCMI Program)	Room 621-A, 6 th Floor Guillermo Arbona Building Medical Sciences Campus GPO Box 365067 San Juan, PR 00936-5067 e.fernandez@upr.edu 787-763-9401 fax 787-758-5206	Confirmed
6.	Dr. Abe Schwartz, Ph.D. President, Center for Quantitative Cytometry	abe@quantcyte.org 787-308-1033	Confirmed Presenter on Challenges and Opportunities in Developing Test for Clinical Use
7.	Dr. Elizabeth Hunsperger, PhD Activity Chief CDC Dengue Branch Immunodiagnostic Development and Research Lab CDC Division of Vector Borne Diseases	CDC Dengue Branch 1324 Calle Canada San Juan, Puerto Rico 787-706-2472 786-280-3839 Enh4@cdc.gov CDC Dengue Branch 1324 Calle Canada San Juan, Puerto Rico 00920-3860	Confirmed Presenter on diagnostic testing and dengue fever
8.	Lic. Ivan Rios, Director Puerto Rico Trust for Science, Technology and Research	Puerto Rico Trust for Science, Technology and Research P.O. Box 363475 San Juan, PR 00936-3475 Antigua Penitenciaría Estatal Carr. #21, Bo Monacillos Rio Piedras, PR 00927 irios@prsciencetrust.org	Confirmed Puerto Rico Trust for Science, Technology and Research
9.	Eng. Lucy Crespo, CEO Puerto Rico Trust for Science, Technology and Research	Puerto Rico Trust for Science, Technology and Research P.O. Box 363475 San Juan, PR 00936-3475 Antigua Penitenciaría Estatal Carr. #21, Bo Monacillos Rio Piedras, PR 00927 lcrespo@prsciencetrust.org	Confirmed Welcome Remarks Puerto Rico Trust for Science, Technology and Research
10.	Dr. Celia Alpuche Aranda, MD Director Center for Infectious Disease Research National Institute of Public Health	National Institute of Public Health Mexico Universidad No. 655 Colonia Santa María Ahuacatitán Cerrada Los Pinos y Caminera C.P. 62100,	CANCELED Clinician's perspective on practical use of rapid diagnostic tests

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	Mexico	Cuernavaca, Morelos México +52 17773293000 Celia.alpuche@insp.mx	
11.	Dr. Lee Gehrke, Ph.D. Hermann von Helmholtz Professor of Health Sciences And Technology, Massachusetts Institute of Technology Harvard Medical School	Massachusetts Institute of Technology Room E25-406B 77 Massachusetts Ave. Cambridge, MA 02139 lgehrke@mit.edu gehrkelab.org 617-253-7608 Lab 617-253-7699 Elizabeth Hoy 617-258-9225 ehoy@mit.edu	Confirmed Presenter Rapid Paperfluidic Diagnostic Devices to Detect and Distinguish Arbovirus Infections
12.	Dr. Brad R. Weiner, Ph.D. Profesor of Chemistry Centro de Recursos para la Ciencias de Ingeniera University of Puerto Rico Rio Piedra Campus	Centro de Recursos para la Ciencias de Ingeniería University of Puerto Rico Rio Piedras Campus PO Box 23334 San Juan, PR 00931-3334 brad@hpcf.upr.edu 787-765-5170 ex.t 2090 787-756-7717 fax Madelyn.aquino@upr.edu	Confirmed Engineering of test technology and the Puerto Rican experience
13.	Leslie Maas Cortés, MHS Brain Trust of Tropical Disease Research and Prevention Trust for Science, Technology and Research	Puerto Rico Science, Technology and Research Trust P.O. Box 363475 San Juan, PR 00936-3475 Antigua Penitenciaria Estatal Carr. #21, Bo Monacillos Rio Piedras, PR 00927 lesliemaas@icloud.com 787-210-5093	Confirmed Coordinator Brain Trust for Tropical Disease Research and Prevention
14.	Dr. Ivan Lugo, Executive Director Industry University Research Center, Inc. (INDUNIV)	PRIDCO Bldg., Suite 101 PO Box 362350 San Juan, PR 00936-2350 787-772-4604 787.772.9272 Fax 787-772-9011 ilugo@induniv.org	Confirmed
15.	Dr. Hal Margolis, MD Former Chief Dengue Branch Centers for Disease Control and Prevention	404-520-0980 mrhepb@earthlink.net	Confirmed Dengue expert

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16.	Jacintha L. Smith, MS CDR, USPHS Microbiologist Doctoral Candidate University of Georgia	678-772-4534 Cvd2@cdc.gov 404-639-3344 Fax 404-718-2096	Confirmed Doctoral candidate UGA Meeting coordination
17.	Dr. Jorge L. Muñoz-Jordán, Ph.D. Chief, Molecular Diagnostics and Research Laboratory CDC	CDC Division of Vector Borne Diseases Dengue Branch 1324 Calle Canadá San Juan, PR 00920 Ckq2@cdc.gov 787-706-2399 787-706-2496	Confirmed Zika and dengue expert
18.	Dr. Kosmas Kretsos, Ph.D. Puerto Rico Consortium for Clinical Investigation Puerto Rico Trust for Science, Research and Technology	Puerto Rico Consortium for Clinical Investigation Puerto Rico Trust for Science, Research and Technology PO Box 363475 San Juan, PR 00936-3475 kkretsos@prsciencetrust.org	Confirmed
19.	Dr. Jack Newman, Ph.D. Contractor Biological Technologies Office Strategic Analyst, Inc. SETA Support to DARPA/BTO	jnewman@consultant.sainc.com 510-847-4496	Confirmed
20.	Renee Galloway, M.P.H. Leptospirosis Expert National Center for Emerging and Zoonotic Infectious Disease Centers for Disease Control and Prevention	Zul0@cdc.gov	Confirmed Presenter on diagnostic testing and Leptospirosis
21.	Dr. Stephen Lindstrom, Ph.D. Team Lead, Diagnostics and Development Team Virus Surveillance and Diagnosis Branch Influenza Division, NCIRD Centers for Disease Control and Prevention	Centers for Disease Control and Prevention 1600 Clifton Road NE Atlanta, GA 30333 404-639-1587 Fax 404-639-0080 sql@cdc.gov	Confirmed Presenter on diagnostic testing and influenza
22.	Dr. Brenda Rivera, MD Epidemiologist Department of Health Puerto Rico	brendarivera@salud.gov.pr briveravm@yahoo.com 787-274-6831	Confirmed State Epidemiologist
23.	Dr. Francis Mandy, Ph.D.	Winnipeg, Manitoba	Confirmed

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	Consultant Winnipeg, Manitoba Canada	Canada	Presenter
24.	Dr. Xiaowu Liang, Ph.D. Board Member President and Chief Executive Officer Antigen Discovery, Inc.	Antigen Discovery, Inc. 1 Technology Drive Suite 309E Irvine, CA 92618 xliang@antigendiscovery.com 949-679-4068	Co-Presenter with Dr. Igancio Pino
25.	Dr. Stephen Fernandez, Ph.D., LTC, MSC Pharmaceutical Systems Project Management Office (PSPMO) USAMMDA	1430 Veterans Dr Ft. Derrick, MD 21702 301-619-7538 Stefan.Fernandez.mil@mail.mil	Department of Defense
26.	Dr. Richard G Jarman, PhD Deputy Director Viral Disease Branch Walter Reed Army Institute of Research LTC USA ARMY MEDCOM WRAIR	Richard.g.jarman.mil@mail.mil 301-319-9223 301-979-5838	Department of Defense
27.	Dr. Stephen Waterman, M.D. Director Dengue Branch Puerto Rico Centers for Disease Control and Prevention	Shw2@cdc.govCenters for Disease Control and Prevention Dengue Branch 1324 Calle Cañada San Juan, Puerto Rico 00920-3860800-CDC-INFO (800-232-4636) TTY: (888) 232-6348	CDC Dengue Branch Puerto Rico
28.	Dr. Raul Castellanos Brana, M.D. Coordinator Puerto Rico Pan American Health Organization World Health Organization	Department of Health Puerto Rico rrcastellano@salud.gov.pr 787-299-5733	WHO and PAHP Representative