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ELSEVIER
Journal of Clinical Virology 32 (2005) 342–346
JOURNAL OF CLINICAL VIROLOGY
www.elsevier.com/locate/jcv

Conference

Meeting report ☆
Part II. Notes from the Twentieth Annual Clinical Virology Symposium

Following directly after the Molecular Virology Workshop, and hosting many of the same active participants, the Twentieth Annual Clinical Virology Symposium (CVS, April 25–28, 2004) was attended by 727 clinical virologists and other interested individuals. Both meetings are organized by the Pan American Society for Clinical Virology (PASCV) and the University of South Florida College of Medicine. Like the workshop, the CVS sessions are characterized by the high quality and focus of the presentations, lively question and answer sessions, and (not least) the same pleasant meeting location in a modest older hotel on a beautiful Gulf of Mexico beach. CVS is distinguished from the workshop by the addition of posters and exhibits and by the panel discussion format of some of the presentations. A CD with the audio portion of all the lectures (unfortunately, no slides – maybe next year?) is available from IntelliQuest Media, Inc. (www.intelliquestmedia.com).

In the summaries that follow here and in the next issue, we offer a brief description of each of 15 sessions that ranged in topics from training virology students in the Rocky Mountain states of the US, to a measles eradication program directed from Geneva, Switzerland, to the elucidation of the SARS coronavirus in several laboratories around the world.

Sessions began on Sunday morning with Practical Issues in Clinical Virology. In Fundamentals of Serology, Diane S. Leland, Ph.D. (DL), Indiana University School of Medicine, described in some detail and with specific examples the three things that good serologists need to know, namely:

What is being measured?
How does the test work?
What do the results mean?

She also responded to several questions:

Q: Are you still doing complement fixation testing?

A: Alas, yes. It is difficult to get reagents, but histoplasma is prevalent in the Midwest and comp fix is still the best test.

Q: What about paired acute and convalescent samples?

A: Since direct detection of organisms has become possible, there is less demand for paired samples, but if requested the testing is still performed.

Q: Is high background in samples from very sick patients a problem with serology tests?

A: Yes, particularly with renal patients.

Q: Are you doing serology tests on oral fluid?

A: No, but the newly licensed anti-HIV test for oral fluid will probably be the first.

Q: Do you track your controls for serology tests?

A: Yes, but without information on the expected reproducibility from the test manufacturers, error detection is a problem.

Next in the session was Christine Ginocchio, Ph.D. from North Shore-Long Island Jewish Health System Laboratories, New York, who spoke on Understanding Tissue Culture. She described the benefits and drawbacks to tissue culture and its laboratory alternatives, rapid antigen testing, direct fluorescence assay (DFA), and nucleic acid tests for respiratory, rectal, CSF and blood samples, and recent improvements in tissue culture reagents like R-Mix (Diagnostic Hybrids) that allow faster, more sensitive and specific culture. R-Mix reduced the mean time to positivity for respiratory syncitial virus (RSV) by 9.6 days, and for other respiratory viruses by less dramatic but still significant amounts. With standardized methods using newer reagents, she stated that most labs can perform viral cultures, and she offered a set of options for optimal results with specific viruses and specimen types.

To end the morning, the two previous speakers and Gregory Storch, M.D., from Washington University, St. Louis, chaired an Interactive Session titled "How do you Handle Exceptions to Standard Procedures?" They began with a discussion of a new SARS outbreak due to laboratory mishandling of the virus in China, and discussed means of preventing similar occurrences, including training and drills on communications and procedures, improved connections between laboratories and public health facilities, and the feasibility of restricting the handling of the SARS virus

☆ Symposium, April 25–28, Clearwater Beach, Florida, USA.

1386-6532/$ – see front matter
doi:10.1016/j.jcv.2004.11.005

to experienced laboratories. Other exceptions discussed included action taken when a test is requested that is not officially performed on-site (suggestions included refusing to do the test, performing the test and reporting the result verbally, and getting informed consent from the patient, then doing the test and record the result in the usual way), and whether it is appropriate to culture if the DFA is positive (depends heavily on the virus; e.g., some culture is appropriate to stockpile strain information for influenza A).

The session on Selecting a System for Performing Real-Time PCR began with an introduction and overview by James Mahoney, Ph.D. (JM), McMaster University and St. Joseph's Hospital, Hamilton, Ont., Canada. He described real-time PCR as a revolution in diagnostic virology that allows us to rapidly identify organisms using a number of different instruments. He reviewed the currently available chemistries and instruments, discussed converting a conventional assay to a real-time format, and mentioned several potential pitfalls, such as failure to optimize $MgCl_2$ concentration, formation of primer dimers that interfere with amplification, and differences in amplification efficiency with different sample matrices.

Elizabeth A. Macias, Ph.D. (EM), from the Air Force Institute for Operational Health Epidemiological Surveillance Division in Texas, reported on performing real-time PCR with the ABI system. Although they also have systems from Perkin Elmer and Roche, they like the ABI 7900, because it is a high throughput system with a 96-well format, the statistical software is easy to use, it is flexible, allowing the use of molecular beacons, different dyes such as SYBRgreen, or Taq Man probes for detection, and it provided the highest sensitivity for their assays with the most reasonable costs per test.

Eric Frost, Ph.D., (EF) Centre Hospitalier Universitaire de Sherbrooke, Quebec, Canada spoke on real-time PCR using the Roche Light-Cycler, which holds 32 samples at a time in the sample carousel with the glass capillaries inserted. The glass capillaries allow a very good heat exchange and a quite small (20 μL) volume. The most expensive parts are the enzymes. EF illustrated the use of the Light-Cycler with SYBR-green on 400 samples of methicillin resistant *Staphylococcus aureus* (MRSA) tested in pools of three on four consecutive days and compared the results to classical bacteriology analyses. Each sample that tested positive was tested twice, thus providing unequivocal results. While classical bacteriology takes 4 days for full analysis, using the Light-Cycler allows you an extra "beach day", because it only takes 3 days. The Roche Light-Cycler advantages are the speed (3 times faster than the PE 9600), the small sample size, the integration with Magnapure purification method, and that the instrument can be purchased via reagent contracts.

Sue C. Kehl, Ph.D., (SK), Department of Pathology, Medical College of Wisconsin and Childrens' Hospital of Wisconsin reported on use of the Cepheid real-time PCR Smart Cycler System. Monthly molecular testing request volume in the Children's Hospital is about 200, or approx. 2500 tests/year. The Smart Cycler System contains 16 independent I-CORE modules with heating plates and fans, so that up to 16 different cycling protocols can be run at a time, allowing for rapid optimization of reagents and test parameters. The system has 4 optical channels, which allows multiplexing. It uses single-use disposable poylpropylene tubes, so problems of breakage are avoided, and it has sealable pressure caps, so amplicon contamination is less of an issue. The run time is about 45 min. The computer allows analysis of other data while the run is in progress. The baseline is monitored, and as in the Light-Cycler, the computer provides the cut-off automatically. The system uses an OmniMix Master Mix (master mix contains all of the necessary reagents for PCR, except primers and probes) with a hot start Taq polymerase, and reached sensitivities for *Bortedella pertussis* of 100 CFU/mL (or 100 copies/mL). Like the Light-Cycler, it allows the use of different detection systems. One disadvantage may be the lack of support for primer and probe design.

The discussion following these talks was extensive and focused less on the individual instrument systems and more on issues of designing test methods. Some of the questions:

Q: Should commercial master mixes be modified?

A: Opinion was divided, but it seemed clear that one does not fit all, and in fact multiple versions of master mix are required for different viruses.

Q: How do you go from traditional to real-time PCR?

A: Very carefully. Try your traditional parameters on your real-time system, but don't expect them to work; everything may need to be re-optimized.

Q: How do you achieve higher sensitivity with real-time PCR?

A: Sample size and primer dimers can present problems, and sensitivity is often lost with multiplexing. A major problem is an inability to trust negatives.

Q: Do you use internal controls?

A: Most commenters did not, but several felt that they should, as inhibition issues are far from being settled. Control of extraction is as important as control of amplification, and reagent quality, particularly for probes, is difficult to control; users must rely on the manufacturer's information. One commenter uses full-length oligonucleotides for controls, but warns against getting these from the same manufacturer that provides your primers and probes.

Q: Is a result of CT = 38 positive?

A: Normally, good positives have critical threshold results in the 20s. Most commenters re-extract the sample after a very high CT result, or ask for a new sample. Some report an indeterminate, but know that this is less useful to the clinician and so do not like to do it.

A second panel discussion followed, on the Role of the Virology Laboratory in Training. Craig Jestila, MBA/HCM, MT(ASCP) (CJ) from Sonora Quest Laboratories in Arizona described the current US laboratory personnel situation: although a 17% growth rate is expected over the next decade in laboratory staff positions, 40% of training programs have closed during the last decade, and more than 50% of current lab personnel are >49 years old. He described an open career

ladder that his laboratory had established based on six levels of increasing responsibility and accessible to employees regardless of previous training. CJ pointed out that employee satisfaction is key to quality productivity, and that a culture of learning with an emphasis on training and process improvement is important in retaining satisfied employees.

Christine Robinson, Ph.D. (CR) from Children's Hospital, Denver, continued the learning culture theme with six ideas, including daily rounds where bench technologists participate with clinical staff to the benefit of patient care, lab employee retention, and long-term learning relationships between the laboratory and clinicians. They also maintain a template for description of every test in both their laboratory manual and their computer so that callers will get consistent answers to questions about methods and results, and a weekly summary of viral detections e-mailed to about 1000 participants.

Mary Beth Baker, M.S., from Pathology Associates in Spokane described programs to reach high school students and spark their interest in laboratory careers, and to provide a mentor to each new employee to assist with training and act as a role model.

From the discussion:

- CDs as teaching tools are available in every laboratory specialty except virology labs, and virologists attending the meeting could possibly remedy this.
- An incentive system for rewarding quality and productivity in the laboratory should have clear metrics. Panelists described successful programs at one laboratory with seven to ten requirements that, if met over a 3-year period, would result in a 4% salary increase for employees, and at another laboratory that involved specific quality metrics evaluated three times a year.
- Because of terrorism concerns, the US government is scrutinizing immigrants more closely, and laboratory personnel have been caught up in this.
- TV programs like CSI are actually creating new interest in lab work among high school students, and potential employers should take advantage.
- Laboratories can motivate their most ambitious people by allowing them to make presentations at meetings organized for continuing education.
- Lab rounds are excellent educational tools, but at least occasional clinical rounds are very useful in connecting lab work with patient outcomes.

Steve Monroe, Ph.D. (SM), Division of Viral and Rickettsial Diseases, CDC, Atlanta, talked on Molecular Epidemiology of Noroviruses.

The family of caliciviridae contains four genera, the genus *Norovirus* with the type species Norwalk virus (alias Norwalk-Like Viruses, NLV and Small Round Structured Viruses, SRSV) and the genus *Sapovirus*, with the type species Sapporo virus (aliases: Sapporo-like viruses, SLV, Classic Human Caliciviruses) and additionally the genus *Vesivirus* and *Lagovirus*. SM talked about the transmission patterns and the molecular epidemiology of Noroviruses, which cause an estimated 23 million infections annually in the US, 9.3 million from food-borne exposures. CDC investigated 233 calicivirus outbreaks in 1997–2002, and found 217 attributable to Norovirus. Cruise ships are required to file a report whenever more than 2% of passengers or crew members come down with gastroenteritis, and more than 3% incidence is considered an outbreak. In 2002 there were nine confirmed Norovirus outbreaks on cruise ships. To get better estimates of Norovirus prevalence, CDC established FoodNet surveillance sites in three states for gastroenteritis. They found that 20% of patients older than 18 presenting with acute gastroenteritis (AGE) had Norovirus. They also obtained paired serum samples (acute and convalescent) and found that an additional 12% were positive by serology who were negative for Norovirus DNA. In the early 1990s, AGE outbreaks were almost 100% bacterial. In the mid-1990s CDC began nucleic acid testing and has found an exponential rise in viral outbreaks, with Norovirus the single largest cause, although data are still patchy, in part because there is no routine testing for Norovirus and no cell culture system available. For PCR, there are primers available for ORF 1 (in the most conserved domain, polymerase) and ORF 2 (see Genbank: M87661). Based on complete capsid sequences in ORF 2, there are 29 distinct clusters of Norovirus broken into 5 major genogroups differing by 44–55% and thus likely to be designated separate species. Examples of recent Noroviruses in food included oysters, raspberries, deli meats at a university cafeteria, pastries in an army mess hall, potato salad at a catered luncheon, frosting on cakes at a grocery store, and wedding cakes. Most probably the contamination occurs at the table or shortly before the food was consumed. The positivity rate by PCR in the outbreak investigations reported did not exceed 70%. It takes very little virus to make someone sick and since there is no cell culture, PCR is the most sensitive method available (apart from the "human bioassay"). The median incubation period was 33 h, until infected people presented with diarrhea and vomiting. The goal of the outbreak investigation program is to create an electronic database (CaliciNet) involving state, local and national labs performing routine RT-PCR and strain identification.

Points from the discussion:

(1) the genogroup II is the most common genogroup associated with outbreaks, although genogroup I strains may be catching up;
(2) viruses can easily be detected 10 days after the onset of symptoms and in some cases after 20 days or more;
(3) serum antibody levels are most likely not protective for more than 6 months, and even then not across genogroups.

David L. Heymann, M.D. (DH) from the World Health Organization (WHO), Geneva, Switzerland, was invited for the Edith Hsiung Lectureship, and he offered a talk that could well have been a movie script (as well as a cautionary tale) on SARS: Tracking an Epidemic as it Emerges. He began with an historical perspective on the continuous emergence and

re-emergence of infectious diseases (ID) and the responses of society, beginning with quarantine for plague in Venice in 1374 and continuing with international efforts at information collection and regulation under the auspices of the WHO since 1947. Information collection has evolved to a highly developed state, with a "network of networks" involving international agencies (UN, WHO), developed and developing countries (the latter supported by WHO in this effort), military laboratories, NGOs and media outlets. The global public health network (GPHIN) in Canada, for instance, searches the worldwide web in seven languages for information relevant to public health, resulting in 5–6 hits/day. Regulation paradigms have been slower to evolve. An outdated set of regulations is put forward in two manuals dating from 1969 that describe ship and airline sanitation measures and require countries to report outbreaks of cholera, plague and yellow fever. This is a passive system, resulting in late detection and delayed response, whereas globalization and vastly increased travel activity requires very early detection and real-time response. A new paradigm for decision making and response for public health events of international importance is being developed in Sweden to replace the old regulations, and the combination of this decision tree and the communications network already in place largely determined the series of events surrounding the SARS outbreak in 2002–2003. On November 16, 2002, the US military and others described a report of an outbreak of respiratory illness in Guangdong, China, in which the government was recommending isolation of anyone with symptoms. WHO requested a report from China, and received one on December 7th indicating normal influenza activity. The next information pickup occurred on February 11, 2003, reporting an outbreak of atypical pneumonia among healthcare workers in Guangdong Province. These reports triggered particular concern at WHO because the 1957 global pandemic of influenza A(H2N2) had emerged from the same region. DH described the subsequent events and the international response to the outbreak of SARS, and praised the 115 experts from 26 institutions in 17 countries who came together to send field teams to five countries, and shared all of their information for the public good, without regard to publication precedence. The availability of cell phone networks, videoconferencing and real-time electronic communications increased the power to control the epidemic. Other strategies that contained SARS outbreaks included case identification via active surveillance, case isolation/hospital infection control, contact tracing, surveillance/quarantine of contacts, international travel recommendations based on epidemiological evidence, collaboration between public health and police systems in Hong Kong, and an element of good fortune: the virus did not spread to countries with the weakest healthcare systems, and had a relatively low secondary attack rate. By June 25, 2003, the cumulative number of probable SARS cases worldwide was 8460 with 808 deaths (almost 10%!). China (5327 cases), Hong Kong (1755 cases) and Taiwan (636) had the highest tolls. The cost of the 6-month outbreak in Asia was estimated by the Asian Development Bank as ranging from 4% of GDP for Hong Kong to 0.5% for South Korea and Mainland China.

While national and international efforts were working to contain the epidemic, teams of scientists provided the information on which the containment efforts were based. The SARS Corona Virus (SARS-CoV) was isolated and sequenced, and its properties and the natural history of the infection were defined. Currently, international efforts continue for case identification, collaborative studies in Guangdong to identify animal reservoirs, research and development of diagnostic tests, vaccines and anti-virals, and continued global surveillance to identify the next major emergence of a new influenza strain or other infection of international importance.

Continuous global surveillance is required because organisms can emerge or re-emerge on any continent, in any country, at any time. Most often pathogens emerge from zoonotic sources, and their severity and transmissibility characteristics become unknown after they have breached the animal–human barrier. Thus, people who handle animals or animal products are at greatest risk to acquire a new infection, but once the pathogen has infected humans, healthcare workers are at risk to acquire and transmit the infection within the hospital but also throughout the community. Occasionally an emerging infection becomes endemic, such as influenza or HIV, and in other cases pathogens may jump species and then die out. WHO believes that influenza remains the largest global pandemic threat, and in that context DH also reported on Influenza A pandemics between 1918 and 2004. While the "Spanish flu" (H1N1) resulted in an estimated 40 million deaths worldwide (all ages affected, origin not clear), the "Asian flu" (H2N2) in 1957 and the "Hong Kong flu" (H3N2) in 1969 (4.5 million deaths in people older than 65 years and under 5 years) most probably emerged as a result of a reassortment of human virus (H1N1, the "Spanish flu") with an avian influenza virus (H2N2, from ducks) in the intermediate hosts, chicken and pigs. The resulting H2N2 finfluenza virus in 1957 carried five gene sgements of human origin and three of avian origin. In 1997 in Hong Kong influenza virus (H5N1) for the first time seemed to move directly from chickens to people rather than infecting people from pigs as the intermediate host. Eighteen people were hospitalized and six died. To prevent the spread of virus, 1.5 million chickens were slaughtered, and since this virus did not undergo human-to-human transmission, the outbreak was contained. In 2003 there were three avian flu outbreaks (February in Hong Kong: 3 infected with H5N1, 2 died; March in the Netherlands: 80 poultry workers infected with H7N7, 1 died; and November in Hong Kong: 1 child infected with H9N2 but recovered). [Authors: By September 2004 there were 42 people infected with H5N1 in East Asia and 30 died (PROMED digest, 49).] An estimated 30 million people are infected with influenza each year, with an estimated death toll of 250–500,000. The known problems of antigenic drift and antigenic shift of influenza viruses in combination with a lack of sufficient vaccine (annual worldwide vaccine production: 250 million doses),

the long production time for vaccines (6–8 months), limited antiviral drug production, and the emergence of resistant influenza strains constitute a major world health challenge.

German F. Leparc, M.D. (GL), from Florida Blood Services and University of South Florida College of Medicine stood in for Michael Busch, M.D., Ph.D. (MB), who could not attend, to take up the Gen-Probe Lectureship. Using MB's slides, GL spoke on Viruses: Implications for Transfusion Medicine. He reviewed the incidence of the major pathogens HIV, HCV and HBV in whole blood donors and the improvements in safety of the blood supply since 1983, when 1% of units collected in San Francisco were HIV-infected. Currently, prior to testing the incidence of HIV in whole blood donors in South Florida is 1/15,000 or 0.0067%. One in five donors is rejected prior to donation on the basis of the questionnaire and other pre-donation screening. Window-period donations (those collected after infection but prior to detection by current screening methods) still cause transmission of HIV, HCV and HBV at a very low rate (1/77,000–149,000 for HBV; 1/1.2–1.7 million for HCV; 1.1.9–2.4 million for HIV) despite testing of pooled sample of all donated units for HIV and HCV RNA. Pool testing for HIV and HCV RNA (nucleic acid testing or NAT) is performed despite a lack of cost-effectiveness. The cost per quality-adjusted-life-year (QALY) for doing HIV/HCV NAT is $4.3M/QALY: economists and policy makers agree that the decision point for QALYs should be $50-100,000/QALY, above which it is not cost-effective to institute the procedure.

Other potential threats to the safety of the blood supply include West Nile Virus (WNV), for which donated blood is being screened by NAT on pools, Hepatitis A, Parvovirus B19, which presents a particular risk to sickle cell anemia patients, who are often transfused, and *T. cruzi*, the etiologic agent of Chagas disease. Because of the inability to eliminate transmission by transfusion of known pathogens, and the potential threat from unknown pathogens, improvements in blood safety are being evaluated for various "pathogen reduction" methods involving chemical treatment of blood components. These measures involve new risks (damage to transfusion product, toxicity to recipient, processing personnel, or the environment, high cost, complexity) as well as potential benefits (reduction of known viruses, bacteria, parasites, and potential reduction of unknown pathogens and need for testing).

Russell Regnery, Ph.D. (RR), Centers for Disease Control and Prevention, Atlanta, talked about monkeypox and human disease. Monkeypox (belonging to the group of orthopoxviruses together with smallpox, cowpox and vaccinia) was first reported in humans in 1970, and is endemic in central and Western African monkeys. Between 1981 and 1986 there was an active surveillance in Zaire and 338 human cases were found. Monkeypox is a zoonosis and no human-to-human transmission has been reported. During June 2003 monkeypox was recognized in North America among mid-western owners of captive prairie dogs. Seventy-two human cases were recognized; all were prairie dog-associated, none were fatal, although the mortality rate in the Zaire outbreaks was 10–15%. Three African (Ghana) rodent species imported to the US were the source of the virus. Co-housing of two of the three species with prairie dogs, which were later recognized to be infected, were the cause of transmission. Thus, prairie dogs may be a novel transitional host for monkeypox, and are obviously a vector for human infection. The genomes of the mokeypox viruses isolated from infected humans and prairie dogs were identical, but also were identical to those endemic in West Africa in contrast to a monkeypox clade in the Congo basin, which is associated with a more severe human disease. The monkeypox viruses were first identified through electron microscopy and subsequently through RT-PCR using standard multiplex monkeypox-specific primers. IgM antibodies could be shown 56 days past the rash onset. PCR from various tissues (blood, brain, feces, liver, lung) of a Gambian rat provided high virus titers (10^5–10^{10} copies/mL), although highest titers were observed in the prairie dogs (eyelids, 10^{10} copies/mL). Live animal import into the US (until 2002) included 28,000 mammals including 28 rodent species, 365,000 birds, 2 million reptiles, 49 million amphibians and 226 million fish. The fact that the wildlife reservoir is not well understood raises concerns for American human and non-human native species, that monkeypox may become an established pathogen. Vaccination for smallpox is estimated to provide >85% protection against monkeypox virus.

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5 November 2004