join us for our

11th annual research colloquium

presenters include

Roberta Mellis
Jonathan Schumacher
Tracey Lewis
Jacob Durtschi
David Crockett
Troy Jaskowski
Leslie Rowe
Buck Lozier
Philippe Szankasi
Aiping Liu
Melanie Mallory
Nana Sono-Koree
Roy Bastien
Mark Ebbert
Shale Dames
Cecily Vaughn
Michelle Wallander
Ha Pham
Julie Ray
Sue Slechta

ARUP INSTITUTE FOR CLINICAL AND EXPERIMENTAL PATHOLOGY®

may 25, 2011 • ARUP matsen conference center • 9:00 am - 4:00 pm

MAY 25, 2011

COLLOQUIUM **AGENDA**

Presentations
ARUP Matsen Conference Center

The order of presentations was determined by a random drawing.

9:00 AM ....Welcome by Harry R. Hill, MD

9:10 AM .... Roberta Melis
9:25 AM .... Jonathan Schumacher
9:40 AM ....Tracey Lewis
9:55 AM .... Jacob Durtschi
10:10 AM ....David Crockett

10:25 AM .... BREAK

10:50 AM .... Troy Jaskowski
11:05 AM .... Leslie Rowe
11:20 AM .... Buck Lozier
11:35 AM ....Philippe Szankasi
11:50 AM ....Aiping Liu

12:05 PM... LUNCH

1:05 PM.. Melanie Mallory
1:20 PM... Nana Sono-Koree
1:35 PM..Roy Bastien
1:50 PM...Mark Ebbert
2:05 PM.. Shale Dames

2:20 PM.. BREAK

2:45 PM..Cecily Vaughn
3:00 PM...Michelle Wallander
3:15 PM... Ha Pham
3:30 PM... Julie Ray
3:45 PM... Sue Slechta

4:20 PM.................. Group Photo at Waterfall
(Judges, R&D Personnel, Medical Directors)

Reception and Awards Ceremony
Marriott University Park Hotel
5:00 p.m. | Social Hour (drinks and hors d'oeuvres)
5:30 p.m. | Awards Presentation

Table of Contents

Personalizing hepatitis C therapy with pharmacogenectics (Melis) 3

A pyrosequencing-based test for quantitative monitoring of the *BCR-ABL* T315I mutation (Schumacher).......... 4

Exon-focused CGH array for x-linked mental retardation (Lewis) 5

Variant identification in multi-sample pools by illumina genome analyzer sequencing (Durtschi) 6

Consensus: A novel predictor model for uncertain gene variant pathogenicity (Crockett) 7

IgA anti-actin antibody in age-specific cohorts with gluten-sensitive enteropathy (Jaskowski) 8

Evaluation of a single platform for copy number, loss of heterozygosity and amplification detection in formalin-fixed paraffin-embedded melanocytic lesions (Rowe) 9

Flow cytometric assessment of acetylcholine receptor modulating antibodies (Lozier) 10

Genetic and epigenetic mechanisms for inhibition of *CEBPA* in acute myeloid leukemia (Szankasi) 11

Evaluation of selected short chain acylcarnitines in blood spots, plasma and urine by UPLC-MS/MS for the differential diagnosis of branched-chain organic acidurias (Liu) 12

Development of a DNA sequencing assay for detecting drug resistance-associated mutations in human cytomegalovirus (Mallory) 13

Platelet washing by BSA-sepharose gel filtration is a practical method to isolate platelets for the serotonin release assay (Sono-Koree) 14

Using the PAM50 breast cancer intrinsic classifier to assess risk in ER+ breast cancers: a direct comparison to Oncotype Dx® (Bastien).......... 15

Characterizing error and uncertainty of centroid-based genomic predictors (Ebbert) 16

Introduction of the first next generation sequencing clinical assays at ARUP: the mitochondria genome and mitochondria 128 gene panel (Dames) 17

Avoidance of pseudogene interference in the detection of 3' deletions in *PMS2* (Vaughn) 18

Comparison of IHC, FISH and RT-PCR for the detection of *EML4-ALK* translocation variants in non-small cell lung cancer (Wallander) 19

Newborn screening for spinal muscular atrophy (Pham) 20

Analysis of cortisol, cortisone and dexamethasone in human serum by LC-MS/MS (Ray) 21

Rapid differentiation of *Cryptococcus gattii* from *Cryptococcus neoformans* (Slechta) 22

Personalizing hepatitis C therapy with pharmacogenectics

Melis R, Fauron C, McMillin G, Lyon E, Shirts B, Hubley L, Slev P

OBJECTIVES

1) Describe two genetic polymorphisms near the interleukin IL28B gene that are associated with spontaneous viral clearance and sustained virologic response (SVR) response to interferon-α and ribavirin therapy in hepatitis C

2) Understand how frequencies of these alleles in several ethnic groups worldwide may explain differing rates of SVR among them

3) Understand how pharmacogenetics could help guide the care of patients with hepatitis C virus infection

Introduction: Recent genome-wide association studies have identified two host single nucleotide polymorphisms (SNPs) near the *IL28B* gene, *rs12979860* C/T and *rs8099917* T/G, that are associated with sustained virologic response in patients infected with hepatitis C virus (HCV). We developed a rapid multiplexed dual-color Fluorescence Resonance Energy Transfer (FRET) probe assay that accurately genotypes for both SNPs simultaneously, as confirmed by single nucleotide extension assay (also developed in house) and sequencing. The FRET assay was utilized to determine allele and genotype frequencies for these two SNPs in different ethnic populations.

Methods: The FRET method on a Lightcycler® was used to genotype 443 samples: 134 DNA samples from the Caucasian (CAU), Japanese, Chinese, South East Asian (AS), Middle Eastern (ME) and African American (AA) ethnicity panels (Coriell Repositories, Coriell Institute for Medical Research, NJ, USA) and 309 clinical samples with self-reported ethnicity from ARUP Laboratories for a total of 125 CAU, 71 AS, 10 ME, 118 Hispanic (HIS), and 119 AA samples.

Results: Six different compound genotypes (*rs12979860* / *rs8099917*) were detected. The rs12989760 CC and *rs8099917* TT favorable genotypes, associated with efficacy in HCV clearance and improved treatment outcome were detected at the following frequencies: CC/TT (39.2% in CAU, 78.9% in AS, 40.0% in ME, 33.9% in HIS, and 16.8% in AA).The presence of either the risk alleles homozygous state *rs12979860* TT and *rs8099917* GG or heterozygous state *rs12979860* CT and *rs8099917* TG , *rs12979860* TT and *rs8099917* TG predicts risk of chronicity and treatment failure were detected at the following frequencies: TT/GG (5.65% in CAU, none in AS, none in ME, 10.2% in HIS, 1.7% in AA), CT/TG (24.0% in CAU, 19.7% in AS, 20% in ME, 39.8% in HIS, 3.4% in AA) TT/TG (8.0% in CAU, 1.4% in AS, none in ME, 3.4% in HIS, 5.9 % in AA), respectively. The presence of the indeterminate compound genotypes with the favorable allele for one SNP and risk allele for the other SNP were also detected in all ethnic groups at the following frequencies: CT/TT (20.8% in CAU, none in AS, 40% in ME, 9.3% in HIS, 37.0% in AA), TT/TT (2.4% in CAU, none in AS, none in ME, 3.4% in HIS, 35.3% in AA) respectively.

Conclusion: As previously reported the correlation between the two single-nucleotide polymorphisms (SNPs) varies with ethnic background, we observed the greatest correlation between the two SNPs in Asians and the lowest in African Americans. Because the causal SNP is unknown, the clinical significance of having a favorable genotype for one but not both SNPs remains unclear and therefore genotyping for both SNPs may be useful.

A pyrosequencing-based test for quantitative monitoring of the *BCR-ABL* T315I mutation.

Schumacher J, Szankasi P, Ho A, Kelley T

OBJECTIVES

1) Describe the clinical significance of the T315I point mutation

2) Describe why a *BCR-ABL* enrichment step is performed prior to *ABL* exon amplification

3) Describe limitations and advantages of pyrosequencing for the T315I point mutation

Introduction: CML patients and those with Ph+ ALL who are treated with tyrosine kinase inhibitors (TKIs) may develop therapeutic resistance from mutations at important drug-binding sites in the kinase domain of *BCR-ABL* fusions. The T315I mutation, known as the "gate keeper", imparts resistance to all currently approved TKIs (imatinib, dasatinib and nilotinib). Patients who develop a T315I mutation often experience disease progression. Currently, there are drugs in development that, in early clinical studies, demonstrate activity in T315I positive patients. Thus, a rapid, sensitive and specific test for identifying and quantifying the T315I mutation would be helpful for on-going patient monitoring and for choosing the appropriate therapeutic option. Pyrosequencing has emerged as a useful technique to detect and quantitate point mutations in many clinically important genes (ie. *KRAS, BRAF*, and *Jak2*) and provides the advantages of being sensitive and inexpensive with high throughput. Here, we describe the development and validation of a pyrosequencing-based assay designed to detect and quantitate the *BCR-ABL* T315I point mutation in CML and Ph+ ALL.

Materials and Methods: 32 samples (10 T315I+, 1 T315F+, 1 F311L+, 20 WT) were subjected to one round of PCR amplification across the *BCR-ABL* breakpoint followed by a second round of PCR using primers, including one with a biotin label, flanking codon 315. Amplicons were subjected to pyrosequencing of codons 315-316. Quantification was expressed as % mutant.

Results: The T315I mutation was detected in 10/10 known T315I+ samples and quantified. The assay also detected a T315F mutation and yielded evaluable codon 315 sequence in a case with a F311L mutation which overlapped with the forward primer binding site. RNA from one T315I-positive case with 100% mutant allele was serially diluted into RNA from a *BCR-ABL* positive donor without the T315I mutation and reverse transcribed. Analysis indicated a sensitivity of approximately 5%.

Conclusion: We describe a quantitative, sensitive, and inexpensive assay for monitoring of the pan-resistant *BCR-ABL* T315I mutation.

Exon-focused CGH array for x-linked mental retardation

Bayrak-Toydemir P, Priest M, Lewis T

OBJECTIVES

1) Understand the clinical significance of X-linked mental retardation

2) Describe the development of a exonic level array for the detection of X-linked disorders

3) Discuss the challenges of variant calling and data analysis

Background: Mental retardation (MR) is estimated to effect 1-3% of the population. Given the excess o fmales observed in large cohorts of patients, defects of genes on the X chromosome are believed to account for greater than 25% of moderate and severe forms and almost 50% of mild forms of mental retardation. There are more than 90 genes on the X chromosome which have been related to MR. However, in other genes copy number changes of variable sizes ranging from single exon of a gene to deletions/duplications of many genes might also cause X-linked MR (XLMR).

Methods: To explore the role of X-chromosome gene deletions and duplications XLMR, a custom 720K NimbleGen CGH array was designed. 1737 transcripts were identified in the USCS Genome Browser and compiled into 954 composite transcripts to account for genes with multiple transcripts. The array contains probes spanning the length of the chromosome, covering the 7722 exons of the 954 transcripts with tiled 60mer probes at 15 bp spacing. Exons represent 2.6Mb, or just 1.7%, of the X chromosome yet 24% of the probes are concentrated in these regions. Probes are placed at a mean interval of 140 bp in introns and 1kb 5' and 3' of each transcript, accounting for 49% of the probes. Probe density in intergenic regions is at a mean spacing of 525 bp. Genomic DNA of nine individuals with known variations were hybridized against sex-matched controls and results compared against the known variations.

Results: Nine patient samples harboring deletions or duplications ranging in size from 1.6 Mb to 1186 bp were analyzed. Three samples with contiguous multiple gene deletions were identified using a 10X averaging window in the SignalMap2.5 software. Deletions and duplications in the four exon and two single gene samples were identified using a 1X-averaging window. Results were concordant with previous studies.

Conclusions: A high-density custom X-chromosome array has been designed for detecting copy number changes ranging from large genomic regions to variations within a single gene. Currently 215 (22%) of the genes on the X chromosome are known to be involved in XLMR, however roughly 40% of the protein coding genes on the X chromosome are expressed in the brain; in principal XLMR could result from copy number changes in any of these genes. Our custom designed exon-focused X chromosome array includes coverage of all known XLMR genes and potential XLMR genes with high sensitivity and specificity.

Variant identification in multi-sample pools by illumina genome analyzer sequencing

Durtschi J*, Margraf R*, Dames S, Pattison D, Stephens J, Voelkerding K

OBJECTIVES

1) Understand the motivation for pooled sample next generation sequencing studies at ARUP and how these methods can be applied to other mixed population analysis

2) Learn the characteristics of Illumina next generation sequencing data and their impact on low frequency allele detection

3 Learn the methods developed in this study to improve low frequency allele detection

Background: Multi-sample pooling and Illumina Genome Analyzer (GA) sequencing allows high throughput sequencing of multiple samples to determine population sequence variation. A preliminary experiment, using the RET proto-oncogene as a model, predicted ≤30 samples could be pooled to reliably detect singleton variants without needing additional confirmation testing.

Methods: This report used 30 and 50 sample pools to test the predicted pooling limit and also to test recent protocol improvements, Illumina GAIIx upgrades, and longer read chemistry. The SequalPrep™ method was used to normalize amplicons before pooling. For comparison, a single 'control' sample was run in a different flow cell lane. Data was evaluated by variant read percentages and the subtractive correction method which utilizes the control sample.

Results: In total, 59 variants were detected within the pooled samples, which included all 22 Sanger identified variants. The 15 known singleton variants due to Sanger sequencing had an average of 1.62±0.26% variant reads for the 30 pool (expected 1.67% for a singleton variant [unique variant within the pool]) and 1.01±0.19% variant reads for the 50 pool (expected 1%). The 76 base read lengths had higher error rates than shorter read lengths (33 and 50 base reads), which eliminated the distinction of true singleton variants from background error.

Conclusions: This report demonstrated pooling limits from 30 up to 50 (depending on error rates and coverage), for reliable singleton variant detection. The presented pooling protocols and analysis methods can be used for variant discovery in other genes and can facilitate molecular diagnostic test design and interpretation, especially when analyzing for variants in mixed cell and molecular populations.

* Equal authorship contribution

Consensus: A novel predictor model for uncertain gene variant pathogenicity

Crockett D, Ridge P, Wilson A, Lyon E, William M, Narus S, Faceli J, Mitchell J

OBJECTIVES

1) Understand importance of genomic data in electronic medical records

2) Know how to leverage existing phenotype data for uncertain gene variant classification

3) List advantages of gene-specific prediction and reference range framework

As electronic medical records incorporate genetic sequence information, gene variant classification is critical to inform clinicians on the most appropriate course of treatment. Accurate interpretation of gene testing is a key component in customizing this patient therapy. While medical geneticists rely on patient history and family segregation, literature review and trusted colleagues to stay informed of the phenotype consequences of a given gene variant, well established computer prediction is also employed. However, there remains a lack of a widely accepted standard computational predictor of mutation severity for novel or uncertain gene variants in clinical use. Gene-specific models from clinically curated gene-disease data sets have been shown to improve prediction algorithms. Here we report Consensus, an implementation of one such clinical predictor along with a weighted sum of other complementary prediction algorithms. Visualization models of this predictor are also presented for augmenting diagnostic decisions. Reliable phenotype classification with a qualitative metric for evaluation of novel or uncertain gene variants may augment current clinical information and assist in improving prediction algorithms as gene variant knowledge increases.

IgA anti-actin antibody in age-specific cohorts with gluten-sensitive enteropathy

Jaskowski T, Hull C, Hamblin T, Book L, Zone J, Wilson A, Tebo A, Hill H

OBJECTIVES

1) Describe the two forms of gluten-sensitive enteropathy (GSE) and assays utilized for diagnosis

2) Illustrate the prevalence of IgA anti-Actin in age-specific cohorts with GSE, and in adult and pediatric healthy controls

3) Review the Marsh scoring system for duodenal biopsy, and the relationship between villous atrophy and IgA anti-Actin in pediatric celiac disease

Objective: To assess the clinical utility of IgA anti-actin antibody (AAA) in serologic and/or biopsy-positive pediatric patients with celiac disease (CD) and to determine the prevalence of AAA in adult patients with dermatitis herpetiformis (DH).

Methods: De-identified sera from 54 pediatric patients with CD (duodenal biopsies graded using modified Marsh criteria), 78 pediatric normal controls, 117 adult patients with DH (duodenal biopsy not performed) and 50 adult healthy controls were included in the study. All sera were assessed for AAA using a commercial enzyme-linked immunosorbent assay (ELISA) from INOVA Diagnostics, San Diego, CA. These studies were approved by the University of Utah Institutional Review Board and met the Health Information Portability and Accountability Act patient confidentiality guidelines.

Results: AAA was detected in 21 of 49 (42.9%) sera from pediatric CD patients with positive Marsh scores (IIIa-c), and in only 1 of 5 (20.0%) sera from patients with negative Marsh scores (0-1); all 78 pediatric normal controls were negative for AAA (PPV = 100.0%). In addition, all pediatric CD patients with positive AAA results were also antibody-positive for one or more well-defined CD serologic markers, namely tissue transglutaminase (tTG) and deamidated gliadin peptide (DGP). In the adult DH group, 17 of 117 (14.5%) patients were AAA positive with a specificity of 94.0% in adult normal controls.

Conclusions: Although less sensitive, our data on AAA and their correlation with Marsh scores IIIa-c in the pediatric CD group further supports a role of this simple non-invasive tool as a reliable marker of severe intestinal mucosa damage. With respect to DH, more studies with biopsy-proven cases are needed to determine the actual prevalence and correlation of AAA with intestinal damage in this type of GSE.

Evaluation of a single platform for copy number, loss of heterozygosity and amplification detection in formalin-fixed paraffin-embedded melanocytic lesions

Rowe L, Chandler W, Jahromi M, Schiffman J, South S

OBJECTIVES

1) Gain an appreciation of the challenges associated with differentiating benign from malignant melanocytic lesions using standard diagnostic tests

2) Recognize the advantages of using molecular inversion probe (MIP) technology for characterizing genomic alterations in formalin-fixed, parafin embedded (FFPE) melanocytic lesions

3) Be familiar with the genomic changes commonly associated with benign versus malignant melanocytic lesions

An estimated 68,130 new cases of invasive melanoma were diagnosed in the United States in 2010, with nearly 8,700 resulting in death. Typically, melanoma can be definitively diagnosed by a combination of clinical observations and microscopic examination. However, a significant number of melanocytic lesions cannot confidently be called benign or malignant using standard diagnostic techniques. For these cases, a sensitive and robust molecular method that provides multiple data points predictive of tumorigenicity would be useful. Previous molecular studies have demonstrated that characteristic copy number alterations occur in melanoma. We have used molecular inversion probe (MIP) technology (OncoScan™, Affymetrix) to identify and characterize genomic alterations in formalin-fixed, paraffin-embedded (FFPE) melanocytic lesions. Sixty-four melanocytic lesions (23 benign nevi, 11 melanocytic lesions of uncertain malignant potential (MLUMP), 27 primary melanoma, 3 metastatic melanoma) were identified for this study. Genomic DNA was isolated from tissue scrolls and processed onto a 330,000 feature MIP microarray. Resulting data was evaluated using Nexus 5.1 Copy Number software (BioDiscovery, Inc). None of the benign nevi samples demonstrated copy number alterations commonly associated with melanoma. Copy number alterations characteristic of melanoma were identified in 24/27 primary (89%) melanoma samples, including gain of 1q, 6p, 7p, 7q and 11q and loss of 6q, 9p, 9q, 10p, 10q, 11q, and 17p. Copy number changes were also identified in 4/11 (36%) MLUMP samples including loss of 1p, 2p, 6q, 7p and chromosomes 3, 9 and 17. Loss of heterozygosity (LOH) was identified in both MLUMP and melanoma samples. In addition, amplification of *CCND1*, *CKS1B* and *MYC* genes was identified in a subset of the melanoma samples. In summary, we have employed a novel technology, using the OncoScan™ array, to generate information on copy number changes, LOH and amplification in melanocytic lesions using within a single molecular platform. This approach will be useful to help classify future melanocytic lesions with unknown malignant potential.

Flow cytometric assessment of acetylcholine receptor modulating antibodies

Lozier B, Haven T, Astill M, Hill H

OBJECTIVES

1) Understand the clinical significance of acetylcholine receptor antibodies in diagnosis of myasthenia gravis

2) Describe the development of a flow cytometric assay for the detection of acetylcholine receptor modulating antibodies

3) Compare the flow cytometric assay with current radioassays

Background: Myasthenia gravis is an autoimmune disorder characterized by antibodies (Abs) against nicotinic acetylcholine receptors (AChRs) at the neuromuscular junction (NMJ). Symptoms include fluctuating and progressive muscle weakness due to AChR dysfunction. Diagnosis can be complicated not only by the variable nature of symptoms but also by the heterogeneity of AChR antibodies. Current clinical tests for AChR Abs include AChR binding, AChR blocking, and AChR modulating Ab radioassays. Acetylcholine receptor modulation results from the loss of AChRs due to Ab cross-linking of receptors and subsequent endocytosis and degradation. Acetylcholine receptor modulation results correlate best with disease severity.

Objective: Assess the clinical utility and performance of an in-house developed flow cytometric assay for the detection of AChR modulating antibodies.

Methods: Serum from 100 self-proclaimed healthy donors and 120 clinical specimens submitted to ARUP were tested for AChR binding, blocking, and modulating Abs by current methods and tested for AChR modulating Abs via a new flow cytometric assay. The new assay utilizes the human rhabdomyosarcoma TE671/RD cell line currently used for clinical testing. Cells are incubated overnight in the presence of control or patient serum to allow Ab binding and occurrence of the modulation effect. The next day cells are dissociated and receptors are first labeled with a monoclonal rat anti-human AChR Ab, and then a goat anti-rat IgG Alexa-488® conjugated secondary antibody. Cells are then analyzed for receptor expression on a BD FACSCanto® cytometer using Diva® software. Reference intervals for the new assay were determined using ROC analysis and optimized for sensitivity and specificity. Results were then compared to those of the current clinical AChR modulating and binding Ab assays.

Results: The positive cutoff for the new assay was determined to be 61% modulation or greater. Modulation results for self-proclaimed healthy donors were all 55% or less, except one individual (62% modulation) that had been previously diagnosed with myasthenia gravis and whose symptoms were in remission. Compared to the current AChR modulating Ab assay, the new assay demonstrates 84% agreement, 91% sensitivity and 81% specificity. Compared to the AChR binding Ab assay, the new assay demonstrates 76% agreement, 66% sensitivity and 93% specificity. The new assay also shows better agreement, sensitivity and specificity compared to the AChR binding Ab assay than the current AChR modulating Ab assay. Of 33 discrepant samples, 27 were negative by the current assay and positive by the new assay with one or more other corroborating positive AChR Ab results.

Conclusions: The new flow cytometric assay demonstrates better performance than the current radiolabeled assay while increasing sample throughput, eliminating the source of radioactivity, decreasing repetitive stress on testing personnel, and decreasing test cost by approximately one half. Furthermore, the levels of modulation observed in the new assay significantly exceed those in the radiolabeled assay with a greater dynamic range while showing greater correlation with AChR binding Ab results. Thus the assay offers a significant technical improvement over current methods, costs less to perform and likely is a better indicator of the presence and prognosis of this severe neuromuscular disease.

Genetic and epigenetic mechanisms for inhibition of *CEBPA* in acute myeloid leukemia

Szankasi P, Bahler D, Ho A, Kelley T

OBJECTIVES

1) Understand the value of detecting gene mutations in the prognosis of AML with normal karyotype

2) Distinguish the effect of different *CEBPA* mutations on AML prognosis

3) Understand the multiple mechanism of *CEBPA* inactivation observed in AML

Acute myeloid leukemia is a heterogeneous disease and prognostic subgroups can be established based on recurrent cytogenetic abnormalities. A large group of AMLs with intermediate prognosis and lacking recurrent cytogenetic abnormalities can be further stratified by determining the mutation status of several genes, including *FLT3*, *NPM1*, *WT1*, *DNMT3A*, and *CEBPA*. Bi-allelic *CEBPA* mutations are associated with improved prognosis in cases of cytogenetically normal (CN) AML. In a subset of AML patients lacking *CEBPA* mutations, core promotor hypermethylation of *CEBPA* has also been observed. We have developed tests for both *CEBPA* mutations by Sanger sequencing and core promoter hypermethylation by quantitative pyrosequencing. Our studies confirm that affected cases with either *CEBPA* double mutations or core promoter hypermethylation show similar phenotypes, such as association with CD7 expression and a lack of concurrent NPM1 mutations. In addition, we show that the observed core promoter hypermethylation results in strongly decreased levels of *CEBPA* mRNA levels. Expression analysis by quantitative PCR indicates that mechanisms other than core promoter methylation may also affect *CEBPA* mRNA levels. We are currently exploring the role of specific miRNAs on *CEBPA* mRNA and protein levels in AML. Together, these analyses may contribute to the development of better prognostic tools for CN-AML.

Evaluation of selected short chain acylcarnitines in blood spots, plasma and urine by UPLC-MS/MS for the differential diagnosis of branched-chain organic acidurias

Liu A, Johnson D, Guymon R, Alston M, Longo N, Pasquali M

OBJECTIVES

1) Overview of branched-chain organic acidurias

2) Overview of screening methods for branched-chain organic acidurias

3) Discuss chromatographic separation of short-chain acylcarnitines to improve specificity of metabolic disorder screening

Branched-chain organic acidurias are a group of disorders that result from an abnormality of specific enzymes involving the catabolism of branched-chain amino acids (leucine, isoleucine, and valine). Common branched-chain organic acidurias include isovaleric acidemia, propionic aciduria, methylmalonic aciduria, 3-methylcrotonylglycinuria, and beta-ketothiolase deficiency. These disorders are biochemically diagnosed by identifying specific acylcarnitines in blood spots and in plasma, by tandem mass spectrometry (MS/MS) and organic acids in urine by gas chromatography-mass spectrometry. MS/MS analysis of acylcarnitines is not sufficient to exclude or confirm the diagnosis of a branched-chain organic aciduria, since multiple acylcarnitine species are isomeric or isobaric with other compounds, including antibiotics and medications used frequently in nurseries. For example elevated C5-carnitine can be observed in isovaleric acidemia, 2-methylbutyrylglycinuria, or in patients receiving certain antibiotics. We have developed a method for the fractionation of short-chain acylcarnitines (C4, C5, C5OH) to assist in the differential diagnosis of branched-chain organic acidurias. The assay is performed on Acquity UPLC coupled with Premier tandem mass spectrometer operated in SRM mode.

We have evaluated samples (blood spots, urine, plasma) from patients with several branched-chain organic acidurias and compared them to normal controls to validate this method. The method was fast (15 minutes injection-to-injection run time) and was effective in resolving different metabolites resulting in elevated C4-, C5-, and C5-OH-carnitines. This assay can be used as a more specific second tier test in differentiating branched-chain organic acidurias and exposure to selected antibiotics in newborn screening.

Development of a DNA sequencing assay for detecting drug resistance-associated mutations in human cytomegalovirus

Mallory M, Hillyard D

OBJECTIVES

1) Describe the clinical relevance of antiviral resistance detection in the management of CMV infection

2) Describe the importance of carry-over contamination prevention as it relates to patient care

3) Discuss challenges related to assaying for CMV antiviral resistance and the advantages of assaying by Sanger sequencing

Background: Human cytomegalovirus (CVM), also known as human herpes virus 5 (HHV-5), is a dsDNA virus that infects up to 80% of the U.S. population. Although most infections are asymptomatic, CMV can cause disease in immunocompromised individuals and in children infected congenitally. Various point mutations and deletions in the CMV UL97 kinase and the CMV UL54 polymerase genes have been documented as conferring resistance to the antiviral drugs ganciclovir, cidofovir, and foscarnet used to treat CMV infections. Detection of relevant mutations requires interrogation of a region spanning approximately 600bp for UL97 and 2,000bp for UL54. Generating sufficiently long amplicons to cover these regions is challenging particularly if dUTP-UNG chemistry, which can negatively impact amplification efficiency, is utilized to prevent carry-over contamination. The aim of this study was to design and validate a DNA sequencing assay that sensitively detects drug resistance in CMV-infected patients and utilizes UNG chemistry for contamination control.

Methods: Viral DNA was extracted from 1mL of EDTA plasma from patient specimens using the QIAsymphony SP platform. A single amplicon spanning codons 457-630 of the UL97 gene and four overlapping amplicons spanning codons 393-1000 of the UL54 gene were generated using a PCR master mix with a 1:1 dTTP:dUTP ratio. The use of a dUTP/dTTP mixture improves amplification efficiency while maintaining UNG activity. Amplicons were cycle sequenced in both directions using ABI BigDye Terminator v1.1. Sequences were assembled and analyzed using ABI SeqScape v2.6. The fully optimized assay was validated using 30 patient plasma samples positive for CMV DNA. Of these, 14 samples were also interrogated using another sequencing-based assay to confirm the results of the ARUP assay.

Results: Of the 30 validation samples, 22 were successfully assayed. Six samples that failed had titers less than 700 copies/mL. Of the 14 samples also assayed using another sequencing-based method, results for 10 were concordant. The four discrepancies included one sample for which the mutation pattern detected differed slightly between the two methods and three low titer samples that were successfully assayed using the ARUP method but failed by the comparative method. In the validation study, the lowest titer of any sample for which both UL54 and UL97 were successfully assayed was 700 copies/mL, although UL97 only was assayed successfully for samples with titers as low as 250 copies/mL. However, in two rare instances samples with slightly higher titers were unsuccessful.

Conclusions: The ARUP CMV drug resistance assay can be used to sensitively interrogate the UL97 and UL54 regions of the CMV genome. Optimized PCR conditions allow for testing of low titer samples while utilizing UNG to prevent carry-over contamination.

Platelet washing by BSA-sepharose gel filtration is a practical method to isolate platelets for the serotonin release assay

Sono-Koree N, Crist R, Rodgers G, Smock K

OBJECTIVES

1) Describe size exclusion gel filtration

2) Discuss the development of the BSA-Sepharose 2B gel column

3) Describe the validation of the BSA-Sepharose 2B gel for obtaining washed platelets for the serotonin release assay (SRA)

Introduction: The serotonin release assay (SRA) for heparin-induced thrombocytopenia (HIT) requires viable washed platelets from healthy donors. However, traditional platelet washing methods may activate platelets. We aimed to determine whether gel filtration using covalently-coupled bovine serum albumin (BSA)-Sepharose 2B gel is practical for obtaining washed platelets for the SRA.

Methods: BSA-Sepharose 2B gel was prepared by covalently coupling thermally deactivated BSA to cyanogen bromide-activated Sepharose 2B and packed into a 20 mL (bed volume) polypropylene column. Platelet-rich plasma (PRP) was prepared from healthy donors drawn into 3.2% sodium citrate. 3.5 mL PRP was layered onto the column and Tyrode-HEPES-1 buffer was added in 350 uL aliquots to elute platelets and plasma proteins. 600 uL fractions were collected and analyzed for platelet count and total protein. Several SRAs were performed using well-characterized positive and negative HIT serum samples to confirm platelet viability.

Results: Platelets were completely separated from plasma proteins with the highest platelet concentrations in fractions 10-14 (3 mL total volume) and proteins eluting after the 20th fraction. Platelet fractions were visibly cloudy and eluted from the column within approximately 6 minutes. Platelet recovery in the pooled fractions was approximately 70% of initial PRP counts. Platelet concentrations obtained were adequate for the SRA (target normalized platelet count 250,000/µL). SRA using BSA-Sepharose 2B gel-filtered platelets and HIT sera yielded expected results, indicating that the platelets were not pre-activated. The same BSA-Sepharose 2B column was used more than 30 times over 4 weeks without loss of integrity.

Conclusions: Gel-filtration using BSA-Sepharose 2B is an efficient and reliable method to produce viable washed platelets for use in the SRA or other assays requiring functional platelets.

Using the PAM50 breast cancer intrinsic classifier to assess risk in ER+ breast cancers: a direct comparison to Oncotype Dx®

Bastien R, Ebbert M, Bernard P

OBJECTIVES

1) Understand the difference in testing and results between the PAM50 and the Oncotype Dx

2) Describe the study design and procedures

3) Discuss what components of the PAM50 most correlate with the Oncotype Dx recurrence score

Background: Multianalyte gene expression assays have proven valuable in the management of breast cancer, however, the methodologies and indications for these assays can differ. In ER+ breast cancer, there continues to be debate about the most important components of these tests for determining risk of relapse. The PAM50 test has genes relevant to ER+ and ER- disease, while the Oncotype Dx recurrence score (RS) was tailored specifically for ER+ breast cancer. The components of the PAM50 that are most relevant to the RS are assessed.

Methods: A total of 151 ER+ breast cancers, that were sent to Genomic Health Inc for Oncotype Dx testing from the University of Texas, MD Anderson Cancer Center at the time of diagnosis (2004-2008), were re-analyzed at ARUP Laboratories using the PAM50 Breast Cancer Intrinsic Classifier. Formalin-fixed, paraffin-embedded tissue blocks containing invasive breast carcinoma were selected after H&E review by a pathologist. RNA was extracted from full-face scrolls and directed punches using the High Pure RNA Paraffin Kit. Multivariate analysis was used to identify components of the PAM50 test that contribute independently to the Oncotype Dx risk score (RS). Linear models were fit to the data. Bootstrap methods were used to correct for bias in the predictions.

Results: After quality control, there were 119 invasive breast cancers that were successfully assayed on both clinical platforms. The subtype distribution consisted of 76 Luminal A (64%), 27 Luminal B (23%), 4 HER2-enriched (3%), 1 Basal-like (0.8%), and 11 Normal-like (9.2%). None of the Luminal A subtype samples had a high RS and 90% (9/10) of high RS samples were Luminal B. There was a negative correlation between the RS and PAM50 Luminal A centroid ($r=-0.44$; $p<0.0001$). The best linear prediction of the RS using PAM50 included subtype – Luminal A vs Luminal B ($p=0.044$), Luminal Score ($p=0.003$), Proliferation Score ($p=2.34e-05$), and PGR Score ($p=0.0014$).

Conclusions: The PAM50 Breast Cancer Intrinsic Classifier and Oncotype Dx use different gene sets and algorithms, however, there is a very large and significant overlap in the ability of these clinical tests to determine risk in ER+ breast cancers.

Characterizing error and uncertainty of centroid-based genomic predictors

Ebbert M, Bastien R, Boucher K, Eilbeck K, Bernard P, Facelli J

OBJECTIVES

1) Discuss the importance of developing a method to validate the reliability of multi-analyte clinical assays, such as the PAM50 Breast Cancer Intrinsic Classifier, for the purpose of improving patient care

2) Determine the degree to which discordant subtype calls from the clinical PAM50 Breast Cancer Intrinsic Classifier are caused by technical variability in gene measurement(s)

3) Propose an in silico model for validating the reliability of multi-analyte clinical assays such as the PAM50 Breast Cancer Intrinsic Classifier using Monte Carlo Simulations

Abstract: Multi-analyte assays are becoming increasingly common to assist clinical decisions, but due to their complexity are considered high risk since the algorithms and potential error for diagnosis are often not well understood. This study focuses on a centroid-based approach to classification in breast cancer subtypes and develops a model for characterizing error in multi-analyte tests.

Introduction: Multi-analyte clinical assays (MCAs) involving gene expression are becoming more common in assisting treatment decisions in medicine, especially in oncology. For example, in breast cancer there is a 55-gene subtype classifier (PAM50 Breast Cancer Intrinsic Classifier), the 21-gene Recurrence Score (Oncotype DX), and the 70-gene prognosis assay (MammaPrint). Due to the increased complexity of MCAs, it is difficult to identify sources of error and assess the potential for misdiagnosis when there is variability in gene measurements. Thus, it is critical to develop methods to account for all sources of error contributing to the uncertainty in the MCA's results. The source of errors to be considered should include uncertainties due to heterogeneity and preparation of the specimen, as well as technical variability, which may be separated into error from measuring devices as well as intrinsic and explicit classification uncertainties. The PAM50 was developed to classify invasive breast tumors into one of four major subtypes using gene expression. The four subtypes are commonly known as Luminal A, Luminal B, HER2-enriched and Basal-like. Centroids for each of these tumor types and "normal" breast tissue were developed using a training set, and patient breast tumors are classified using Spearman's Rho correlation as a measure of the distance between gene expression profiles. The purpose of this study is to determine how tolerant subtype assignment is to technical variability in gene expression measurements.

Methods: Technical variability was assessed by assaying cell lines and breast cancers with 4 different biologies (Luminal A, Luminal B, HER2-enriched, Basal-like) a total of 12 times each. The overall error was measured as the total range of values for each gene. Using the error data for each gene, Monte Carlo simulations were employed to explore whether the inherent error in the data significantly affected the subtype prediction. Specifically, both a uniform and normal distribution were used in the Monte Carlo simulations for each gene to estimate the worst-case scenario for subtype misclassifications. Exactly 100,000 samples were generated using both the uniform and normal distributions for each gene of the 4 samples run, for a total of 800,000 simulations. The effect of the tumor subtype prediction is measured by the percentage of simulations that changed the subtype prediction. Furthermore, a preliminary analysis of the error data was performed to develop a model by which the error can later be imposed on an independent sample set to further study the effects of laboratory error on subtype prediction stability.

Results and Conclusions: None of the 800,000 samples generated using the uniform and normal distributions were misclassified, suggesting that laboratory error has minimal effect on stability of the subtype prediction. However, the results only apply to the four samples tested thus far. In order to better understand the effects of laboratory error on the stability of subtype prediction, the measured error must be imposed on an independent data set.

A preliminary analysis of the error data shows that error increases as the level of expression in a gene decreases. Furthermore, error between genes is significantly correlated, suggesting there are other biases to be accounted for.

Introduction of the first next generation sequencing clinical assays at ARUP: the mitochondria genome and mitochondria 128 gene panel

Dames S, Margraf B, Durtschi J, Mao R

OBJECTIVES

1) Introduce the mitochondrial next generation sequencing process

2) Describe alignment, variant calling, and data analysis

3) Provide details on validation of mitochondrial assays

In the past six years, next generation sequencing (NGS) has been broadly adopted and used in many areas, including gene discovery and clinical testing. ARUP Labs has acquired an Illumina HiSeq NGS platform and the serviceable required infrastructure for research and clinical applications. The HiSeq allows for > 200 Gb of sequence from a single, eight-day experiment. To put this number into perspective, 200 Gb allows for approximately 60-fold average sequence coverage of the human genome. This high sequencing output potentially allows for parallel sequencing of up to 192 samples per experiment. To leverage the throughput provided by the HiSeq, the first clinical application is for mitochondrial disorders, a condition with a reported frequency of 1:8000. Mitochondrial disorders involve mutations in both the mitochondrial genome and potentially up to as many as 1,500 human nuclear genes. Since the condition is highly variable and can present with non-specific clinical features, patients with mitochondrial disorders are presumably under diagnosed. Current assays that examine lactic acid concentration in plasma and CSF, or biochemical interrogation of oxidative phosphorylation in biopsy samples may be ambiguous or falsely negative. Sanger sequencing is available for mitochondrial genome for a limited number of nuclear genes, however, the tests are expensive and the sensitivity is low. To take advantage of NGS, we have developed the first clinical assay at ARUP for mitochondrial disorders. The assay includes whole mitochondrial genome sequencing and a 128-gene nuclear panel specific for selected mitochondrial disorders. The assay requires amplification of the entire mitochondrial genome and the 128 nuclear gene panel. Both libraries are barcoded, providing the ability to run multiple samples in a given lane.

Five overlapping, long-range PCR (LRPCR) amplicons are used to redundantly amplify the entire 16.5 Kb mt genome. Processing of the amplicons involves equimolar pooling, shearing, and SPRI-TE automated HiSeq library preparation. Afterwards the libraries are loaded into a cBOT for HiSeq cluster generation and subsequently placed in the HiSeq for single-end, 100-base reads. The mt 128-gene nuclear panel uses exon-specific amplification for enrichment. A total of 1,304 amplicons representing 680 Kb of DNA are amplified using the RainDance emulsion PCR platform. The RainDance derived amplicons require a different processing workflow than the LRPCR assay, but the basic processes and times required are similar. We currently estimate that samples can be sequenced using 100-base reads to decrease turnaround.

Post-sequencing bioinformatic analysis is required for alignment and to find single nucleotide polymorphisms (SNP) and small deletion/insertions polymorphisms (DIP) using CLCBio software. The major difference between the mt nuclear and mt genome assay is the amount of bioinformatic analysis required. The 128 genes are currently aligned to a masked human genome reference and visually interpreted to ensure all exons are sequenced. The number of SNPs/DIPs range from 25,000-45,000 per sample. Filtering of nonsynonymous variants reduces this number dramatically to 40-60 variants. Currently we are manually interpreting the variants until internal variant databases are established.

Overall, this test is the most comprehensive and cost effective assay that can be developed for mitochondrial disorders. It significantly improves the sensitivity of heteroplasmic mutation detection in the mitochondrial genome, and provides the ability to examine multiple nuclear genes that would be technically and financially prohibitive. We believe it will have a great impact in the patient care.

Avoidance of pseudogene interference in the detection of 3′ deletions in *PMS2*

Vaughn C, Hart K, Samowitz W, Swensen J

OBJECTIVES

1) Discuss the current limitations of MLPA for detecting *PMS2* deletions

2) Describe the components of the new deletion detection methodology

3) Utilize the MLPA ratios and sequencing algorithm to identify *PMS2* deletions in patient samples

Lynch syndrome is characterized by mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6*, and *PMS2*. While detection of mutations in *MLH1*, *MSH2*, and *MSH6* is straightforward, evaluation of the *PMS2* gene is greatly complicated by numerous pseudogenes. In particular, detection of deletions in the 3' region of the gene has been virtually impossible due to the pseudogene *PMS2CL*, which shares homology with *PMS2* exons 9 and 11–15. Extensive gene conversion between *PMS2CL* and *PMS2* at the 3' end has resulted in a lack of gene-specific sequences for exons 12–15. We have developed a methodology for detection of deletions in this region utilizing a newly-designed multiplex ligation-dependent probe amplification (MLPA) kit for *PMS2*. Included in this kit are probes for variants found in both *PMS2* and *PMS2CL*. This design provides for detection of deletions, but not localization of a detected deletion to the gene or pseudogene. To address this, we have incorporated control samples with known copy numbers of gene and pseudogene variants, and paired MLPA results with sequencing of the gene and pseudogene in patient samples. Using this method, we tested a subset of clinically-indicated samples for which mutations were either not previously identified or not fully characterized using existing methods. We identified eight unrelated patients with deletions encompassing exons 9–15, 11–15 (2 patients), 13–15 (2 patients), 14–15 (2 patients), and exon 15. Five of these patients (those with deletions limited to exons 12, 13, 14 and/or 15) harbor mutations undetectable by existing methods. For the remaining three patients, the 3' extent of the deletion was previously undeterminable. By incorporating specific, characterized control samples and sequencing the gene and pseudogene in patient samples it is possible to use the newly designed *PMS2* MLPA kit to identify deletions in this region of *PMS2* and provide clinically relevant results. This methodology represents a significant advance in the diagnosis of patients with Lynch syndrome caused by *PMS2* mutations.

Comparison of IHC, FISH and RT-PCR for the detection of *EML4-ALK* translocation variants in non-small cell lung cancer

Wallandar M, Geiersbach K, Tripp S, Layfield L

OBJECTIVES

1) Describe the molecular subtypes of lung cancer and available therapies

2) Describe the *EML4-ALK* gene fusions and associated clinical characteristics

3) Discuss the advantages and limitations of each *EML4-ALK* fusion detection method

Background: *EML4-ALK* gene fusions have been detected in 3-13% of non-small cell lung cancers (NSCLCs) and are associated with adenocarcinomas, lack of *EGFR* and *KRAS* mutations, and younger patient age. Patients with tumors harboring *EML4-ALK* fusions are candidates for targeted therapy with ALK inhibitors. An accurate diagnostic test for the detection of *EML4-ALK* gene fusions would be of great utility.

Design: Fifty-two formalin-fixed, paraffin-embedded lung adenocarcinoma specimens were selected from the University of Utah surgical pathology files. The study population was enriched for specimens with wild-type *EGFR* status (WT: n = 46; Mutant: n = 6). Specimens were screened for the presence of *EML4-ALK* fusions by three methods: ALK immunohistochemistry (IHC) (Dako), ALK FISH (Abbott Molecular), and RT-PCR (*EML4-ALK* variants 1 and 3a/b). FISH and IHC were scored by three viewers to determine agreement. Concordance between methodologies was determined for the detection of *EML4-ALK* variants 1 and 3a/b, which account for approximately 80% of reported cases

Results: *EML4/ALK* variant 3a/b was detected in 2 of 52 (4%) specimens. Concordance between IHC, FISH, and RT-PCR methodologies was 100% for the detection of variant 3a/b. Complete agreement among FISH and IHC viewers was obtained for *EML4/ALK* variant 3a/b. *EML4/ALK* variant 1 was detectable by RT-PCR in 9 of 52 (17%) specimens. IHC and FISH were both highly discordant with RT-PCR for the detection of *EML4-ALK* variant 1. Complete agreement among IHC viewers for variant 1 was achieved but the sensitivity of IHC was poor (11%). Agreement among FISH viewers was poor for the detection of *EML4/ALK* variant 1 as only one of nine (11%) specimens was scored FISH positive by all three viewers. Sensitivity of ALK FISH for the detection of *EML4/ALK* variant 1 ranged from 11-78%, depending on the viewer.

Conclusions: RT-PCR demonstrated the best sensitivity and specificity for the detection of the two most commonly reported *EML4-ALK* variants in lung carcinoma. In addition, RT-PCR was much less subjective to interpret than FISH. Implementation of *EML4-ALK* testing by RT-PCR will aid in the identification of lung cancer patients that could benefit from ALK inhibitor therapy.

Newborn screening for spinal muscular atrophy

Pham H, Swoboda K, Lyon E, Reyna S, Dobrowolski S

OBJECTIVES

1) Develop a test to prospectively screen for spinal muscular atrophy within the context of a routine newborn screening laboratory

2) Demonstrate that the assay clearly distinguishes SMA affected patients from wild types and SMN1 deletion carriers

3) Validate assay performance, test characteristics, and initiate pilot studies of the SMA screening system

Spinal muscular atrophy (SMA) is a progressive neuromuscular disease inherited as an autosomal recessive trait. New treatment modalities provide means to improve the quality of life for affected patients. To realize the maximum benefit from treatment, it must be initiated immediately after birth. To identify SMA-affected newborns and facilitate their early enrollment in a treatment regimen, a test to perform prospective newborn screening for SMA was developed. Defects in the survival motor neuron 1 gene (SMN1) are causative in SMA and ~95%-98% of SMA patients have a homozygous deletion of SMN1 that involves the 7th exon. The test to prospectively identify those SMA affected patients with a homozygous deletion of SMN1 leverages the subtle differences between SMN1 and the highly homologous SMN2 gene. A strategy involving calibrated short-amplicon genotyping and post-PCR high resolution melt profiling was applied. The assay showed complete concordance with previously characterized samples from known SMN1 affected or carrier individuals (N=30). In addition, the assay was validated directly from blood spots (N=1200). The assay is robust and possesses test characteristics (simplicity, easy of interpretation, low cost, high sensitivity, high specificity) that make it suitable for the high-throughput newborn screening laboratory.

Analysis of cortisol, cortisone and dexamethasone in human serum by LC-MS/MS

Ray J, Kushnir M, Rockwood A, Meikle A

OBJECTIVES

1) Understand the metabolism and physiopathology related to Cortisol, Cortisone and Dexamethasone

2) Describe the LC-MS/MS method developed for this assay

3) Recognize the importance of simultaneous clinical testing of cortisol, cortisone and dexamethasone in samples from ICU vs non-ICU and Cushing's patients

Background: A high sensitivity LC-MS/MS method was developed for simultaneous measurement of cortisol, cortisone and dexamethasone in human serum. Using this method, we compared concentrations of cortisol, cortisone and their ratios in samples from intensive care unit (ICU) and non-ICU patients. Dexamethasone suppression test is used to diagnose and differentiate among the various types of Cushing's syndrome and other hyper-cortisol states. Using this method, we also evaluated cortisol and dexamethasone concentrations in patients with suspected Cushing's syndrome.

Methods: 200 µL of human serum aliquots were extracted using solid phase extraction and analyzed using Liquid Chromatography-Tandem Mass Spectrometry.

Results: The limits of quantification for cortisol and cortisone were 0.3 µg/L and for dexamethasone it was 0.5 µg/L. Total imprecision was less than 10.9%. Median cortisol to cortisone ratio in ICU patient samples was found to be 2 times higher than non-ICU samples. Among the samples submitted to ARUP since implementation of the method for routine use, 54% of patients after 1mg dose of overnight dexamethasone could be categorized as consistent with Cushing's syndrome.

Conclusions: The method has high sensitivity and specificity. High cortisol to cortisone ratios in samples from ICU patients suggests change in activity of 11β-hydroxysteroid dehydrogenase in modulation of systemically available cortisol. Simultaneous measurement of dexamethasone and cortisol is effective in differentially diagnosing diseases causing elevated concentrations of cortisol.

Rapid differentiation of *Cryptococcus gattii* from *Cryptococcus neoformans*

Slechta S, Barker A, Siady S, Hanson K

OBJECTIVES

1) Understand the importance of differentiating *Cryptococcus neoformans* from *Cryptococcus gattii*

2) Describe the different testing methods currently used to differentiate these two species

3) Demonstrate that MALDI-TOF is a reliable, inexpensive alternative to other methods that identify *Cryptococcus gattii*

Background: *Cryptococcus gattii* is an emerging infectious disease in the United States. Recently, outbreak of a hypervirulent strain in the Pacific Northwest was linked to several deaths in relatively young, otherwise healthy individuals. *C. gattii* appears to differ from its sibling species, C. *neoformans*, both in its clinical aspects (e.g., less responsive to antifungal drugs and more likely to cause tumor-like lesions called cryptococcomas) and in its ecologic niche. Differentiating between the two is important for management and prognosis. Currently, the laboratory relies on expensive selective media to differentiate the two species, which takes several days.

Objective: To test two alternative methods of identifying *C. gattii*, MALDI-TOF mass spectroscopy, and sequencing of the Intragenic Spacer (IGS) region.

Methods: Yeast isolates previously identified as *C. neoformans* were removed from permanent storage and tested on Canavanine-Glycine-Bromothymol (CGB) Blue agar (Hardy Diagnostics) to confirm their identity. All isolates identified as *C. gattii*, as well as a subset of C. *neoformans*, underwent IGS rRNA sequencing. PCR amplification used primer pairs against IGS1a. Sequence analysis was performed using BLAST searches in GenBank. Yeasts were also extracted for MALDI-TOF analysis using formic acid and acetonitrile. Mass spectra were acquired on a microflex LT MALDI-TOF spectrometer (Bruker Daltonics) in linear positive ion mode using FlexControl 3.0 software.

Results: A total of 189 isolates were subcultured to CGB agar. Of these, 8 were phenotypically consistent with *C. gattii*. Sequence analysis of the IGS region confirmed the *C. gattii* identification for all 8 isolates. An additional 20 randomly selected, CGB negative organisms were identified as *C. neoformans* by their IGS sequence. In a follow-up blinded study of all 189 isolates, using an identification cutoff score of ≥ 1.8 , MALDI-TOF spectral analysis differentiated *C. gattii* from *C. neoformans* 100% of the time.

Conclusions: Both IGS sequencing and MALDI-TOF analysis gave accurate results. MALDI-TOF is more cost effective and rapid. We conclude that MALDI-TOF should be the default method for Cryptococcal identification, with sequencing or CGB agar as backups.