IAAM - INVASIVE ASPERGILLOSIS ANIMAL MODELS: Fourth Annual Meeting

The University of Texas Health Science Center at San Antonio
Academics and Administration Building Room 114
7703 Floyd Curl Drive
San Antonio, TX 78229-3900
Telephone (210) 567-4627

Thursday, October 18, 2007
8:00 AM - 1:00 PM

- **Welcome & Introductions**
  - Dennis Dixon / Rory Duncan **(view presentation)** / Tom Patterson **(view presentation)**
    - Welcome / Meeting Expectations
  - Ultimate Goal – FDA Cleared Diagnostic(s) for Early Detection and Treatment Monitoring of Invasive Aspergillosis
  - Statement of Work
    - Develop standardized animal models
      - Detect organisms
      - Evaluate targets and surrogates (PFGRC – microarrays)
        - http://www.niaid.nih.gov/dmid/genomes/pfgrc/default.htm
      - Estimate fungal burden
      - Distinguish infection from colonization
    - Need for a reference standard
      - Historical perspective: Critical needs in antifungal susceptibility
      - Method reproducible
      - Provides reference standard for comparison of other methods
      - Validated for clinical correlation
      - Highest priority for Aspergillus and new diagnostics
      - Standardized models aimed at identifying new targets for diagnosis & monitoring of disease progression
    - Molecular toolbox
      - Target identification
      - Strain analysis
  - Timeline
    - 2 years to develop and standardize
      - Immunosuppressed mouse model
      - One larger animal model (up to rabbit) to allow continuous blood sampling
      - Develop molecular tools
      - Produce SOPs
    - 2 years of community posed “Key questions” addressed with the models and samples provided to industry or other interested community members for evaluating new diagnostic approaches.
    - Years 5 – 7
      - Identify and pursue new diagnostic approaches
      - Provide community support
      - www.sacmm.org/iaam.html
        - Provide samples
        - Provide training
      - Adjust or add models
        - Accommodate diagnostic discoveries
        - Refine for different at-risk populations
  - New diagnostic initiatives
    - Award of a new contract (DxIA) to Dr. John Wingard, U. Florida, Project Officer – Marilyn Tuttleman
    - Potential new diagnostics for IA premature for processing through DxIA funneled to IAAM
    - Promising new diagnostics in IAAM go to DxIA
  - Contract Deliverables/Model refinement
    - SOPs online

- Alternative Af strains
- Role of host responses
- Distinction of colonization vs disease
- Impact of sample types, collection, storage

- New target identification
  - Gene product(s) in disease
  - Host gene responses
  - Novel approaches
- Diagnostic development
  - Industry partners
  - Pre-clinical support for diagnostics
  - Community awareness/interaction
- Developmental Status Report
  - Completion of Phase I—continuation of phase 2 (end of year 4)
    - Deliverables: Standard operating procedures
      - Molecular techniques
      - Animal models
      - www.sacmm.org/iaam.html
    - How to identify "key" questions/new diagnostics
      - Interactions with Steering Committee, Scientific Community & Commercial Partners
    - Procedures for receiving requests and establishing priorities
    - Development of new diagnostics
      - New targets
      - New platforms
      - Provide tissues/samples; testing
    - Qualified investigator
      - Researcher with interest in Aspergillus
      - Trained to safely perform requested research
      - Qualifications: Principal investigators; Trainees (Post-doctoral fellows, students); Industry researchers
    - Key Questions
      - Gene/gene product as diagnostic target
      - Evaluation of surrogate marker(s)
      - Effects of therapy on disease progression/gene expression
      - Role of virulence determinants in diagnosis
    - Prioritization of key questions
      - High likelihood of commercialized diagnostic product
      - Data to support development of diagnostic product
      - Pilot studies to test theoretical diagnostic target
        - NIH funded research
        - Preliminary data to support NIH application with favorable priority score on review
        - Pilot studies to evaluate investigator initiated concept
        - Industry sponsored research

- **Session 1 MODEL UPDATES**
  - Scott Filler **(view presentation)** & Laura Najvar **(view presentation)**, Murine Models
    - Murine Model introduction
      - Infection:
        - Acrylic Chamber:
          - nebulized conidia *A. fumigatus* 10^9 conidia/ml of fluid for 1 h
          - simultaneous challenge of 50 mice
        - Madison Reference Chamber:
          - nebulized conidia *A. fumigatus* 10^{10} conidia/ml of fluid for 1h
          - simultaneous challenge of 120 mice
      - Immunosuppression:
        - Day -2: Cortisone acetate 250 mg/kg SC+ Cyclophosphamide 250 mg/kg IP
        - Day +3: Cortisone acetate 200 mg/kg SC + Cyclophosphamide 250 mg/kg IP
    - Key questions addressed using the mouse

- Influence of the Mouse:
  - Outbred ICR vs Inbred C57, DBA, and Balb/c
- Gender influence on outcome:
  - Male vs Female
- Interlaboratory Correlation:
  - UCLA-Harbor,
  - UTHSCSA
- Aspergillus strain differences:
  - AF293 vs CEA10
  - Antifungal Impact on Diagnostics

- Analysis parameters
  - Course of infection analyzed by length of survival (Logrank test)
  - Semi-quantitative tissue culture of lungs done on serial days 1, 3, 5, 7 and 11 after infection (Mann Whitney U test)
  - Additional assessments (Mann Whitney U test)
    - qPCR
    - Galactomannan
    - β D glucan
  - $P<0.05$ required for statistical significance
- Conclusions
  - Outbred ICR and Inbred Balb/c mice are equivalent in this model of invasive pulmonary aspergillosis.
  - Inbred DBA and C57 mice have specific limitations and therefore, specific uses.
  - In both chambers, there is no difference between gender in ICR or Balb/c mice.
  - Good interlaboratory correlation.
  - No differences were seen in survival, tissue burden (CFU) or drug response between isolates AF293 and CEA10 in this model.

- Comparison of IA in Neutropenic vs. Non-neutropenic Mice
  - Gliotoxin does Not Contribute to Virulence in Neutropenic Mice
  - Gliotoxin Contributes to Virulence in Non-neutropenic Mice
  - Gliotoxin Causes Neutrophil Death in Mice
- Future Studies of IA in Non-neutropenic Mice
  - Test intermediate doses of steroids
  - Determine pulmonary and serum galactomanan levels
  - Determine the profile of host inflammatory response
    - MPO levels
    - Cytokine levels
  - Test other diagnostic assays

- Rick Kirkpatrick **(view presentation)** Guinea pig model / Peter Warn **(view presentation)** Rat Model
  - Introduction to Guinea Pig Model
    - Based on Filler / Sheppard mouse model
    - Madison chamber reference standard
      - Simultaneous exposure of up to 18 Guinea pigs
    - Acrylic Chamber
      - Simultaneous exposure of up to 7 Guinea pigs
    - Serial serum/blood samples
    - Larger tissue volumes
      - Histology
      - Tissue burden assessments
      - Blood volume
    - Suitable for evaluation of surrogate markers
    - Provides a venue by which “key” questions in IA may be addressed
  - Large Model benefits
    - Recognition of significance for serial assessments
    - Early diagnosis or detection following exposure
    - Therapeutic monitoring
      - Residual fungal burden
      - Metabolites

        - Antigens
        - Gene Expression
    - Provide preclinical evaluation for newer technologies prior to testing on clinical samples
- Model Design
    - Male Hartley Guinea pigs, 500 g
        - Immunosuppressed
            - Cortisone Acetate and Cyclophosphamide
                - 250 mg/kg each CA and Cy on day -2
                - 250 mg/kg CA & 200 mg/kg Cy day +3
    - Organism: *Aspergillus fumigatus* AF 293
    - Establishment in "Reference standard" chamber, the Madison Chamber
        - Dose response
        - Inoculum determination experiments
    - Reproducibility in acrylic chamber
        - more easily available
        - less costly!
    - Quantification of fungal burden
        - Tissue / tissue extracts: lung, kidney, spleen, brain, liver; serial blood samples
        - Colony counts
            - Proteomics
            - PCR
                - Quantitative
                - Taq-Man
        - Surrogate Markers of infection
            - Galactomannan
            - β-D-Glucan
            - PCR
            - HPLC
            - Chitin Assay
- Guinea Pig Model Conclusions
    - Uniform delivery of conidia in:
        - Acrylic chamber
        - Madison Chamber
    - Serial sampling is available
    - Assessment by survival, CFU, CE, GM and…..more…....
    - IPA from pulmonary infections may have utility in assessment of new diagnostic tests or therapeutic regimens for IPA

- Rat Model Introduction
    - Large size relative to mice
    - Low cost and good availability relative to guinea pigs
        - UK costs:
            - 25 g Swiss mouse = £5
            - 250g rat = £26
            - 400g Guinea pig = £106
    - Simple venous access suitable for multiple daily samples
    - Rats tolerate multiple procedures better than guinea pigs
- Experimental Design
    - Rats strain: Sprague Dawley (250-300g)
    - Rats immunosuppressed with cyclophosphamide IP (75-250mg/kg) OPTIMUM DOSE 75mg/kg every 5 days
    - Rats immunosuppressed with either cortisone acetate SC (150-250mg/kg) OR Depo-medrone (long acting prednisolone) IM (10mg/kg) nb cortisone acetate >150mg/kg every 5 days toxic
    - Rats treated with Baytril (enrofloxacin)10mg/kg SC daily from day -2 ceftrioxone 50mg/kg IM daily added from day 3 post infection
    - *Aspergillus fumigatus* CEA10 prepared as for mice and guinea pigs
    - Rats infected using the aerosol chamber as for mice and guinea pigs
- Rat Model Conclusions

- Uniform pulmonary infection
- Serial sampling is available
- Assessment by survival, CFU, CE, GM
- Amenable to other assessments

- **Session 2 SURROGATE MARKERS AND GENE EXPRESSION**
  - Ana Vallor **(view presentation)** PCR & Nathan Wiederhold **(view presentation)** Beta-D-glucan
    - Comparison of DNA Extraction Methods Utilizing Real Time PCR-based Amplification for the Detection of *Aspergillus fumigatus* in Murine Specimens
      - Background
        - The lack of externally validated PCR methods remains a limiting factor for standardization of PCR for early diagnosis of invasive aspergillosis.
        - This pilot study provides an opportunity to identify and address variables present in current methodolologies for assessment of fungal burden and how they may or may not contribute to result variation.
        - We describe a bi-center comparison of different DNA extraction methods to detect *A. fumigatus* 293 (AF 293)-DNA in murine lung and serum samples.
        - Centers were located at University Clinics of Wuerzberg, Germany (CTR 1) and the University of Texas Health Science Center at San Antonio, USA (CTR 2).
      - Results
        - The use of recombinant lyticase for extraction of AF293 DNA present in infected serum appeared to be more efficient as shown by quantitative PCR.
        - Significant differences in the amount of serum fungal DNA were detected when comparing the two real time platforms. Further studies utilizing the same primer-probe combinations should be performed to further address sensitivity issues.
        - Using either enzymatic or mechanical DNA extraction methods, qPCR as performed on the Applied Biosystems 7300 PCR system demonstrated no significant difference in the detection and amount of pulmonary fungal DNA.
      - Conclusions
        - Due to the differences found in this study the necessity for external validation is critical in PCR standardization.
        - IAAM model plays a crucial role by providing ample amounts of biological fluids and tissue to allow pre-clinical investigations for optimization of current diagnostic methods as well as assessments of new diagnostic methods without expending human/patient samples.

    - Assessment of Serum 1,3-β-D-glucan as a Measure of Disease Burden in Invasive Pulmonary Aspergillosis
      - Background
        - Two surrogate markers accepted by EORTC/MSG
          - Galactomannan & 1,3-β-D-glucan
          - Little clinical experience with 1,3-β-D-glucan in high risk patients for diagnosis of IPA
        - Measure the serum kinetics of 1,3-β-D- glucan in murine and guinea pig models of IPA
        - Examine assay as a surrogate marker of disease burden
          - Presence of antifungal therapy
      - Methods
        - Serum separated and transferred to 96 well cell culture tray
          - 5 mcL per well
          - Each sample in duplicate
        - Alkaline serum treatment
          - Incubation 37ºC
        - Fungitell™ reagent
          - Mean rate ΔO.D. (405 nm) over 40 minute period
        - Unknowns compared to standard curve

        - Range 0 - 500 pg/mL
    - Fungal burden
        - Lungs harvested at Day +5, homogenized and bead beaten
        - DNA extracted using proteinase K incubation and Qiagen DNeasy columns
    - DNA analyzed by real-time qPCR assay using probes and primers specific for DNA encoding Aspergillus FKS gene
    - Unknowns compared to standard curve generated from Aspergillus genomic DNA
        - Conidial equivalents/g
    - Samples were run in duplicate
- Results and Conclusions
    - Serum 1,3-β-D-glucan detectable earlier in course of infection in murine model
        - > 60 pg/mL by day +3
        - Later detection in guinea pig model (day 7)
    - Early decreases in serum 1,3-β-D-glucan were predictive of survival in murine model
        - Median values of < 60 pg/mL on day +5 associated with 100% survival in posaconazole group
        - Reductions associated with improved survival in amphotericin B groups
    - Median serum 1,3-β-D-glucan concentrations less predictive of outcome in guinea pigs
        - Reductions to <60 pg/mL in serial samples in same animal from day 7 to day 11 observed in animals treated with posaconazole
        - Increases in serial samples in same animal observed in animals who failed therapy
    - Results suggest potential use of 1,3-β-D-glucan assay for screening and early diagnosis of IPA
        - Concern for discordance between residual tissue burden and serum 1,3-β-D-glucan concentrations
    - Potential use of serial assessment of 1,3-β-D-glucan as a measure of treatment efficacy
    - Additional pre-clinical studies warranted
        - Comparison with other surrogate markers
        - Clarify time required for assay to become positive

- Don Sheppard **(view presentation)** In vivo Galactomannan Release
    - Effects of Caspofungin on Galactomannan Kinetics in vivo
        - Paradoxical Effect theory
            - Platelia EIA for Aspergillus
                - Commercial EIA for circulating galactomannan (GM)
                - GM is both released by remodeling hyphae and bound to 1,3-β-D-glucan in the cell wall
            - Echinocandins inhibit 1,3-β-D-glucan synthesis in the cell wall
            - Echinocandins could potentially
                - Increase GM release by reducing GM binding to cell wall
                - Decrease GM release by inhibiting fungal growth
        - Implications
            - Alter the utility of following GM levels to monitor response to therapy with echinocandins
            - May suggest a role for improving diagnostic sensitivity of GM assay
                - Echinocandin “stimulation” test
        - Study design
            - In vitro confirmation of GM release
            - Evaluate effects of single and multiple doses of antifungals on GM in a guinea pig model
            - Relatively high doses of each agent used
                - Caspofungin 6 mg/kg/d
                - Amphotericin B 1.3mg/kg/d

        - Voriconazole 10 mg/kg bid
    - Inhalational model
        - Guinea pigs to allow for repeat sampling
        - Immunosuppressed with cortisone acetate and cyclophosphamide
        - Infected by inhalation on Day "0"
        - Develop progressive invasive pulmonary disease with 100% mortality
        - One time dosing with antifungal
        - Serial samples time 0h, 4h, 24h 48h,72h, 96h
    - Results and Conclusions
        - Although exposure of A. fumigatus to caspofungin causes increased GM release in vitro, a single dose does not cause increased serum GM in neutropenic animals
        - Instead, a single dose of antifungals actually causes long-lasting suppression of serum GM
        - Therefore, even a short exposure to antifungal agents may markedly reduce the sensitivity of the GM assay for up to 4 days

- **Session 3 MOLECULAR TOOLBOX**
    - Brian Wickes **(view presentation)** Aspergillus Strain Identification / David Denning
        - Common/Recent Typing Methods for *Aspergillus fumigatus*
            - Random Amplification of Polymorphic DNA (RAPD)
                - *PCR-based, easy, no sequence information needed, little DNA needed*
                - *poor reproducibility (intra-interlaboratory)*
            - Afut1 hybridization of Restriction Fragment Length Polymorphism (Afut RFLP)
                - easy to interpret, most basic of all molecular techniques
                - isotopic, good quality DNA needed in large amounts, cloned probe
            - Microsatellites
                - same as RAPDs, semi quantitative, portable data, reproducible
                - (genome) sequence data needed, instrumentation, data analysis
            - Additional Methods
                - Sequence-Specific DNA Primer analysis (SSDP)
                - Multi-locus Enzyme Electrophoresis (MLEE)
                - Amplified Fragment Length Polymorphism (AFLP)
                - Multilocus Sequence Typing (MLST)--developed for bacteria, useful for *C. albicans*
        - There are a variety of epidemiological methods that are useful for typing *A. fumigatus*. Some are more practical than others for clinical studies, however, others are more powerful in terms of data that are generated
        - The most common typing methods used for the major fungal pathogens (RAPD, hybridization, microsatellites), also work for *A. fumigatus*.
        - In order of preference, we utilize gel-based microsatellites, RAPDs, and finally hybridization.

- **Session 4 KEYNOTE LECTURE : CLINICAL NEEDS**
    - John Wingard *University of Florida Shands Cancer Center*, New Diagnostic Targets: Clinical Needs **(view presentation)**
        - Clinical Laboratory Diagnostics for Invasive Aspergillosis (IA)
            - **As**pergillus **Te**sting **C**onsortium (AsTeC)
        - Goal of Study
            - Link clinical specimens from pts with IA to approved & experimental laboratory tests for diagnostic proof of principle & test comparisons
        - Methods
            - Establish & maintain repository of clinical samples from pts at high risk for &/or infected with IA
            - Assess new IA diagnostic assays by performance of:
            - replication studies
            - comparison evaluations of experimental IA assays with approved tests
            - studies to determine what conditions may interfere with test performance
        - Sample plan
            - Two strategies
                - Longitudinal collection of samples from those at highest risk (Group 1)
                    - Allogeneic BMT

        - Acute leukemia
        - Lung transplant
            - Pros: baseline samples before onset IA
            - Cons: Very inefficient

    - Collection of samples from others who become infected (etiology suspected to be IA) (Group 2)
        - Any pt group
            - Pros: More efficient, data in pt groups not in the highest risk
            - Cons: no baseline uninfected samples
- Group 1 sampling expectations
    - Longitudinal samples from 42 IA-infected patients/yr
    - Longitudinal samples from 67 pts with probable IA/yr
    - Baseline samples from 902 pts not infected/yr; follow-up sampling is driven by the occurrence of clinical factors that put pt at risk for IA (about 30-40% of pts enrolled at baseline)
- Group 2 sampling expectations
    - Pts with suspected IA/yr
    - 30-40% will have documented IA
    - Of IA cases 30-40% will have proven IA (the rest probable)
    - To collect a target of 30-50 pts with proven IA, we will collect samples from 188-556 pts with suspected IA
- Plan for test evaluation
    - SOPs for test evaluation will be designed using Clinical Laboratory Standard Institute (CLSI) Reference Procedures and FDA Guidance Documents
    - Pre-IDE packet including draft SOPs will be submitted for FDA review and comment
- Planned Analyses
    - Replication (Reproducibility) Studies
        - Initial Repeatability Study
        - Precision
        - Limit of Detection
        - Linearity
    - Accuracy
    - Comparison Studies
    - Interfering Medical Conditions
    - Possible Inter-laboratory Reproducibility Studies
- Technical issues
    - Specimen Stabilization and Integrity
        - Particular issue for RNA and DNA samples
        - Minimum manipulation at collection site preferred
            - reduces risk of contamination
            - less technical expertise and equipment at site
    - Transporting and Storing Fungal DNA
        - May loose sensitivity by storing whole blood
        - Will assess RNAlater® and NucliSENS Lysis Buffer® for stabilization
            - Work with Animal Models group to assess acute stability
            - Perform series of studies to compare DNA stability overtime using an existing repository of samples (some stored up to 6 years in Lysis Buffer)
            - Work to improve storage strategies overtime….*dynamic process*
    - Detecting Fungal DNA
        - Breaking open fungal cell walls for DNA release
            - Requires complex, time-consuming enzymatic or mechanical manipulation
            - Einsele method considered standard in literature, but VERY labor-intensive, open to contamination
        - Fungal DNA contamination of reagents and supplies is a MAJOR, well-documented problem

- No PCR Assay standardized or commercialized for use as a reference method
- Timeline of Study
  - IRB approved protocol: early to mid November 2007
  - Collection of samples to begin January 2008
  - Estimated collection of 3 proven IA cases per month
  - Start of repeatability studies spring 2008
  - Start of comparison studies summer 2008
- Proposed Interactions with IAAM
  - Divided responsibilities for testing diagnostics
    - Early work with manufacturers: IAAM
    - Preparatory for licensure: AsTeC
  - IAAM will provide standards for repeatability and reproducibility testing
  - Coordinated meetings

- Session 5 NEW DIAGNOSTIC PLATFORMS AND TARGETS
  - Margo Moore, *Simon Fraser University,* Siderophores for IA Diagnosis **(view presentation)**
    - Serum inhibits the growth of most pathogenic fungi
    - Iron-binding proteins (e.g., transferrin, lactoferrin) maintain free iron levels at ~10-18 M
    - Strategies for iron acquisition by pathogenic fungi:
      - Secretion of low MW reductants
      - Ferric reductases
      - Siderophores
        - low molecular weight ferric iron chelators
        - Hydroxamate siderophores are secreted by *Aspergillus spp*.
    - Molecules <10 kDa remove iron from transferrin in vitro
    - *A. fumigatus* secretes siderophores in iron-limited media
    - Siderophores purified from *A. fumigatus* grown in serum-containing media
    - Siderophore secretion is required for *A. fumigatus* virulence in a mouse model of invasive aspergillosis
    - Summary
      - *A. fumigatus* can survive and grow in vivo because it produces hydroxamate siderophores which can remove iron from human transferrin.
      - TAF is stable in human serum.
      - TAF is the major siderophore secreted by *A. fumigatus*.
      - TAF is secreted just after germination and does not require fungal lysis.
      - The siderophore biosynthetic pathway is not present in the host.
      - Siderophores have been detected in all strains of *A. fumigatus* tested thus far, and in *A. flavus* and *A. niger*, two other causative agents of invasive aspergillosis.

  - Annette Fothergill & Wieslaw Furmaga, UTHSCSA, Proteomics in IA Diagnostics **(view presentation)**
    - Is Proteomics a Potential Tool?
    - Pilot Study design
      - Proteomic analysis extremely limited by available departmental funds
      - 5 Groups – 3 Animals each group
        - Group 1 - Control
        - Group 2 - 1 hour post exposure
        - Group 3 - 3 days post exposure
        - Group 4 - 5 days post exposure
        - Group 5 - 7 days post exposure
      - Guinea Pigs immunosuppressed at D-2
        - Cyclophosphamide + Cortisone Acetate
        - Repeated at D+3
      - Sera – triplicate testing
      - Urine – triplicate testing
        - Extremely difficulty to collect
        - Samples with obvious blood were discarded
    - Detection Technology

        - Surface-Enhanced Laser Desorption Ionization – Time Of Flight (SELDI-TOF)
        - Qualitative analysis of protein mixtures
        - Utilizes stainless steel chips
            - 1-2 mm in diameter
            - Chemical Bait
            - Hydrophilic
            - Hydrophobic
            - Biological Bait
            - Antibody
            - Enzyme
    - Chip overview
        - Permit differential capture of proteins
        - Uses small volumes of sample (50-150 µl)
        - Bound proteins are laser desorbed & ionized
        - Mass calculated based on time-of-flight
        - <1000Da ->300Kd
        - Unique sample signatures are identified
        - Patterns of masses vs. actual protein ID
        - Possible to ID diseased from healthy specimens
    - CM10 Chip
        - Weak Cation Exchanger
        - Used for protein profiling/biomarker discovery
        - Attracts positively charged proteins
        - Binding is pH dependant
            - Low pH results in more binding
            - High pH results in less binding and thus ↑ specificity
    - Q10 Chip
        - Strong Anion Exchanger
        - Used for protein profiling/biomarker discovery
        - Attracts negatively charged proteins
        - Binding is pH dependant
            - High pH results in more binding
            - Low pH results in less binding and thus ↑ specificity
    - IMAC30 Chip
        - Immobilized Metal Affinity Capture
        - Used for protein profiling/biomarker discovery
        - Analysis of tagged/metal-binding proteins
        - Binding occurs through coordinated metal interaction
        - Selectivity generated by increasing concentrations of competitors
    - H50 Chip
        - Reversed phase/hydrophobic interactions
        - Used for protein profiling/biomarker discovery
        - Proteins partition between lipophilic array surface and binding to buffer
        - ↑ hydrophobic = ↑ binding
    - Summary
        - Screening study only
        - Animal numbers statistically insignificant
        - Peaks of interest were identified
        - Further investigation warranted

- Steven B. Kleiboeker, Viracor, Real time PCR of *A. fumigatus,* **(view presentation)**
    - Project Goals
        - Develop sensitive and specific qPCR assays for the detection of *Aspergillus* genus and species in clinical specimens.
        - Utilize systematically developed animal models of infection for *A. fumigatus*, *A. terreus*, *A. flavus*, and *A. niger* to assess the diagnostic characteristics of real-time (quantitative) PCR assays
        - Implement these qPCR assays into clinical diagnostic use
            - Sensitive and specific detection
            - Speciation
            - Rapid turnaround

- Methods
  - Nucleic acid extraction:
    - Bead-beating followed by standard extraction methods (e.g. MagNA Pure automated extraction)
  - qPCR amplification:
    - Pan *Aspergillus* qPCR – a single TaqMan (hydrolysis probe) assay to detect and quantify DNA from all potentially pathogenic *Aspergillus* species
    - *Aspergillus* species specific qPCR - Multiple TaqMan assays that provide species-specific detection and quantitation of DNA from medically important *Aspergillus* species
      - *A. fumigatus*
      - *A. niger*
      - *A. terreus*
      - *A. flavus*
      - *A. nidulans*
  - Rabbit model
    - Organism: *A. fumigatus*
    - Blinded study on BAL specimens from 24 rabbits in a retrospective neutropenic rabbit model study initiated by Dr. Thomas Walsh's group at NIH
    - Rabbits in this study were infected with *A. fumigatus* only, and some rabbits were treated with AmB as part of the original drug efficacy study
    - Real-time PCR was performed with Pan-Aspergillus assay and 5 species-specific assays
    - Real-time PCR results were compared with culture, CT, and galactomannan data collected during the original study
  - Guinea Pig Model
    - Aerosol guinea pig model: *A. fumigatus*
    - Blinded study on serum and whole blood samples from *A. fumigatus* infected, immunosuppressed guinea pigs in a prospective model initiated by Dr. Tom Patterson's group at UTHSCSA
    - 8 – 12 animals sacrificed at 1 h, 3 d, 5 d and 7 d p.i.; uninfected immunosuppression controls sacrificed at 7 d p.i.
    - qPCR for *A. fumigatus* was performed with and without a pre-amplification step
    - Comparative results (e.g. galactomannan, culture) not yet available
  - Conclusions
    - qPCR detection of *Aspergillus fumigatus* from BAL samples in a neutropenic rabbit model is sensitive and specific (relative to fungal culture) using both a species-specific and pan-Aspergillus assay.
    - qPCR detected *Aspergillus fumigatus* in both serum and whole blood in an immunosuppressed guinea pig model.
    - Early detection post infection was not routinely achieved.
    - Both sensitivity and specificity will require significant improvement.

- Marta Feldmesser, Albert Einstein College of Medicine, Diagnostic Tests for *A. fumigatus*
  - Monoclonal Antibodies in *A. fumigatus* Diagnostics
    - Galactomannan Antibody Engineering in progress
    - Alternative Applications sought
    - Alternative Targets to be investigated
  - Conidial Surface Binding MABs under investigation
    - Galactomannan binding in 5 MABs
      - Bind to JP Latge's purified GM
    - Other binding sites
      - b-1,3-glucan

- 50 (90) kDa protein
- Unknown

- Limitations to Platellia:
  - Sensitivity
  - Specificity
    - Broader than *A. fumigatus* may be OK
- GM binding Monoclonals tested
  - Binding patterns to other microbes
    - Reactive
      - *A. fumigatus*
      - *A. flavus*
      - *A. niger*
      - *Penicillium spp.*
      - *Fusarium spp.*
      - *T. rubrum*
      - *S. pneumoniae* serotype 34
    - Non-reactive
      - *A. terreus*
      - *C. abicans*
      - *C. neoformans*
      - *W. dermatitidis*
      - *E. faecalis*
      - *S. epidermidis*
- Goals of MAB engineering
  - Sensitivity
    - Desire higher affinity
    - Sequence variable regions
  - Specificity
- Affinity Enhancement methods
  - Several noted
  - Each may enhance affinity 5 – 400 fold
  - In combination, may achieve 10000x enhancement
  - Detection to femtomolar range
- Related studies
  - Examine immunotherapeutics
    - Works in Cryptococcus
  - Reexamine other MABs
    - Detection of secreted molecules
    - Detection of other targets

- Tom Walsh, National Institutes of Health/National Cancer Institute, Host/Pathogen Proteomics of Experimental IPA
  - Proteomic Biomarkers in Experimental Invasive Pulmonary Aspergillosis
    - Introduction
      - Models of experimental pulmonary aspergillosis can be powerful tools in the characterization of new biomarkers and in the development of new platforms for detection and therapeutic monitoring.
      - Proteomic technologies carry the potential for a new era in molecular medicine for understanding pathogenesis, improving molecular detection and elucidating host-pathogen interaction.
      - Little is known, however, about the proteomics of invasive pulmonary aspergillosis.
      - Invasive pulmonary aspergillosis may generate a distinctive proteomic signature in serum and bronchoalveolar lavage fluid
    - Methods
      - Proteins in serum and BAL were selectively retained on chip surfaces
      - Analyzed by surface enhanced laser desorption ionization time of flight (SELDI-TOF)
      - Two persistently neutropenic rabbit models of invasive pulmonary aspergillosis (*Aspergillus fumigatus*) and of *Pseudomonas aeruginosa* pneumonia.

- Plasma Processing
  - sampled Day 0 to post-inoculation day (PID) 14
  - denatured (9M Urea, 2% CHAPS)
- Samples applied in duplicate to ProteinChip weak cation exchange arrays (Ciphergen Biosystems)
- Time of flight mass spectra read on PBS-IIc (Ciphergen, Inc)
- Spectra analysis (Ciphergen Express 3.0 Software)
  - peak picking and hierarchichal clustering
  - (>10x signal-to-noise, $p<0.001$)
- Exported data (Matlab 5.1, The Mathworks)
- Student's t-test at each spectral point to identify significant peaks ($p<0.001$)
- Principal Components Analysis (PCA)
  - (JMP 5.1, SAS, Inc)
  - log transformed averaged spectra across group
- ANOVA Aspergillus Time Course (JMP 5.1)

- Results
  - Principal component analysis (PCA) of serum proteins in pneumonias caused by *Aspergillus fumigatus* or *Pseudomonas aeruginosa* demonstrated good separation of groups.
  - Early infections caused by *A. fumigatus* or *P. aeruginosa* clustered together and were not well separated by serum PCA.
  - Analysis of the top 30 spectral points distinguished between aspergillus and pseudomonas pneumonia in later infection with 100% sensitivity and specificity.
  - Analysis of the time course of each separate infectious etiology:
  - The spectral analysis of IPA versus that of pseudomonas pneumonia showed 20 spectral regions with significant differences.
  - A similar spectral analysis of plasma from the aspergillus and pseudomonas groups revealed 28 spectral regions with significant differences.
  - Three significant peaks were common to both plasma and BAL
- Conclusions
  - Proteomic analysis of serum and BAL proteins of experimental aspergillus and pseudomonas pneumonias demonstrates principal components and spectral regions that are shared in early infection and that distinguish the later stages of infection.
  - SELDI mass spectroscopy can identify candidate biomarkers of invasive pulmonary aspergillosis
  - Plasma biomarkers of pneumonia can distinguish between *Aspergillus fumigatus* and *Pseudomonas aeruginosa* pneumonia in neutropenic rabbits.
  - Several candidate biomarkers vary significantly over the time-course of invasive pulmonary aspergillosis and warrant identification with confirmatory techniques.