Molecular Pathology/Probes

Wednesday, August 2, 2017

Poster Session: 9:30 AM - 5:00 PM
Molecular Pathology/Probes

B-204

SHOX2 and SEPT9 genes hypermethylation as biomarkers for plasma-based discrimination between malignant and nonmalignant colorectal lesions

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Background: Colorectal cancer (CRC) is a common malignancy and the fourth leading cause of cancer deaths worldwide. It results from the accumulation of multiple genetic and epigenetic changes leading to the transformation of colon epithelial cells into invasive adenocarcinomas. In CRC, epigenetic changes, in particular promoter CpG island methylation, occur more frequently than genetic mutations. Most cases of CRC are curable if diagnosed early enough. There is a variety of procedures for CRC screening that may be divided into invasive (colonoscopic and sigmoidoscopic examination) and non-invasive methods (fecal occult blood testing (FOBT)). Significant efforts have been invested to develop biomarkers that identify early adenocarcinomas and adenomas with high-grade dysplasia. methylated SEPT9 has been proved to be assay for CRC detection by many studies. However, different analysis methods used currently in data interpretation led to variation in test sensitivity. The aim of the present study was to determine the specificity and sensitivity of SEPT9 and SHOX2 genes hypermethylation as biomarkers for colorectal cancer (CRC). Furthermore, usefulness of these circulating methylated genes will be compared to colonoscopy which is considered as the gold-standard investigation of CRC screening.

Methods: A total of 106 selected individual (25 CRC negative and 81 CRC positive; 50 to 78 years old, 71 male and 35 female) undergoing screening by colonoscopy were included in the study. Circulating DNA was extracted from 3.5 mL plasma samples using Abbott mSample preparation system DNA kit automated on Abbott m2000sp instrument, treated with bisulfite using Abbott Real-time Bisulfite Modification Kit, purified, and assayed by real-time polymerase chain reaction for assessment of DNA methylation of (SHOX2) and (SEPT9) genes, these assays were validated, optimized and evaluated before processing of patient samples. A multiplex polymerase chain reaction combination either SHOX2 or SEPT9 and the reference gene beta gene (ACTB) was performed in triplicate for all specimens.

Results: SHOX2 and SEPT9 genes methylation was significantly higher in patients with malignant colorectal lesions than those with nonmalignant lesions (P<0.001). In detecting malignant colorectal lesions, SHOX2 showed higher sensitivity (97.5% vs. 88.8%) and specificity (92.6% vs 73.5%) than SEPT9. SHOX2 revealed a better sensitivity than SEPT9 in detecting stage I (92% vs 72%) and II (100% vs 93%) CRC, while both markers showed similar sensitivity (100%) in detecting stages III and IV CRC.

Conclusion: SHOX2 and SEPT9 are frequently methylated in CRC patients. Promoter hypermethylation of SHOX2 and SEPT9 may therefore serve as minimally invasive biomarkers for detection CRC. SHOX2 methylation was found to be more sensitive than SEPT9 in detecting stages I and II of malignant CRC lesions.

B-205

Genomic DNA extraction from whole blood: A comparative study between modified salting out technique and spin - column based method

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Background and Objective: DNA extraction from different sources is the essential primary step in any genomic research. Methods used for extraction are evaluated based on the duration, feasibility and cost - effectiveness. Generally speaking, all methods used in DNA extraction should involve disruption of cells / tissues, denaturation of nucleoprotein complex, inactivation of DNase in the sample, and removing contaminants from the extracted DNA. The used method is judged based on the quantity and quality of the yielded DNA. The traditional salting - out technique remained the standard DNA extraction method for years. Researchers started to develop several modifications to this method in order to improve the DNA yield, decrease the extraction time and minimize the cost. Over the past years, spin - column DNA extraction kits became widely used in molecular biology labs. This could be attributed to the simplicity of the technique and the possibility of producing a better quality DNA. In the present study we aimed at comparing the extracted genomic DNA using a modified salting out technique versus that produced from the same peripheral blood samples using a commercially available spin - column DNA extraction kit.

Methods: Peripheral blood was collected from 100 volunteers, in standard EDTA tubes, and DNA extraction from leukocytes was performed using both a modified salting out technique and a commercially available spin - column kit. In this - house modified salting out technique, proteinase K was not used, 1% sodium dodecyl sulfate was used as a detergent in the white blood cells lysis solution, and protein precipitation was performed using ammonium acetate in high concentration. The concentration of the resulting DNA from both methods was measured using Nanodrop spectrophotometer, and the 260 / 280 ratio was checked for all samples.

Results: Comparing the DNA extracted from peripheral blood leukocytes using both mentioned techniques showed a significantly higher concentration using the spin column kit (p = 0.003) than the resulting DNA from the modified salting out technique. On the other hand, the effective deproteinization of both methods (Using the 260 / 280 ratio) did not show any significant statistical difference (p = 0.134). Correlation was tested between the resulted DNA concentration using both methods but it was insignificant (p = 0.7).

Conclusion: Using spin - column based genomic DNA extraction method from peripheral blood results in a yielded DNA with higher concentration than that produced from salting out technique, although the quality (purity) of DNA resulted from both methods is comparable. Further work is needed to assess whether the difference in concentration is cost effective or not taking into consideration that the spin - column technique is more expensive especially when used on a large scale.

B-206

Multi-variant Genetic Panel for genetic risk of opioid addiction

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BACKGROUND
Over 116 million people worldwide have chronic pain and prescription dependence1. In the US, opioids account for the majority of overdose deaths, and in 2014, almost 2 million Americans abused or were dependent on prescription opioids2,3. Genetic factors may play a key role in opioid prescription addiction.

OBJECTIVES
Describe genetic variations between opioid addicted and non-addicted populations and derive a predictive model determining risk of opioid addiction.

METHODS
Design: Case cohort study comparing the frequency of sixteen single nucleotide polymorphisms involved in the brain reward pathways in patients with and without opioid addiction. Data were modeled with TreeNet 10-fold cross validation (Salford Systems, San Diego, CA), and used to generate a weighted score.

Setting: Thirty-seven patients with prescription opioid or heroin addiction and thirty age and gender matched controls were used to design the predictive score. Generalizability of the prediction score was tested on an additional 138 samples.

RESULTS
Method Derivation: Observed data: ROC statistic=0.92 , sensitivity=82% (95% CI: 66-90), specificity=75% (95% CI: 56-87). TreeNet “learn” data: ROC statistic=0.92, sensitivity=92%, specificity=90%, precision=92%, and overall correct=91%. Generalizability: Sensitivity=97% (95% CI: 90 to 100), specificity=87% (95% CI: 86 to 93), positive likelihood ratio=7.3 (95% CI: 4.0 to 13.5), and negative likelihood ratio=0.03 (95% CI:0.01 to 0.13).
Preclinical validation of fluorescence in situ hybridization assay for detection of 5p deletions associated to Cri-Du-Chat syndrome

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Background: Cri-du-chat syndrome is a chromosomal disorder caused by a deletion of the short arm of chromosome 5, which may be visible or submicroscopic. The most important clinical features are a high-pitched cat-like cry, facial dysmorphisms, microcephaly, severe psychomotor and mental retardation. The patients with 5p deletion show a high variability phenotypic and cytogenetic. A critical chromosomal region involved in the cat-like cry is mapped to proximal 5p15.3, while the region involved in the remaining features of the syndrome mapped to 5p15.2. The CTNND2 gene, mapped in this region, is potentially involved in cerebral development and the neurodevelopmental delay that may be associated with mental retardation. The first test to perform is karyotype analysis, which will confirm the diagnosis. In doubtful cases, when there is a conflict between the clinical suspicion and an apparently normal karyotype result, Fluorescence in situ Hybridization (FISH) analysis should be performed. Although the performance of FISH probes has been evaluated by the manufacturer prior to marketing, they also must be validated prior to implementation of assay. Objective: To validate a FISH assay for detection of 5p15.2 and 5p15.3 deletions associated to Cri-Du-Chat syndrome. Methods: We used Cri-Du-Chat and Sotos probe combination manufactured by Cytoceq®. Metaphase cells obtained from 5 karyotypically normal male blood samples were used to localize the probe and determine its sensitivity and specificity. To establish a normal cutoff was estimate the false positive rate from 10 cultured normal blood samples. Two analysts score 200 cells (100 per analyst). All probe signal patterns were recorded. The cutoffs were calculated using the beta inverse (BETAINV) function. FISH analysis was also performed with a sample whose 5p15 deletion previously detected by the karyotype. Results: The Cri-Du-Chat probe presents the FLJ25076 (5p15.31) and CTNND2 (5p15.2) probes labelled respectively with green and red fluorophores. The SOTOS probe (NSD1gene) is labelled in green and is used as a control. In the normal cell, there should be fusion of the red and green signals (2F) and two green signals (2G), whilst a deletion of FLJ25076 probe results in 1F1R2G signal pattern, a deletion of CTNND2 results in 1F3G. The probe demonstrated 100% specificity and analytical sensitivity. In the analysis of 10 normal blood samples were did not observe false positive cells. The normal cutoff for the positive signal pattern was 1.5%. The FISH analysis performed in a sample with 46,XY,del(5)(p15.2) karyotype showed the 1F2G2R signal pattern in 100% of the cells. In this case there was only loss of FLJ25076 probe (5p15.31). Discussion: FISH analysis confirmed the previously identified 5p15 deletion, allowing more accurate detection of the deleted region. The occurrence of mosaicism is a very rare finding. Although we did not observe false positive cells, resulting in a 1.5% cutoff, a case with low number of positive cells should be carefully evaluated. The probe specificity and sensitivity was higher than recommended by the ACMG. This FISH assay showed high quality and reproducibility and was approved for use in our laboratory.

Validation of dual-color, dual-fusion fluorescence in situ hybridization assay for the detection of PML/RARA translocation

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Background: Acute promyelocytic leukemia (APL), comprises 5% to 8% of cases of acute myeloid leukemia (AML). It is typically characterized by neoplastic proliferation of cells in the bone marrow with a promyelocytic phenotype and presence of the fusion gene PML/RARA created by the t(15;17)(q24;q21) translocation. The detection of this translocation by conventional cytogenetic can be hampered by low quantity and quality of metaphases. In this context the fluorescence in situ hybridization (FISH) is applied as an usual and rapid diagnostic tool. FISH can be performed in dividing and nondividing cells, which is important when dealing with leukemia cell with low proliferation. Although the performance of most FISH probes has been evaluated by the manufacturer prior to marketing, each clinical laboratory must individually validate its FISH. Objective: To validate a fast FISH assay for detection of translocation PML/RARA following recommendations from the American College of Medical Genetics (ACMG). Methods: We use the FAST PML/RARa translocation, dual fusion probe manufactured by Cytoceq®. Metaphase cells obtained from 5 karyotypically normal male blood samples were used to localize the probe and determine its analytical sensitivity and specificity. To establish a normal cutoff (1.5) were estimate the false positive rate from 10 uncultured normal bone marrow samples and 10 uncultured normal blood samples. Two analysts score 500 interphase cells (250 per analyst). All probe signal patterns were recorded. The cutoffs for each signal pattern were calculated using the beta inverse (BETAINV) function. The probe hybridization time was only one hour. Results: The FAST PML/RARa probe presents the PML (15q24) and RARa (17q21) probes labeled respectively with red and green fluorophores. A normal result should show 2 green and 2 red signals (2G2R). Two fusion signals in addition to the one green and one red signals (2F1G1R) indicate the presence of the classical translocation. The probe demonstrated 100% specificity and analytical sensitivity. In the analysis of bone marrow and blood samples were identified six atypical signal patterns, but we did not observe false positive cells with the typical positive signal pattern (2F1G1R). The normal cutoff for the 2F1G1R signal pattern was 1.5%, both bone marrow and blood samples. The cutoffs obtained with BETAINV function were validated for counting 200 cells. The analyses of normal and abnormal samples by FISH were in agreement with the karyotype results. Discussion: Immediate treatment of patients carrying the t(15;17) translocation is critical due to the risk of early death. FAST PML/RARa FISH probe allows rapid detection of the rearrangement, with only one hour of hybridization required. The probe specificity and sensitivity was higher than recommended by the ACMG. Adopt a protocol without cell culture using the FAST PML/RARa probe will allow optimizing the process and reducing the release time of the result with the same quality and reliability obtained with the conventional probe. This FISH assay showed high quality and reproducibility and was approved for use in our laboratory.
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Confidence interval (CI) 7.23-23.23, P<0.001 comparing the lowest to the highest quartile of LTL. After search, 6 studies using qPCR to detect telomere length were found, meta-analysis indicated telomere length was significantly shortened in PD (random SMD=0.63, 95% CI 0.03-1.24, P=0.041).

Conclusion: This is the first study exploring the relationship between telomere length and PD in Chinese. Our study indicated telomere length is shortened in Chinese PD patients, and the result is consistent with the pooled result of meta-analysis. These observations suggest that telomere is accelerating shortened in PD patients in comparison to the normal population and shorter telomeres were associated with increased PD risk.

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**B-210**

Multiplex Ligation-Dependent Probe Amplification (MLPA) as a diagnostic tool for detection of a large deletion on the MECP2 gene in Rett Syndrome.

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Background: Rett syndrome (RS, MIM #312750) is a neurodevelopmental progressive disorder, X-linked that occurs almost exclusively in females. It is caused by mutations in the MECP2 (MIM #300005), gene located on chromosome Xq28, which comprises four exons. The prevalence of RS has been estimated to be between 0.25 and 1 per 10,000 female live births. The disorder is characterized by arrested development between 6 and 18 months of age, followed by developmental regression with loss of acquired skills. The patients first lose purposeful hand movements and an interest in the surrounding world, along with speech. They develop apraxia with characteristic stereotypical hand-wringing movements that resemble hand washing, autistic behaviour and learning disabilities. With increasing age, they can also develop additional complex neurologic findings. The most of the pathogenic mutations described in MECP2 gene are located in exons 3 and 4. About 5-10% of the pathogenic mutations described are large deletions spanning whole exons of the MECP2 gene. Multiplex Ligation-Dependent Probe Amplification (MLPA) has been introduced into DNA diagnostic laboratories for the detection of molecular genetic alterations that are of diagnostic and prognostic significance, such as gene large duplications and deletions.

Aims: Validate the MLPA kit P015 in Brazilian patients with RS. Methods: All of the patients included in this study had a consistent clinical diagnosis for this disorder. Thirteen patients with RS were tested using the commercial MLPA kit P405 version A1 (MRc-Holland), following manufacturer’s instructions. Three patients were also tested with Sanger sequencing and one patient was tested by array-comparitive genomic hybridization (CGH). The analysis was performed using the Coffalyser v.140721.1958 software. Results: The MLPA results were concordant in all patients tested with same kit. Three patients presented MECP2 mutations. One of them presented deletion in exons 1 and 2. Another patient presented deletion in exon 3 and one of them presented duplication in exons 1, 2, 3 and 4. Two patients tested by Sanger sequencing presented pathogenic MECP2 point mutations. The same patients not presented alterations in MLPA because the test detect large deletions or duplications. Array-CGH identified duplication in MECP2 gene in one patient. This finding was also confirmed by MLPA. Conclusions: Until recently, no suitable screening method for detecting whole-exon deletions was available. MLPA has become available for the detection of a large deletion on the MECP2 gene allowing genetic confirmation of previously unconfirmed cases of clinical Rett syndrome.

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**B-212**

Cytogenetics findings in a brazilian male population with infertility

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Background: The causes of infertility are diverse, and can occur in men, women, or both. We can mention among the genetic causes of male infertility the aneuploidies, such as Klinefelter Syndrome, translocations between the pseudo-autosomal regions of sex chromosomes, additions or deletions of chromosomal material, mosaicism and inversions.

Objectives: To report the incidence of cytogenetic findings in men referred for karyotype to investigate infertility or gestational loss in a brazilian clinical laboratory.

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**B-213**

An evaluation of Roche cobas MRSA/SA test on the Roche cobas 4800 system


Introduction: Routine screening of patients for methicillin-resistant Staphylococcus aureus (MRSA), an important nosocomial or hospital-acquired pathogen, followed by cohorting of patients tested positive for MRSA, is one of measures to prevent and control spread of MRSA in healthcare institutions.

We evaluated cobas® MRSA/SA test, a qualitative polymerase chain reaction (PCR) assay for detection of MRSA and S. aureus (SA) on cobas 4800 system (Roche Diagnostics, Switzerland), against MRSAselect™ II chromogenic agar plates (Bio-Rad Laboratories, USA).

The cobas 4800 system consists of software-driven fully automated sample processing and pre-PCR preparation module and PCR thermo-cycler.

Materials and Methods

Total of 53 anonymised nasal, axillary and groin swab samples that have been suspended into Mswab™ liquid media (Copan Diagnostics, USA) were tested for MRSA concurrently using PCR and chromogenic culture methods. Data was analysed using binary matrix.

Limit of Detection (LOD) study was conducted using serially diluted liquid media that was spiked with ATCC MRSA strain. LOD was defined as lowest concentration detectable by method.

Within-run precision studies were conducted using assay quality control material and variance of MRSA, SA and Internal Control (IC) cycle threshold values (Ct) were analysed.

For interference studies, liquid media with MRSA concentration 3 times the LOD was spiked with P. aeruginosa and E.coli at up to 1 McFarland equivalent concentrations, methicillin-susceptible SA (MSSA) at greater than 4 McFarland equivalent concentration and 0.1 g/L haemoglobin. Difference in Ct as well as the obtained response of test and control samples were evaluated.

Cefoxitin-resistant S. epidermidis and S. lugdunensis at up to 1 McFarland equivalent concentrations were used to assess analytical specificity.

Results: cobas MRSA/SA test yielded diagnostic sensitivity and specificity of 100.00% and 96.43% respectively compared with chromogenic culture. Concordance between both methods was 98.11%. Discrepant results, when investigated, agreed with the PCR findings. Assay LOD was assessed at 1950 CFU/mL.
Precision studies gave coefficients of variation of 0.821%, 0.690% and 0.707% respectively for positive control MRSA, SA and IC Ct and 1.208% for negative control IC Ct.

Compared with control sample, difference of 0.5, 0.3 and 0.3 Ct for MRSA were observed with test samples with haemoglobin, E. coli and P. aeruginosa respectively. In presence of high MSSA concentration, assay was able to detect MRSA in test samples. Difference of 0.1-0.3 Ct between test and control samples was obtained. The assay did not yield any false negative results. All tubes of cefoxitin-resistant Staphylococcus epidermidis and S. lugdunensis yielded ‘not detected’ for both SA and MRSA.

**Conclusion.** Our data suggests that cobas MRSA/SA test correlates well with chromogenic culture method with good specificities and sensitivities. MRSA detection in samples was also not affected by common contaminants such as haemoglobin, high concentration of MSSA and commonly isolated organisms such as P. aeruginosa and E. coli. No cross reactivity was observed with cefoxitin-resistant coagulate negative staphylococcus. As the cobas MRSA/SA test is largely automated and has high throughput, laboratory productivity is increased which translates to cost savings. cobas MRSA/SA test therefore can play an important role in the epidemiological control of MRSA in hospitals.

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**B-214 Application of Matrix Assisted-Laser Desorption Ionization Time-of-Flight Mass Spectrometry for CYP2D6 Genotype and Copy Number Analysis**

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**Background:** Cytochrome P450 (CYP) 2D6 enzyme activity is known to affect individual responses to pharmacological treatments, particularly variation in drug levels and risk of dose-related adverse reactions. The prediction of CYP2D6 phenotype from genotype is complicated by more than 100 single nucleotide variants (SNV), copy number variations (CNV), presence of pseudogenes and hybrid rearrangements. Recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) has been used to analyze CYP genes including 3A4, 3A5, 2C9, 2C19 and 2D6. The objective of this study was to use MALDI-TOF/MS to identify CYP2D6 SNVs and CNVs in samples collected from venlafaxine treated patients. Of note, venlafaxine is a serotonin-norepinephrine reuptake inhibitor used for the treatment of anxiety and major depressive disorders. SNVs and CNVs of CYP2D6 have been reported to associate with venlafaxine pharmacokinetics and optimal dose.

**Analytical Methods:** Seventeen whole blood samples from venlafaxine treated patients were collected pre-dose at steady state from consenting patients. Concentrations of venlafaxine and metabolites were determined and therapeutic doses were recorded in a previous study. Genomic DNA was extracted (PureGene) and samples were de-identified according to institutional protocols. The MALDI-TOF/MS based MassARRAY® System combined with iPLEX® CYP2D6 panel assay (Agena) was used for CYP2D6 SNV and CNV analysis. The iPLEX® CYP2D6 assay includes a panel of 35 pre-designed SNV assays and 5 CNV assays. Each DNA sample was subjected to a multiplexed PCR amplification followed by shrimp phosphatase treatment to neutralize unincorporated dNTPs. Subsequently, samples were subjected to the iPLEX reactions. In an automated Chip prep module the extension products were desalted, and a nanoliter of each reaction was dispensed onto a SpectroCHIP® Array (Agena). Mass of each allele was detected via MALDI-TOF/MS. Data were analyzed using the Typer™ software (Agena). Report tables were generated with presumed haplotypes to predict allele calls and copy number. CYP2D6 genotypes identified by MALDI-TOF/MS were compared to those obtained previously using the Luminex Bioscience Tag-It Assay.

**Results:** We identified one poor metabolizer (PM) with two copies of nonfunctional alleles (*3/*4) and 4 intermediate metabolizers (IM) with one copy of nonfunctional allele and one copy of decreased functional allele (*5/*4, *5/*4 and *5/*9). Furthermore, 12 out of 17 samples were identified as extensive metabolizer (EM) with two copies of functional alleles (neg/neg or *1/*1). One copy of decreased functional allele (*5/*4, *5/*4, *2A/*4, and *2A/*9) or one copy of one functional allele and one copy of nonfunctional allele (*5/*4, *5/*4, *2A/*4, and *2A/*9). Among seventeen samples five samples were detected with only one copy of CYP2D6 and one sample was detected with more than 2 copies. MALDI-TOF/MS results were found to be 100% concordant with the findings obtained by Luminex technology.

**Conclusion:** The MassARRAY® System combined with iPLEX® CYP2D6 panel assay reported here is a suitable and reliable platform for CYP2D6 SNV and CNV analysis.
Non-invasive fetal sex determination using cell-free fetal DNA isolated from maternal capillary blood obtained by fingertip puncture: the elimination of exogenous male DNA from the collection site is crucial.

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Background: Non-invasive fetal sex determination is a test capable to indicate the baby’s sex at the beginning of pregnancy. This assay searches the Y-chromosome in a maternal blood sample drawn by venipuncture. Its presence indicates the gestation of a boy and its absence indicates a girl. Despite its clinical indications, the greatest demand is from mothers eager to know the sex of their unborn child. With the intention of bringing comfort to the mothers and evaluate the presence of fetal DNA in maternal microcirculation, we idealized to perform it in the capillary blood. Thus, the aim of the present study was to investigate whether fetal sex determination performed on plasma isolated from capillary blood is comparable to venous blood. The latter is a well-established method in our laboratory.

Methods: This study enrolled 101 pregnant volunteers. The gestation weeks ranged from 8 to 20 weeks, and the median was 11 weeks. After asepsis with isopropyl alcohol, venous and capillary bloods were collected at the same occasion in appropriated EDTA tubes. DNA was isolated within two hours (150 uL for capillary and 1mL for venous bloods) and submitted to an automated DNA extraction. The multiplex sequence DYS1-14 was assessed in quadruplicate by qPCR. DNA samples were co-amplified with Invigene’s RNAse P as reference. Nucleic acid was extracted by using QIAamp mini (Qiagen) from capillary blood and with QIAamp DNA blood kit (Qiagen) for venous bloods. The presence of the X and Y chromosome marker was determined by qPCR and relative quantification comparison revealed: R² = 0.99 and bias of -0.0073 log).

Conclusion: Fetal DNA is present in the maternal microcirculation allowing the execution fetal sex determination on the capillary blood. The capillary blood collection is much less invasive than venipuncture bringing comfort to the mother. Moreover, it offers the possibility of home self-sampling. However, exogenous male DNA could be present at the women’s fingertips and for a reliable fetal sex determination by using the above-described method, the elimination of exogenous male DNA from the collection site is critical. Furthermore, the knowledge gained in this study can also impact the forensic sciences, specifically, the touch DNA field.

B-217

Non-invasive fetal sex determination using cell-free fetal DNA isolated from maternal capillary blood obtained by fingertip puncture: the elimination of exogenous male DNA from the collection site is crucial.
Incidence of Turner syndrome in a brazilian female population with infertility


Background: Turner syndrome affects approximately 10% of women between the ages of 15 and 44 and the female factor accounts for 30% of the causes of infertility. Some of the causes of female infertility are: anatomical abnormalities, genetic alterations and age. Among the genetic alterations, we can mention Turner syndrome, translocations between the pseudo-autosomal regions of the sex chromosomes, mosaicism and inversions. For every 2,500 girl born, 1 is a carrier of Turner syndrome, often discovered when there are recurrent miscarriages or amenorhea.

Objectives: To report the incidence of cytogenetic findings in women referred for karyotype to investigate infertility or gestational loss in a brazilian clinical laboratory.

Methods: Analysis of the cytogenetic findings of female karyotype between the ages of 18 and 40, referred for the investigation of infertility, abortion and amenorrhea during the years of 2015 and 2016.

Results: 3,330 cases were referred to the laboratory. We identified 60 altered karyotypes (1.8%), with 24 cases (0.7%) related to the X chromosome, 4 cases (0.1%) of women with karyotype 46,XY and 32 cases (0.9%) with other changes such as inversion, translocation, Robertsonian translocation or the presence of a marker chromosome. Among the related to X, 9 cases were 45,XO (0.27%), 8 cases of mosaicism (0.24%), 2 cases with a deletion on Xq (0.06%), 1 delX (0.03%), 1 isoX (0.03%), 1 dupX (0.03%), 1 translocation (X;5) (0.03%) and 1 mosaic of Turner with a delX (0.03%).

Conclusion: The karyotype is essential for the discovery of chromosomal alterations related to infertility, such as Turner syndrome. Genetic counseling becomes a possibility for these patients with the presence of mosaicism for Turner syndrome. Only 2-10% of syndromic women achieve spontaneous pregnancy, but anomalies on the X chromosome can be transmitted to offspring, increasing the risk of miscarriages, congenital malformations and chromosomal abnormalities.

PRKAR1A Sanger Sequencing and Deletion/Duplication Analysis for the Clinical Diagnosis of Carney Complex


Background: Carney Complex (CNC) is an autosomal dominant condition, characterized by spotty skin pigmentation, endocrine hyperactivity, and myxomas (both cardiac and cutaneous). Cardiac myxomas result in an obstruction of blood flow, embolism, and/or heart failure. The phenotype overlaps with Cushing Disease, Primary Pigmented Nodular Adrenocortical Disease (PPNAD) and other adrenal hyperplasias. The majority of CNC is due to pathogenic variants in PRKAR1A, coding for the type 1-alpha regulatory subunit of protein kinase A. More than 125 pathogenic variants in PRKAR1A have been identified. The estimated prevalence is 1:1,000,000 with 100% penetrance and approximately 160 index cases reported hitherto. Approximately 70% of individuals diagnosed with CNC have an affected parent, and approximately 30% have a de novo pathogenic variant. Sequence analysis of PRKAR1A will detect a pathogenic variant in 60% of probands, while an additional 10% of probands may harbor a large deletion or duplication variant. While immunohistochemical (IHC) staining analysis may be used to screen for CNC, identification of a PRKAR1A pathogenic variant can confirm diagnosis.

Materials and Methods: We have developed and validated a clinical assay for PRKAR1A variants using Sanger sequencing and qPCR for Deletion/Duplication. In this procedure, genomic DNA is first extracted from whole blood, followed by polymerase chain reaction (PCR) amplification of all exons regions and intron/exon boundaries of the gene. Following enzymatic digestion to purify the PCR product and remove it from unincorporated primers and nucleotides, bi-directional Sanger sequencing is performed using universal primers and fluorescent-dye terminator chemistry. Sequencing products are separated on an automated sequencer and trace files are analyzed for variations in the exons and intron/exon boundaries of all exons, using Mutation Surveyor™ software and visual inspection. Deletion/Duplication Analysis consists of a very short (80-150 bp) PCR amplification in a reference gene and in all PRKAR1A exons. During PCR, SYBR Green intercalates into the double stranded DNA structure and the fluorescence increases greatly. The fluorescent signals are used to calculate a relative copy number.

Results: To validate the accuracy of the assay, we compared our Sanger sequencing results with results reported in the 1000 Genomes Project for 4 Corriell samples, and we compared our Deletion/Duplication results with array-CGH results for 3 samples that were previously identified with large deletions or duplications. Assay imprecision was also assessed.

Conclusions: In summary, we have developed and validated a clinical assay to detect PRKAR1A variants including large deletions and duplications. This test will aid in the clinical diagnosis of CNC in individuals, specifically in confirmation of those cases that exhibit loss of immunohistochemical PRKAR1A expression.

Cytogenetics findings in Jacobsen Syndrome

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Background: Jacobsen syndrome is rare and caused by the terminal deletion of the long arm of chromosome 11, more specifically in the 11q23 band. This region is home to a hereditary folate-sensitive site. The first time this disorder was observed was in 1973 by Jacobsen. This syndrome results in a complex phenotype characterized by delayed neuropsychomotor development, craniofacial anomalies, varied cardiac defects, and blood dyscrasias. The diagnosis is initially based on karyotype analysis when the deletion involving the q23 band is identified. It is also possible to obtain confirmation through molecular studies (FISH), where the absence of the FLI-1 and JAM-3 genes is visualized. Studies confirm that only 15% of these alterations may have been inherited and 85% occur de novo.

Objective: To report the efficiency of cytogenetics in the diagnosis of Jacobsen Syndrome in a Brazilian population.

Methods: A survey of karyotypes performed at DASA Cytogenetic Laboratory of São Paulo from January 2014 to January 2017, with a deletion in the 11q23 region visible by microscopy.

Results: During this period, 6,286 constitutional karyotypes of patients up to 20 years old were performed, with the diagnostic hypothesis compatible with some of the
features found in Jacobsen Syndrome. Of these karyotypes, only 3 cases presented the 11q23 deletion.

**Conclusion:** The results demonstrate the small incidence of Jacobsen Syndrome. We can conclude, due to the size of the deletion, that it is possible to be visualized through classical cytogenetics in the analysis of the karyotype with resolution of at least 400Mb, confirming the Jacobsen Syndrome, and guaranteeing the efficacy of the analysis method. However, deletions less than 7.5 Mb which involve the genes for the development of the syndrome may not be identified in the cytogenetic study, being necessary the complementation with the analysis by FISH.

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**B-224**

Minimal Residual Disease Monitoring in AML by RT-qPCR of NPM1 mutations

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**Background:** While many markers exist for cytogenetically abnormal AML, standard-risk AML offers a limited number of markers with which to base treatment decisions. In order to offer physicians at our hospital a better tool for prognostication and treatment, we recently investigated the use of a newly published method to track NPM1 mutation status in peripheral blood as a marker of residual disease. **Methods:** An RT-qPCR method was devised based on work by Ivey et al published last year. We modified the protocol by analyzing various PCR conditions on positive and negative cell line DNA to determine the best conditions for the RT-qPCR reaction. This was then followed by patient samples with various mutational statuses confirmed by the Illumina TruSight Myeloid Sequencing Panel. **Results:** We determined that the RT-qPCR reaction run with primers designed by Ivey et al were able to determine the presence of mutated NPM1 transcripts in peripheral blood of positive patients, though it was not able to distinguish Type A (TCTG insertion) from Type B (CATG insertion). Efficiency of the PCR reaction was determined to be 102% and 97% for the NPM1 and Abl reactions, respectively, with linear correlations of greater than 0.99 across over 1000-fold dilution of RNA. Limit of detection was determined to be 0.1% mutated RNA in wild-type RNA background, with potential for lower limits. Concordance with the TruSight Myeloid panel was 100% between wild-type and Type A mutation patients, with an N of 5 and 2, respectively. In addition, we determined that the RT-qPCR reaction could be run at a single annealing temperature for both the NPM1 and control ABL reactions as well as with a simpler intercalating dye reaction instead of the costlier quenched probe assay without sacrificing specificity or sensitivity. A larger cohort study is now underway in our institution. **Conclusion:** We now have a successful pre-validation assay for use in determining minimal residual disease in NPM1-mutated AML patients. While it is unfortunate that the assay cannot distinguish Type A and Type B mutations, the prognostic information is currently identical for both types, allowing this assay to be used for additional patients. We have also successfully simplified the assay through several means from the original paper, reducing necessary tech time and potential mistakes.

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**B-225**

Association between angiotensin-converting enzyme gene insertion/deletion polymorphism and risk of recurrent miscarriage in Middle Eastern population

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**Background:** Angiotensin-converting enzyme (ACE), a key enzyme in the renin-angiotensin-aldosterone system, converts angiotensin I to angiotensin II. Recurrent pregnancy loss (RPL) has said to be related to the angiotensin converting enzyme insertion/deletion gene polymorphisms (ACE I/D). But the conclusions were controversial. This study was conducted to investigate the real association in ACE I/D polymorphisms and RPL. **Method** A total of 786 women (496 with history of RPL and 290 with no history of RPL; 21 to 36 years old) from 4 different Middle Eastern countries (Egypt [213], Saudi Arabia [297], Qatar [173] and United Arab Emirates [103]) whose knew to be wild type for both factor V Leiden and factor II (prothrombin) genes mutation included in the study. Genomic DNA extraction was performed using Qiagen DNA blood extraction kit, ACE I/D genotype was assayed using validated reverse hybridization polymerase chain reaction (PCR) kit from ViennaLab Diagnostics GmbH. **Results** We found that the genotype frequencies of ACE I/D (24.1% vs. 18.3%) and D/D (59.7% vs. 4.5%) were more seen in women with RPL compared with the women with no RPL history, the difference between the two groups is statistically significant (p<0.001). **Conclusion:** Our data shows a significant association between ACE I/D polymorphism and recurrent miscarriage risk. ACE polymorphic D allele contributes to increased risk of recurrent miscarriage.
Molecular Pathology/Probes

Wednesday, August 2, 9:30 am – 5:00 pm

Confirmation of CGG Repeats in Brazilian Women Tested for Fragile X Syndrome
DASA - DIAGNOSTICOS DA AMERICA, BARUERI, Brazil

Background: Fragile X syndrome (FXS) is a common genetic condition that causes intellectual disabilities, including deficits in development. It affects both males and females of all ages, but the frequency of the disease is higher in males. The repetition of the trinucleotide CGG in the 5’UTR region and the methylation status of FMR1 (Fragile X Mental Retardation 1) gene are the major causes for this disorder. Clinical interpretation is based on the number of CGG repeats and alleles with more than 200 CGG characterizes a full mutation with FMR1 gene inactivation, 55-200 CGG is considered a premutation, 45-54 CGG is considered a grey-zone and in healthy people, this segment is repeated around 5-45 times. Diagnostic testing for FXS usually relies on FMR1 gene PCR and conventional sequencing.

Objective: Here we report the incidence of the identification of just one allele in blood samples from female patients submitted to FXS diagnostic test in a large Brazilian private laboratory.

Methods: 199 samples collected during the year of 2016 from women from zero to 66 years old were evaluated using the Amplidyne™ FMR1 PCR kit. Briefly, a PCR was performed using specific primers that span the FMR1 repeat region. PCR products were sequenced at the 3730xl DNA analyzer and the electropherograms analyzed using the GeneMapper v4.0 software. The archives originated in the 3130xl were analyzed using GeneMapper and the sizes of the alleles were plotted in an excel file that converted sizes to number of CGG repeats using a specific formula.

Results: From all 199 samples, 40% (79) presented only one peak, which represented only one allele; 54% (107) were considered normal; 4% (8) were classified as premutated; 2% (3) grey-zone and 1% (2) fully mutated.

Conclusion: Since it was not possible to define whether the other allele was fully mutated or the difference between the amounts of CGG in both alleles was so small making it impossible to distinguish the difference between them, we established a routine to solve this issue. The injection time during sequencing was decreased from 5 to 2.5 seconds, in order to avoid signal saturation, and after decreasing injection time, it was possible to confirm that the samples that showed only one peak in fact represented signal saturation and looked like CGG repeats of only one allele. After the decrease of injection time, it was possible to observe a small but clear difference of CGG repeats between the two alleles with the separation of the peaks and ensuring the correct result.

Development and Validation of a Quantitative Digital Droplet PCR Assay for Detection of KRAS Mutations in Codons 12, 13, 61 and 146 in Plasma cfDNA
Mayo Clinic and Foundation, Rochester, MN

Introduction: Approximately 30-50% of colorectal cancers (CRC) have mutations in KRAS. Most occur in hotspot regions in codons 12, 13, 61 and 146. These mutations lead to constitutive activation of the RAS/MAPK pathway downstream of EGFR, limiting the effectiveness of anti-EGFR therapies, such as cetuximab and panitumumab, which inhibit ligand-mediated activation of EGFR. Therefore, identification and quantification of these mutations is critical in selecting the appropriate therapy. Liquid biopsies might provide a less invasive and cost effective alternative to tissue biopsies to establish KRAS status.

Objective: To assess the analytical performance of three commercially available KRAS assays: A screening KRAS12, 13 assay for detection of mutations in codon 12 and 13 (G12A, G12C, G12D, G12R, G12S, G12V, G13D), a screening KRAS 61 assay for detection of mutations in codon 12 and 13 (G12A, G12C, G12D, G12R, G12S, G12V, G13D), a targeted assay for detection of KRAS A146T (range 97-117%) and 105% for KRAS A146T (range 92-121%). Plasma DNA from 60 normal donors was negative for each KRAS mutation tested, while all FFPE and reference standards were KRAS mutation positive.

Conclusions: The tested KRAS assays are fast, reliable and accurate in detecting and quantitating KRAS mutations in liquid biopsies for therapy selection and might also prove useful in assessing patients’ responses to treatment.
Performance characteristics study comparing Roche Cobas AmpliPrep/Cobas TaqMan and Cepheid GeneXpert Real-Time PCR-based Hepatitis C Virus (HCV) Assays

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Background: Hepatitis C virus (HCV) RNA detection and quantification are the key diagnostic, prognostic and monitoring tools for the management of hepatitis C. The accurate and sensitive measurement of HCV RNA is essential and critical for the clinical management and treatment of infected patients and as a research tool for studying the biology of HCV infection. The aim of this study was to evaluate the performance characteristics and manufacturer claims of the new Cepheid GeneXpert HCV assay in comparison with the FDA approved Roche Cobas AmpliPrep/Cobas TaqMan (CAP/CTM) assay for HCV RNA quantification.

Method: Accuracy study between the two HCV assays CAP/CTM and the GeneXpert were performed on 167 available samples covering the 6 known HCV genotypes to determine whether the methods are equivalent within Allowable Total Error 20%, specimens were compared over a range of 0.00 to 6.75 Log IU/mL. Commercial HCV controls (genotypes 1, WHO fourth International standard) were used to evaluate linear range, Limit of detection (LOD), Limit of Quantification (LOQ) and precision, analytical specificity (Exclusivity) was evaluated by adding potentially cross reactive organisms with different concentrations into HCV negative EDTA plasma.

Results: The comparison (accuracy) study has been passed; difference between the two methods was within allowable error for 167 of 167 specimens (100%). The average Error Index (Y-X)/TEa was 0.00, with a range of -0.02 to 0.03. The largest Error Index occurred at a concentration of 5.27 Log IU/mL. The reportable range was verified near the manufacturer claimed range and found to be acceptable. The LOD evaluation was performed according to CLSI guideline E17-A2 and HCV RNA concentration that can be detected with a positivity rate of greater than 95% was determined to be 5.0 IU/mL. The LOQ analysis demonstrates that the HCV VL Assay can determine 10 IU/mL (1.0 log10) with an acceptable precision. The precession study passed (SD: 0.082, 95% confidence for SD: 0.057 to 0.150, CV: 1.5) within allowable total error of 20% and allowable random error of 10%. The linearity of the assay was accepted over a measured range of 0.0 to 6.9 log IU/mL within allowable systematic error (SEa) was 10.0%.

Conclusion: Cepheid GeneXpert HCV assay shows excellent correlations and produces highly comparable results if compared to the FDA approved Roche Cobas TaqMan (CAP/CTM) HCV assay. All performance characteristics of Cepheid GeneXpert HCV assay were verified and found to be within manufacturer claim. Cepheid assay is much easier and time saving than Roche assay. GeneXpert instrument modules should be considered as a separate Real-time PCR machines and the validation process should include all modules before testing patient samples.

Incidence of inversion of chromosome 1 as a cause of infertility

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Background: Infertility can be defined as the inability to achieve a pregnancy within one year or the repeated failure to bring a pregnancy to term, most cases of infertility and abortion are not related to chromosomal abnormalities, but in absence of another causal factor, cytogenetic studies of the couple are indicated and may demonstrate infertility caused by changes in sex chromosomes not investigated and/or manifested during puberty or a balanced structural rearrangement.

Objective: To verify the incidence of cases with inversion of chromosome 1 in Brazilian patients with infertility and referred to perform a karyotype in a clinical laboratory.

Methods: Statistical survey of cases from 2014 to 2016 with diagnostic hypothesis of infertility and abortion.

Results: A total of 8,166 normal patients with only 165 altered were analyzed, and only 3 of them present the inversion of chromosome 1. Two male patients and one female patient with diagnostic hypothesis of infertility and repetitive abortion.

Conclusion: The inversion of chromosome 1 corresponds to a very low percentage of the alterations found, being less common than the inversion of chromosome 9. The importance of cytogenetics and the increase of the research on infertility help in the identification, for example, of the polymorphisms of the regions of constitutive heterochromatin, considered normal variations in the population, but that can be decisive for the determination of the cause of reproductive incapacity. The enormous variation of heterochromatin observed among the homologous pairs led several researchers to consider the idea that this difference could hinder the pairing or non-disjunction of the chromosomes, thus predisposing the carriers to alterations in the reproductive capacity.

Detection of an atypical 22q11.2 deletion not including the critical region related to the DiGeorge and Velocardiofacial syndrome

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Background: Deletions of chromosome 22q11.2 comprise the most common microdeletions in humans; it is associated with a highly variable phenotypic spectrum including Velocardiofacial and DiGeorge syndromes. The majority of patients (~97%) with the 22q11.2 microdeletion have either a recurrent 3 Mb deletion from LCR22-A to LCR22-D that contain at least 30 genes, or a less frequent smaller 1.5 Mb deletion that occurs between LCR22-A and LCR22-B. Both deletions include TUPLE1 and TBX1 as candidate genes to the major syndrome phenotypes. The presence of several highly homologous low copy repeats (LCRs) at the proximal end of the long arm of chromosome 22 predisposes to these rearrangements. However, a limited number of patients with atypical deletions have been described. Objective: To report a case of atypical distal 22q11.2 deletion detected by array-based comparative genomic hybridization (array-CGH) using the Agilent platform. Case report: We report a three-year-old girl with neuropsychomotor development delay, malformation of the central nervous system and typical dysmorphic features. The karyotype analysis at 450 band resolution shows 46,XX normal result. Fluorescence in situ hybridization (FISH) analysis was negative using the commercially available TUPLE1 probe. Array-CGH revealed a 369 Kb interstitial microdeletion: arr 22q11.2(19,425,275-
Assessment of six susceptibility variants of LRRK2 on the risk of Parkinson’s disease: a case control study in China

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Background: Leucine-rich repeat kinase 2 (LRRK2) is identified as a candidate gene linked to both familial and sporadic Parkinson’s disease (PD). A number of variants of LRRK2 have been reported to affect the risk of PD but the results were not always consistent in Chinese.

Methods: In this study, we conducted a case-control study comprised 296 PD patients and 297 matched controls to investigate the prevalence of three well-known pathogenic variants (R1441C/G/H, G2019S, I2020T) and three Asian-prevalent (R1398H, R2385R, R1628P) variants, and to assess their roles in the susceptibility to PD. All the patients were recruited from the Department of Neurology of Peking Union Medical College Hospital with standard diagnosis. Controls were age and gender-matched healthy people with no history of neurodegenerative diseases. DNA samples were extracted from peripheral blood and amplified for sequence analysis. Chi-square test was performed to compare frequency distribution of genotypes and alleles, and Hardy-Weinberg equilibrium was verified.

Results: The results showed that all the three pathogenic variations were absent in our study, indicating they were not common pathogenic SNPs in Chinese. PD patients carried a higher frequency of variant R2385R than control subjects (10.8% vs. 5.7%); AA+AG vs. GG OR=2.0 (0.61-3.68); P=0.027; A vs. G OR=1.89, 95%CI 1.05-3.39, P=0.003). However, no significant difference was found in the prevalence of variant R1398H (15.5% vs. 16.2%) and R1628P (2.4% vs. 1.0%) in PD patients and controls. In addition, we found a patient carrying both R1398H and R1628P variants.

Conclusion: Our study demonstrated that R2385R was a risk factor associated with increased PD susceptibility in Chinese, and called for larger samples or comprehensive systematical reviews for the other two Asian-prevalent variations for further confirmation.
Conclusion
We observed an overall diagnostic yield of 30% after unsuccessful initial standard-of-care molecular investigation, suggesting that additional molecular investigation by WES is relevant for identifying pathogenic mutations linked to rare genetic conditions after failure of standard-of-care investigation.

B-235

Disease targeted NGs coupled with homozygosity mapping improves the efficiency of mutation identification in patients with epidermolysis bullosa

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Background: Epidermolysis bullosa (EB) comprises a phenotypically and genotypically heterogeneous group of blistering disorders with the clinical hallmark of skin and mucosal fragility. The lack of definitive clinical findings suggestive of a candidate gene for molecular diagnosis of EB makes patient skin biopsies for electron microscopy and/or immune-epitope mapping necessary as a screening method before mutational analysis. However, antigen mapping is a complicated and demanding technique in terms of expertise and cost and is restricted to a few referral EB centers.

Methods: To circumvent these problems, we developed an EB-targeted next generation sequencing (NGS) panel consisting of 18 genes reported to be causative EB genes (COL1A1, COL1A2, DSP, DST, EXPH5, FERMT1, ITGA3, ITGA6, ITGB4, LAMB3, LAMA3, LAMB1, LAMB2, PKP1, PLEC1, TGM4) and 3 (CD51, CD8D, CHST8) causing a skin fragility disorder in the differential diagnosis. In addition, to find the appropriate candidate genes for sequencing, in consanguineous pedigrees we applied homozygosity mapping with genome-wide single nucleotide polymorphism (SNP) arrays consisting of 550,000 markers.

Results: A total of 94 patients with clinical diagnosis of EB (and two controls) were sequenced. In 50 patients, pathogenic variants were found in one candidate gene and for the others several variants of unknown significance were found in more than one gene. SNP-based homozygosity mapping identified runs of homozygosity of ≥2 Mb, and in 43 probands there was at least one candidate gene identified by co-alignment of the gene with a homozygosity block. Using this approach pathogenic variants were found in 86 out of 93 families (detection rate of 94.6%) in 17 different EB-associated genes. All mutations were confirmed by Sanger sequencing. The most common EB mutated genes were COL1A1, COL1A2, PLEC and LAMB3 (23%, 16%, 13% and 10% of all EB cases, respectively); and, collectively they comprise about 62% of mutated genes. A previously unreported splice junction mutation, the second pathogenic variant in CD51, was found in a patient with pretilial EB and nephropathy. The pathogenic splice mutation leads to exon skipping and was confirmed by RT-PCR from whole skin and Sanger sequencing. The other 5 mutations were located in LAMA3 (5%), LAMB4 (2%), EXPB5 (2%) and FERMT1 (1%). Collectively, genome-wide SNP-based homozygosity mapping facilitates identification of candidate genes in EB families.

Conclusion: The specific mutation information forms the platform for prenatal testing and preimplantation genetic diagnosis, as well as for development of allele-specific therapies in the realm of precision medicine for this group of currently intractable disorders. We developed a disease-targeted next generation sequencing approach which is rapid, minimally invasive and cost-effective in identifying mutations in patients with EB.

B-236

Clinical Utility of Genetic Testing for Mitochondrial Disorders in Adult Patients and the Importance of Tissue-Specific Analyses


Background and Objectives: Mitochondrial diseases are a group of clinically heterogeneous disorders, typically of childhood onset, caused by mutations in mitochondrial DNA (mtDNA) or in nuclear genes encoding mitochondrial proteins. DNA sequencing is considered the gold standard for diagnosis and, historically, the preferred specimen was a muscle biopsy. Over time, however, practitioners have come to favor less invasive screening. Whole mitochondrial genome analysis by next generation sequencing (NGS) was launched one year ago by the Mayo Clinic Molecular Genetics Laboratory and, to date, 110 unique patient samples have been received for clinical testing. Surprisingly, nearly 70% of our specimens were received from adults (≥18 years old). Here we present our data on the clinical utility of mtDNA testing in adult patients, and highlight two unique cases in which a diagnosis could only be made when tissue biopsies were analyzed rather than blood.

Methods: The mitochondrial genome was amplified by long-range PCR, and NGS was performed on the PCR products using a TruSeq Nano library preparation sequenced on an Illumina MiSeq (primers) and an Ion Plus Fragment library preparation sequenced on an Ion Torrent PGMI (comparative). Large deletions detected by the MiSeq were further confirmed by gel electrophoresis. A retrospective study of all patient results and clinical information, when provided, was then performed. Samples received for verification or proficiency testing were excluded from our analyses. All variants detected were classified according to the 2015 American College of Medical Genetics and Genomics (ACMG) guidelines. A positive diagnosis was defined as having one or more likely pathogenic or pathogenic mtDNA mutations as per ACMG guidelines.

Validation: Accuracy was demonstrated for our NGS assay by testing 127 DNA samples extracted from blood, cultured cells, and muscle biopsies that had been previously genotyped using established methods. Results were 100% concordant. Precision was assessed using eight samples run in triplicate both on the same run and across three separate runs. All results were 100% concordant among replicates down to the limit of detection (LOD). The LOD was determined to be 1.875 ng of input DNA for the long-range PCR and 25 ng input PCR product for library preparation. The minimum detectable variant frequency was 6% for single nucleotide variants and insertions/deletions, and 20% for large deletions.

Results: Our diagnostic yield was approximately 15% (16/110). Eighty-eight percent (14/16) of these positive cases were adults and, interestingly, 43% (6/14) of these were above age 60. Of note, two patients who were negative for mtDNA mutations in blood were found to be homoplasmic and 62% heteroplasmic for pathogenic variants in their muscle and renal biopsies, respectively. One case has the oldest known onset of Kearns-Sayre Syndrome, and the other extends the phenotypic spectrum of MT-ND5 mutations beyond Leigh syndrome, MELAS, and optic atrophy.

Conclusion: Next generation sequencing of the whole mitochondrial genome may be especially useful in adult patients suspected of having a mitochondrial disease. Due to heteroplasy, patients with negative testing on blood may benefit from additional testing of other tissues.

B-237

Pathogenic genomic findings revealed by aCGH in a boy with Klinefelter Syndrome (47, XXY) and loss of Xq28: Rett Syndrome or inactive X chromosome?

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Background: Klinefelter Syndrome is a common chromosomal disorder affecting 1/500 live birth males affecting physical and cognitive development. Rett Syndrome (RTT) is a X-linked condition that affects almost exclusively females showing learn and motor skills delay. This condition is mainly caused by loss of functional copy of MECP2 gene (Xq28). The probability for the simultaneous occurrence of Klinefelter and RTT events is very low (about 1/10 to 15,000,000 live births). Here, we described a boy (9y) previously diagnosed by karyotyping with Klinefelter Syndrome, showing a 18 year old. Here we present our data on the clinical utility of mtDNA capillary electrophoresis. A retrospective study of all patient results and clinical information, when provided, was then performed. Samples received for verification or proficiency testing were excluded from our analyses. All variants detected were classified according to the 2015 American College of Medical Genetics and Genomics (ACMG) guidelines. A positive diagnosis was defined as having one or more likely pathogenic or pathogenic mtDNA mutations as per ACMG guidelines.

Methods: Next generation sequencing of the whole mitochondrial genome may be especially useful in adult patients suspected of having a mitochondrial disease. Due to heteroplasy, patients with negative testing on blood may benefit from additional testing of other tissues.

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patients with IQ42-q2 duplications present craniofacial dysmorphisms but a quite normal psychomotor development. However, Xq28 deletions including MECP2 gene is lethal in men and clinically relevant in women. The present case has an extra X chromosome that allows its compatibility with life. To our knowledge, this event is uncommon in the literature. All these findings lead us to hypothesize that proband must present RTT phenotypic characteristics. If not, is possible to assume that extra X chromosome with Xq28 deletion must be preferentially inactivated. Conclusion: Based on these results it is necessary follow-up proband’s endophenotype and family members to investigate eventual familial common alteration. The next step is to investigate X inactivation to confirm our hypothesis that suggests a preferential X inactivation as important modulator factor of the severity of the RTT phenotype in these patient. The use of AGH allows the identification of new genomic findings and helps clinicians at diagnosis of rare genetic syndromes. The familial study is essential for genetic counseling and a better understanding of neurological disorders.

B-238

Validation of a Quantitative Digital Droplet PCR Assay for Assessment of EGFR T790M Mutation Status using Plasma cfDNA


Background: The release of tumor-derived cell-free DNA (cfDNA) into circulation allows for rapid and non-invasive plasma-based tumor genotyping. Plasma-based genotyping provides a solution to some of the limitations of traditional tissue-based genotyping, which include slow turnaround time (TAT), limited tissue biopsy material, and the risk of failure due to sampling bias. In the case of non-small-cell lung cancer (NSCLC), determining the EGFR p.T790M mutation status is critical for treatment decision-making. Two-thirds of NSCLC patients who carry activating EGFR mutations and experience disease progression after being treated with an EGFR-TKI have developed the T790M resistance mutation. Early detection of the T790M mutation may provide benefit by allowing an earlier switch to an alternative therapy without necessity of a repeat biopsy. The objective of this study was to evaluate the analytical performance of the Bio-Rad EGFR T790M digital droplet PCR (ddPCR) assay for the identification and quantification of EGFR p.T790M mutations in plasma cfDNA. Methods: A high-throughput semi-automated ddPCR assay was developed using commercial reagents from Bio-Rad. Plasma cfDNA was extracted using Qiagen’s QIAamp Circulating Nucleic Acid kit. ddPCR was performed in a 96-well plate format using an automated droplet generator (AutoDG, Bio-Rad), a standard thermocycler and a fluorescence droplet reader (QX200, Bio-Rad). A Bio-Rad multiplexed TaqMan probe-based assay, designed to detect both the EGFR c.2369C>T (p.T790M) mutation and the corresponding wild-type nucleotide, was utilized. Analytical performance was assessed using reference standards (Horizon Discovery), cell-line derived EGFR p.T790M mucosal DNA (ATCC #H1975), tissue-derived tumor DNA, and cfDNA from patients and healthy donors. Using these materials, the following parameters were measured: specimen stability, intra- and inter-assay imprecision, linearity, accuracy, carryover, analytical specificity and cross-reactivity, LOD and LOQ, analytical turnaround time and reference intervals. Results: Plasma specimens were stable up to 21 days stored at -80°C. Intra- and inter-assay imprecision of twenty replicates were measured at five different concentrations of mutant DNA (EGFR p.T790M) spanning the anticipated analytical measurement range. Probit regression analysis of this data determined that the LOD was five mutant droplets (15 copies/mL of plasma). CVs were <20% at concentrations as low as 40 mutant DNA copies/mL of plasma. Dilution linearity was demonstrated from 3750 to 30 copies/mL of plasma. The lowest reportable copy number (LOQ) was determined to be 40 mutant DNA copies/mL of plasma. Increasing wild-type DNA (over two orders of magnitude) had no impact on the accuracy of mutant DNA concentrations. A reference interval study failed to find any detectable mutant DNA copies in 60 healthy individuals. A blinded method comparison study was performed using tumor-derived DNA and demonstrated 100% concordance between the Bio-Rad ddPCR assay and the FDA-approved Roche Cobas EGFR v2 plasma cfDNA assay (n=20). Conclusion: The Bio-Rad EGFR T790M ddPCR assay performed using the AutoDG/QX200 system is a robust, economical and rapid method for determining EGFR T790M status from plasma cfDNA.

B-240

Performance of a multi-level, multi-analyte external assayed quality control developed for the detection of Healthcare-associated infections (HAIs)


Background: Bio-Rad recently obtained FDA clearance for Amplichek II as an external assayed quality control material to monitor the performance of the Cepheid GeneXpert assays targeted for HAI detection. The Amplichek II quality control product consists of 4 levels targeted for HAI assays manufactured by Cepheid: Level Negative containing Methicillin sensitive S. epidermidis (MSSE), Level 1 containing Methicillin Sensitive S. aureus (MSSA), Level 2 containing low concentrations of Methicillin Resistant S. aureus (MRSA), C. difficile (Cdiff), and Vancomycin resistant Enterococci (VRE), and Level 3 containing high concentrations of MRSA, Cdif, and VRE. The performance of the product was tested on the appropriate Cepheid assays over 24 months, on multiple control and reagent kit lots. The Amplichek II product was also tested on four additional HAI testing platforms.

Although the Cepheid assays provide qualitative assessments for the presence of HAIs, we used the data from a precision study to assess whether the semi-quantitative C values could be used to track assay performance. Here, we show an example of the potential use of C values to track performance over a period of 24 months on the Cepheid Cdif assay.

Methods: The Amplichek II product, stored at 2-8°C for the length of the study, was tested on the appropriate Cepheid assays at the following time points: 4, 8, 12, 16, 20, and 24 months. The qualitative data results were analyzed as % agreement with the expected results for each of the time points. Additional platform testing of Amplichek II was assessed for Level Negative and Level 3 only. The additional platforms in the study were the BD GeneOhmTM/Max (VRE and MRSA), Nanosphere Verigene (Cdif), Luminex xTAG (Cdif), and Meridian Illumigene (Cdif), for respective analytes. The results were analyzed for % agreement with the expected results. A precision analysis was performed by monitoring C values on the Cepheid assays according to recommendations provided by CLSI document EP5 (2 replicates tested twice a day, over a period of 10 days, 40 total replicates). The data was analyzed using Microsoft excel’s Analyze-IT software package. The Cepheid assays %CV analyzed using semi-quantitative Ct values is ~6%. Based on this finding, the semi-quantitative C values, from the Real Time stability study (for a representative analytic, Van A, with 6 %CV), was assessed using a Levey Jennings plot.

Results: Amplichek II was in 100% agreement of expected results throughout the 24 month shelf life of the product for all levels and all analytes. Additional platform testing shows that Level Negative and Level 3 were in 100% agreement for all analytes, on all platforms.

Conclusion: Bio-Rad’s Amplichek II control provides an independent assessment of the performance of in vitro laboratory nucleic acid testing procedures developed for the detection of HAIs.

B-241

Performance of a bi-level, multi-analyte external unassayed quality control across multiple sample type configurations, for molecular diagnostic platforms detecting sexually transmitted infections


Background: The CDC estimates that the incidence of sexually transmitted infections (STIs) is 20 million new cases every year in the United States, accounting for almost $16 billion in healthcare costs. Part of the prevention and treatment plan includes the surveillance of STIs through the use of numerous FDA cleared molecular diagnostic tests. Unlike molecular diagnostic tests of other diseases, tests targeted for STIs utilize multiple patient specimen types ranging from swab to cytology specimens, which require additional precaution during validation and quality assurance. Bio-Rad Amplichek STI external unassayed quality control was developed to monitor the performance of these assays in the various specimen or sample types, to add confidence in the reliability of test results. The use of quality control materials is indicated as an objective assessment of the performance of methods and techniques in use and is an integral part of good laboratory practice. Amplichek STI consists of a negative level (negative for CT, NG, and HPV, while containing HPV negative human cells) and a positive level (positive for CT, NG, and HPV), developed for use across multiple STI molecular diagnostic assays and various specimen type configurations (e.g. swab, urine, PreservCyt).
Molecular Pathology/Probes

Methods: A comprehensive performance evaluation study was conducted on the AmplexRed STI product, in order to assess its performance across the various specimen types validated for use by the assay manufacturer, for 8 commonly tested STI assays for the detection of CT, NG, and HPV. The study was conducted using 3 separate testing sites, with 2 replicates per day, over a period of 5 days (n=10 replicates per site, for a total of 30 replicates).

Results: The results were within 100% agreement for both the Level negative and Level positive, on all assays, across all sample type configurations.

Conclusion: Bio-Rad’s AmplexRed STI is the first molecular control formatted for use across multiple sample type configurations on common molecular assays used in the detection of STIs, providing an independent assessment for laboratories to maintain good laboratory practice.

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Precision of a multi-level, multi-analyte external assayed quality control for molecular diagnostic platforms monitoring viral load of blood borne pathogens.


Background: Monitoring the viral load for the blood borne pathogens, HIV, HCV, HBV, facilitates in assessing the prognosis and viral response to treatment. The Abbott RealTime and Roche COBAS AmpliPrep/COBAS TaqMan are FDA cleared to monitor the viral load of these blood borne pathogens. Bio-Rad Laboratories’ AmplexRed 1 is an external independent assayed quality control developed to monitor the performance of these assays to add confidence in the reliability of test results obtained for unknown specimens. The routine use of assay agnostic quality control materials is indicated as an objective assessment of the precision of methods and techniques in use and is an integral part of good laboratory practices.

Methods: Precision tests were performed for AmplexRed 1 based on the guidelines recommended by CLSI document EP5, in which two replicates were tested twice a day, over a period of ten days (n=40 total). AmplexRed 1 Level Negative (negative for HIV, HCV, and HBV), Level 1 (low positive for HIV, HCV, and HBV), Level 2 (low positive for Roche) and Level 3 (high positive for HIV, HCV, and HBV, targeted for both platforms), were tested on the Roche assays for the respective analytes. AmplexRed 1 Level Negative (negative for HIV, HCV, and HBV), Level 2 (low positive for HIV, HCV, and HBV, targeted for Abbott and Level 3 (high positive for HIV, HCV, and HBV, targeted for both platforms), were tested on the Abbott assays for the respective analytes. Precision analysis was performed using the Microsoft Excel “Analyze-it” tool package for precision measurements.

Results: The precision results were within the expected assay performance documented in the respective manufacturers’ package inserts, for all the analytes tests.

Conclusion: Bio-Rad’s AmplexRed 1 provides an independent assessment of the performance of in vitro laboratory nucleic acid testing procedures for the quantitative detection HIV-1, HBV and HCV on the Abbott RealTime and Roche COBAS molecular diagnostic platforms.

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Electrochemically Modified Sensitive Nitric Oxide Sensors for Detecting Nitric Oxide at the Level of Single Cells.

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Background: Nitric oxide (NO) is an important biological molecule that has diverse functions in human physiology. The concentration of NO in tissues and cells are of vital importance and the presence of too low NO concentration is the source of a variety of diseases. Measurement of NO in biological samples is a challenging task because of its rapid chemical reactions with a wide range of biomolecules, its nano-molar concentrations in tissues and its very short half-life of approximately a few seconds. In general, NO measurement techniques can be classified as direct (measuring NO itself) and indirect methods. Analytical techniques for direct measurement of NO include electrochemistry; fluorometry and electron paramagnetic resonance (EPR). Amongst them electrochemical tools are most promising because they allow fabrication of miniaturized probes with electro-catalytic surfaces which greatly enhances the sensitivity and selectivity for direct, real time and accurate measurement of NO in cell lines and tissue samples along with enabling very low limit of detection.

Methods: In this work, we fabricated combined reference/working carbon fiber electrodes with 7-micron diameter tips for direct placement near the cell lines. The exposed surface of the fiber tip was electrochemically modified with ruthenium oxide and Poly(3,4-ethylenedioxythiophene) (PEDOT). To improve the selectivity of our sensors we coated the surface with an ionic liquid composite and measured the response to NO using Differential Pulse Amperometry to differentiate NO response from other interferents present in biological samples. Madin-Darby Canine kidney (MDCK) epithelial cell lines were used to detect NO release with our sensor. To get a detailed analysis of the temporal and spatial resolution of the NO release, simultaneous monitoring by fluorescence and electrochemical method was performed.

Results: With our method of sensor modification we attained a normalized sensitivity of 2.82E-4 pA/nM/cm² and 4.31E-4 pA/nM/cm² towards NO in the linear range of 0.1 - 3.2 μM and 2-16 nM (biologically relevant range) respectively, with a R² value of 0.993. The limit of detection (LOD) of our modified sensor to NO with a signal to noise ratio of 3 was as low as 250 pm. Such an enhanced LOD is unique to our method of sensor modification in comparison to other work done in this field. In terms of selectivity our sensors could effectively discriminate amongst the major biological interferents that are present near NO producing systems. The interference (%) was 0.0003 for 1.0 mM L-Arginine; 0.0002 for 1.0 mM NO; 0.11 for 0.1 mM NO, and 0.015 for 0.1 mM ascorbic acid.

Conclusion: The relevance of our work to clinical laboratory medicine is the development of a highly sensitive and selective NO sensor capable of direct and accurate measurement of very low concentration of NO at the level of single cells and biological samples due to its excellent linearity in a relatively wide concentration range as well as selectivity towards NO in the presence of interferents. Thus, our sensors can be used to study clinical pathology of diseases where NO release is significantly decreased.

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Microparticles derived from tissue factor, leukocyte, endothelium and neuron are associated with Alzheimer’s Disease

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Background: In the last few years, microparticles (MPs) have been studied as new specialized structures for intercellular communication. MPs are important messengers in cell-cell communication and contribute to the induction of endothelial damage, inflammation, and angiogenesis, carrying signaling molecules, such as chemokines, cytokines, enzymes, growth factors, receptors, adhesion molecules, mRNAs and microRNA. This study aimed to evaluate the levels of MPs derived from platelets, leukocytes, endothelium, tissue factor and neuron in Alzheimer’s Disease (AD) patients compared with cognitively healthy individuals.

Methods: 54 individuals were recruited and classified as probable AD (29 patients - 15 men and 14 women, age 72.9±7.0 years) and cognitively healthy individuals (25 controls - 8 men and 17 women, age 73.2±7.7 years). Blood samples (citrate plasma) were collected and MPs were isolated by ultracentrifugation and measured by flow cytometry. Trucount control tubes were included as a quality control. Statistical analyses were performed using Mann-Whitney test on SPSS program version 13.0. Values of p < 0.05 were considered significant.

Results: The median (interquartile range) levels of MPs (MPs/μL) derived from tissue factor [78.8 (82.2)], leukocytes [109.9 (86.9)], endothelium [40.9 (76.5)] and neuron [200.3 (362.4)] are significant higher in AD group than in control group [37.4 (13.4), 39.0 (27.3), 21.8 (20.8) and 41.4 (72.4), respectively; all p<0.05]. MPs derived from platelet did not differ between the groups (p = 0.167).Conclusion: The results suggests that MPs derived from tissue factor, leukocytes, endothelium and neuron could be associated with the physiopathology of AD and, in the future, the MPs may be included as diagnostic biomarker for AD in the clinical routine.