Development of a Multiplex CYP21A2 Genotyping Assay for Congenital Adrenal Hyperplasia Screening

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Forms of Congenital Adrenal Hyperplasia (CAH)

- **Classic CAH**
  - Salt Wasting: Severe to complete loss of 21OH activity
    - Elevated Stress response
    - Loss of electrolyte homeostasis
    - Adrenal crisis can lead to hypotension and cardiac arrest
  - Simple Virilizing: Partial 21OH activity
    - Normal sodium balance
    - Elevated androgen production, partial to complete masculinization in females

- **Non-Classic CAH**
  - Late-onset: slight decrease of 21OH activity
  - Not life-threatening but results in significant quality of life issues
Primary CAH Newborn Screen

- Primary newborn screening assay by time-resolved fluoroimmunoassay (FIA) for 17-α OHP

- FIA high false-positive rate
  - 17-α OHP levels are high in premature and/or stressed babies
    - Birth weight or gestational age stratification for 17OHP cut-offs
    - Second specimen screening programs
    - Second-tier LC-MS/MS steroid profiling

- Lack of specificity with FIA
  - Second-tier organic extraction of DBS
Rationale for CAH Molecular Second Tier

- Minnesota Department of Health identified classic CAH children missed by newborn screening
- Many of false negative results had 17OHP levels below FIA assay cutoff
- Steroid profiling and organic extraction 2\textsuperscript{nd}-tier methods reduce false positive rate but do not improve false negative rate

Pilot to test if lowered 17OHP cutoffs and 2\textsuperscript{nd}-tier mutation detection increases overall sensitivity for detecting CAH while retaining assay specificity
CAH Molecular Second Tier Screening Study

- “Can molecular testing improve newborn screening performance and outcomes for CAH?”
  - University of Minnesota Masonic Children’s Hospital
  - Minnesota Department of Health
  - CDC’s Newborn Screening & Molecular Biology Branch

- Project funded by

- Three major goals:
  - Determine Minnesota population CYP21A2 mutation panel
  - Develop genotyping assay appropriate for NBS laboratory
  - Pilot test molecular second-tier method and evaluate assay performance and cost effectiveness
Goal 1: CYP21A2 Mutations in Minnesota

- Enrolled 83 families affected by CAH
  - 200 total specimens
  - ~70% with prior genotype information

- Long-range PCR and DNA sequencing
  - Confirm CYP21A2 genotypes and genotype unknown samples
  - Characterized 30kb Deletion and Gene Conversion samples

- Identified 22 CYP21A2 mutations
  - 12 common diagnostic panel mutations
  - 10 additional non-panel mutations
  - Novel IVS9+1 G > T splice site mutation
PCR-Based Detection of Chromosome Deletion and Gene Conversion Alleles

Most-common chromosome arrangement in normal population

30Kb Deletion

Gene Conversion

Pseudogene
Common Diagnostic CYP21A2 Mutation Panel

Non-Classic
- P30L

Simple Vir.
- Ivs2 G

SW-CAH
- 30kb Δ
- Intragenic Δ
- Gene Conversions

E1
- E2
- E3
- E3Δ8
- I172N
- E6 Cluster: I236N V237E M239K
- E6
- V281L
- E7
- F306 + t
- Q318X
- E8
- R356W
- E9
- E10
- P453S

Recombination events account for ~30% of CAH-causing mutations
Expanded Minnesota CYP21A2 Mutation Panel

Non-Classic
- P30L
- H62L
- Ivs2 G

Simple Vir.
- E3Δ8
- I172N
- V237E
- M239K
- V281L
- F306 + t
- L307G
- Q318X
- R356W

SW-CAH
- 30kb Δ
- Intragenic Δ
- Gene Conversions

E6 Cluster:
- I236N
- V237E
- M239K
- F306 + t
- L307G

Poster 60: Minnesota Population Spectrum of Congenital Adrenal Hyperplasia Causing Mutations in the CYP21A2 Gene; Detwiler
Goal 2: NBS Genotyping Method Development

- Detection of 30 kb deletions and gene conversion alleles
  - Benchtop capillary electrophoresis and automated data capture

- Multiplex mutation detection method
  - Allele-Specific Primer Extension for 21 mutations
  - Luminex instrument also used for CFTR at MDH laboratory

- Validation and accuracy of lab-developed method compared to provided genotypes
  - Sensitivity
  - Specificity
  - Positive Predictive Value and Negative Predictive Value
Automated capillary electrophoresis and data capture of CYP21A2 functional gene, 30kb Deletion, and Gene Conversion alleles

100% reproducibility for same day and between day repeats
Performance of PCR-Based Assay to Detect 30kb Deletions and Gene Conversions

<table>
<thead>
<tr>
<th></th>
<th>CYP21A2 Detection</th>
<th>30kb Deletion Detection</th>
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<tbody>
<tr>
<td>True Positives</td>
<td>131</td>
<td>59</td>
</tr>
<tr>
<td>True Negatives</td>
<td>9</td>
<td>122</td>
</tr>
<tr>
<td>False Positives</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>False Negatives</td>
<td>1*</td>
<td>2**</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.992</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>(0.958 – 0.999; 95% CI)</td>
<td>(0.881 – 0.991; 95% CI)</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>90%</td>
<td>93.8%</td>
</tr>
</tbody>
</table>

*CYP21A2 false negative paired with 30kb deletion
**Both 30kb false negatives with potential hemizygous CYP21A2 allele
Allelic Ratios for Robust ASPE Genotyping

- Normalized plot representing the signal for each allele

  Mutant signal
  
  \[(\text{Mutant signal} + \text{Normal signal})\]

- Luminex default ratio values
  - \[0.75 \leq 1.00 = \text{Mutant only}\]
  - \[0.25 \text{ to } 0.75 = \text{Heterozygous}\]
  - \[0.00 \leq 0.25 = \text{Normal only}\]

- Final ratios determined empirically for each probe using inter and intra day repeats
Performance of Genotyping Assay to Detect CYP21A2 Mutations

- Highly specific and accurate on initial test of 190 patient and family samples
  - No False Positive Calls
  - No False Negative Calls

- 186 samples passed for all probe sets (97.9%)
  - 4 out of 190 samples gave an equivocal result
    - 3 normal specimens with EQ-Low for p.Arg426Cys
    - 1 specimen EQ-Low IVS2-13 A/C > G and EQ-High p.Ile172Asn
  - 99.9% robustness with 100% accuracy per genotype
Conclusions and Next Steps

- CYP21A2 genotyping method is sensitive and accurate
- Method transferred in February 2016 to MDH
- Validation for use with newborn DBS ongoing
- MDH pilot of molecular second tier to start in 2016
  - Describe assay efficacy and utility in newborn screening
  - Analyze cost effectiveness
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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.
CYP21A2 Genotyping Assay Workflow

DNA extraction and reagent preparation

- CYP21A2 PCR: 1.5 hours
- 30kb Deletion PCR: 1.5 hours
- Gene Conversion PCR: 1.5 hours

Capillary Electrophoresis to detect CYP21A2 gene, 30kb deletion, or Gene Conversion alleles: ~1 hour

Secondary PCR, clean-up & ASPE Reaction: 4 - 5 Hours

Bead Set Hybridization

Targeted ASPE Luminex