Overview of CF and CF Genotyping Platforms

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APHL Molecular Training Course
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Presentation Overview

- **Part 1**
  - Brief summary of cystic fibrosis
  - Newborn screening for CF
  - Biochemical assays vs. molecular assays
  - CF screening algorithms in U.S.

- **Part 2**
  - CFTR gene structure
  - Standard vs legacy mutation nomenclature
  - Description of methods – advantages & limitations
What is Cystic Fibrosis?
Disease and Symptoms

- **Chronic disease of the lungs and digestive system**
  - Mutations in the *CFTR* gene (encodes a chloride channel)
  - CFTR channel found in cells producing mucus, sweat, saliva, tears, and digestive enzymes
  - Imbalance of chloride ions into & out of the cell affects mucus consistency
  - Mutations affect production, structure, or stability of the channel

- **Symptoms**
  - Thick, sticky mucus
  - Salty sweat
  - Failure to thrive (pancreatic insufficiency)
  - Many more
From Mutations to Symptoms: Cause & Effect

- **CFTR GENE**
- **CFTR PROTEIN (CHANNEL)**
- **ION TRANSPORT**
- **ALTERED SECRETIONS**
- **BLOCKED DUCTS & IMPAIRED MUCOSAL DEFENSE**
- **INFECTION, INFLAMMATION**
- **CYSTIC FIBROSIS**
Lung Disease
(Chronic infection, inflammation, and airways obstruction)

Salt Loss
(high sweat electrolytes--diagnostic test)

Gastrointestinal Abnormalities
(pancreatic insufficiency, malabsorption, and malnutrition)

CYSTIC FIBROSIS
Autosomal recessive disorder
(1/4000)*

Other Clinical Manifestations
(intestinal obstruction, cirrhosis, diabetes, etc.)

Sweat chloride ≥60 mEq/L traditionally used for diagnosis, although lower levels are compatible with CF
(Farrell and Koscik, Pediatrics 1996;97:524-528)

*Estimated incidence by ethnic/genetic background:
White Americans ~ 1/3000
Hispanic Americans ~1/6000
African Americans ~1/10,000
(Comeau et al, Pediatrics 2004;113:1573-1581)
Treatments*

- **Improve Protein Function**
  - FDA review of Kalydeco/Lumacaftor combination for people who have 2 copies of F508del

- **Airway Clearance**
  - Manual or mechanical techniques
  - Inhaled medication – mucolytics or hypertonic saline

- **Antibiotics**
  - Oral, intravenous, or inhaled

- **Nutrition**
  - Pancreatic enzymes
  - Monitoring calories

Why Is CF One of the NBS Disorders?

- **1997 CDC Workshop**
  - Evidence for nutritional benefit; more research needed

- **2003 CDC Workshop**
  - Recommend CF as newborn screening disorder
  - MMWR October 15, 2004 / 53(RR13);1-36

- **2006 Recommended Uniform Screening Panel**
  - CF included as a primary condition

Scientific evidence demonstrated that early diagnosis of CF resulted in better nutritional and health outcomes due to early intervention.
Public Health Benefit*
Median Predicted Age of Survival was 40.7 years in 2013
How CF Molecular Assays Complicated Your Lives

- New concepts to understand
- New nomenclature & terms
- New methods to learn (DNA extraction, PCR, assays, interpretation)
- Multiple techniques to detect mutations
- Multiple mutation panels
- Unique unidirectional workflow requirements
- Specific environmental Burden of contamination
- Detection of carriers
- Multiple algorithms available to adopt
Comparison of U.S. Mutation Panels

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CF NEWBORN SCREENING ALGORITHMS*
THE GOOD, THE BAD, AND THE UGLY

IRT/IRT
IRT/DNA
IRT/IRT/DNA
IRT/DNA/EXTENDED GENOMIC ANALYSIS (EGA)

Algorithm 1: IRT/IRT

If IRT level is elevated, a second sample is collected and tested

Advantages

- Carrier status is not determined
- Does not require carrier genetic counseling
- Biochemical test easily incorporated into NBS laboratory

Limitations

- Best suited to second specimen states
- Complicating variables
  - IRT level variation (increasing age, sick and low birth weight, race/ethnicity)
  - Issues with assay kits have been documented
- Difficulty setting cut-off limits due to IRT variation
Algorithm 2: IRT/DNA

If IRT level is elevated, DNA from the blood spot is tested

Advantages

- Second specimen is not required
- Less time to final result (about 1 week)
- Improved detection sensitivity
- Facilitation of follow-up planning
- Facilitation of interpretation of sweat chloride test results
- Reduction of false negatives from high IRT not due to CF

Limitations

- Increased cost for testing and genetic counseling
- More sweat tests for CF carrier infants
- Mutation panel may not reflect population
Algorithm 3: IRT/IRT/DNA

If IRT level is elevated, a second sample is collected and, if it is still elevated, DNA is tested from the second spot.

Advantages
- Improved detection sensitivity by lowering IRT cut-offs
- IRT can be done on a subset of second specimens
- Fewer CF carrier infants detected
- Screening can be completed without a second specimen

Limitations
- Best suited to second specimen states
- Need for genetic counseling
- Mutation panel may not reflect population
Algorithm 4: IRT/DNA/EGA

If IRT level is elevated, DNA from the blood spot is tested. If only one mutation is detected, sequencing is performed to determine if a second mutation exists.

Advantages

- Only babies with two or more CFTR mutations and/or variants are considered screen positive
- CF carrier infants detected but not referred for sweat chloride testing
- With time, have better understanding of mutations in the population

Limitations

- Higher cost
- Longer time until final screening result
# Algorithm Summary

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<td>Baseline</td>
<td>Increased</td>
<td>Somewhat increased</td>
<td>Decreased</td>
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* Theoretically, increase in cost is recouped or decreased if only a subset of 2nd specimens are tested for IRT.
THERE IS NO RIGHT WAY OR WRONG WAY FOR CF NEWBORN SCREENING
Cystic Fibrosis Key Points – Part 1

- CF is caused by mutations in the *CFTR* gene (chromosome 7)
- Kalydeco is a drug therapy now available for some CF patients
- NBS algorithms used to detect CF
  - IRT/IRT (no molecular component)
  - IRT/DNA & IRT/IRT/DNA: elevated IRT → *CFTR* mutation(s)
  - IRT/DNA/EGA (elevated IRT → *CFTR* mutations → gene sequencing when only 1 mutation is found)
- There are several different panels of mutations used by NBS labs that perform a molecular test.
  - Programs that use a panel include at a minimum the recommended ACMG 23 *CFTR* mutations
CFTR Gene Structure
CFTR Gene Structure
CFTR Gene Structure
CFTR Gene Structure
**HGVS vs. Legacy Nomenclature**

- **Human Genome Variation Society guidelines** facilitate uniform and standard nomenclature of DNA and protein sequence variants

  - **HGVS nomenclature recommends**
    - Sequence variations should be described at the DNA level
    - DNA name: “g” for genomic or “c” for cDNA followed by nucleotide number(s) affected by the change
      - may be an insertion, deletion or substitution
    - Protein name: “p” followed by the affected amino acid, the aa number and the substitution

- **Legacy nomenclature**
  - DNA names used for intron mutations, deletions, and insertions
  - Protein names used for both substitution and nonsense mutations
### CFTR HGVS Nomenclature

#### Legacy Exon # vs. Exon #

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<th>xTAG CF Kit Name/ Legacy Name</th>
<th>HGVS DNA Name</th>
<th>HGVS Protein Name</th>
<th>Legacy Exon or Intron</th>
<th>Exon or Intron</th>
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#### Exon Changes

# CFTR HGVS Nomenclature

## Insertions

**Ex:**

AGGTACCTG ATCGCTGAA

AGGTACCTGGATCGCTGAA
**CFTR HGVS Nomenclature**

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<th>xTAG CF Kit Name/Legacy Name</th>
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**Deletions**

Ex: 
AGGTACCTCTTGCTGAA
AGGTACCT     GCTGAA
CFTR HGVS Nomenclature

Frameshift Deletion
Ex:

Thr  Glu  Gly  Gly  Asn  Ala  Ile  Leu  Glu
ACA GAA GGT GGA AAT GCC ATA TTA GAG

Lys  Lys  Val  Glu  Met  Pro  Tyr  STOP
A-AG AAG GTG GAA ATG CCA TAT TAG AG
### CFTR HGVS Nomenclature

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#### Substitutions

**Ex:**

AGGTACCTGATCGCTGAA

AGGTACCTAATCGCTGAA
Assays to Detect Mutations in CFTR

- **Single mutation detection (F508del)**
  - Gel based assays to discriminate size differences
  - Fluorescent detection Taqman assay

- **Multiplex mutation detection**
  - xTAG CF Assay – Luminex Corporation
    - xTAG 39
    - xTAG 60
  - InPlex CF Assay – Hologic (Invader technology)
    - InPlex – 23 mutations
    - InPlex – 40 mutations
  - DNA sequencing
    - Unlimited within amplicons
xTAG Cystic Fibrosis Assay Technology
Luminex Corp

Multiplex PCR Rxn
Amplicon Treatment

Allele-specific Primer Extension

Bead Hybridization

Reporter Addition

Data Acquisition

https://www.luminexcorp.com/Products/Assays/ClinicalDiagnostics/xTAGCysticFibrosis/
Probes and Invader oligos bind to specific DNA sequences creating a flap which is then cleaved when the desired sequence is present. Flaps combine with fluorescence resonance energy transfer (FRET) probe generating a fluorescent signal. Fluorescent Detection

http://www.inplexcf.com/laboratory/inplextechnology.html
CFTR DNA Sanger Sequencing

When a dideoxy DNA base is incorporated, the DNA synthesis stops.
Case Study of a Newborn with Elevated IRT

- DBS was detected with elevated IRT above the 4% Cutoff → Reflex to 2\textsuperscript{nd} Tier Mutation Testing

- Initial Assay: Luminex xTAG 39
  - Two probes representing mutations Y1092X C>G and Y1092X C>A failed both the initial and repeat run
  - Repeat specimen was requested with the same results

- Secondary Assay: Inplex CF - 40
  - No mutations detected – both Y1092X probes gave a normal result
Case Study of Newborn with Elevated IRT

- DBS was detected with elevated IRT above the 5% Cutoff → Reflex to 2\textsuperscript{nd} Tier Mutation Testing
- Assay: Luminex xTAG 39
  - Two probes representing mutations Y1092X C>G and Y1092X C>A failed both the initial and repeat run
  - Repeat specimen was requested with the same results
- Sample sent for DNA sequencing of Exon 20
  - Baby was “homozygous” for Y1092H T>C
Known Mutations in CFTR Exon 20

CFTR Mutation Database: http://www.genet.sickkids.on.ca/
Y1092H’s proximity to Y1092X resulted in a failure of the Luminex Y1092X probes to hybridize.
Is this Case Study Done??

- Was the baby **homozygous** or **hemizygous** for Y1092H T>C?
  - hemizygous is when there is only 1 member of a chromosome segment rather than the usual 2

- Could there be a large deletion of Exon 20????

- How could this be determined????
  - **Approach 1:** Sequence Exon 20 in both parents to see if they both have Y1092H T>C
  - **Approach 2:** Perform a molecular deletion assay such as MRC Holland’s MLPA which can detect 1 versus 2 copies of Exon 20
Case Study Take Home Messages

- Assay failures can offer important information
- No assay can catch everything
- Assays used in newborn screening labs do not detect most large deletions
- Know your state’s policies
  - What is your program responsible for and what is diagnostics responsible for in your state?
  - How do you communicate your findings in the most meaningful way to diagnostic partners?
Cystic Fibrosis Key Points Part 2

- HGVS nomenclature describes the nature of the mutation which is different from the legacy nomenclature previously used for CFTR mutations.
  - Eg. F508del (legacy) vs. c.1521_1523delCTT (HGVS)
- There are two commonly used technologies used in the U.S. to detect a panel of CFTR mutations
  - InPlex CF Assay from Hologic – probe hybridization and invader technology
  - xTAG CF Assay from Luminex – primer extension
Thank you!

Newborn Screening

Saving Lives.
Promoting Healthier Babies.
Protecting our Future.

For more information please contact Centers for Disease Control and Prevention

1600 Clifton Road NE, Atlanta, GA 30333
Telephone: 1-800-CDC-INFO (232-4636)/TTY: 1-888-232-6348
Visit: www.cdc.gov | Contact CDC at: 1-800-CDC-INFO or www.cdc.gov/info

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.

Use of trade names and commercial sources is for identification only and does not imply endorsement by the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, the Public Health Service, or the U.S. Department of Health and Human Services.
**CFTR HGVSNomenclature**

- **Exon changes**
- **Deletions**
- **Insertions**
- **Substitutions**
- **Frameshifts**

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<td>pThr1220LysfsX8</td>
<td>Exon 19</td>
<td>Exon 22</td>
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CFTR Gene Structure