Development of a PZA Pipeline to Improve Phenotypic and Molecular-Based Susceptibility Testing for PZA

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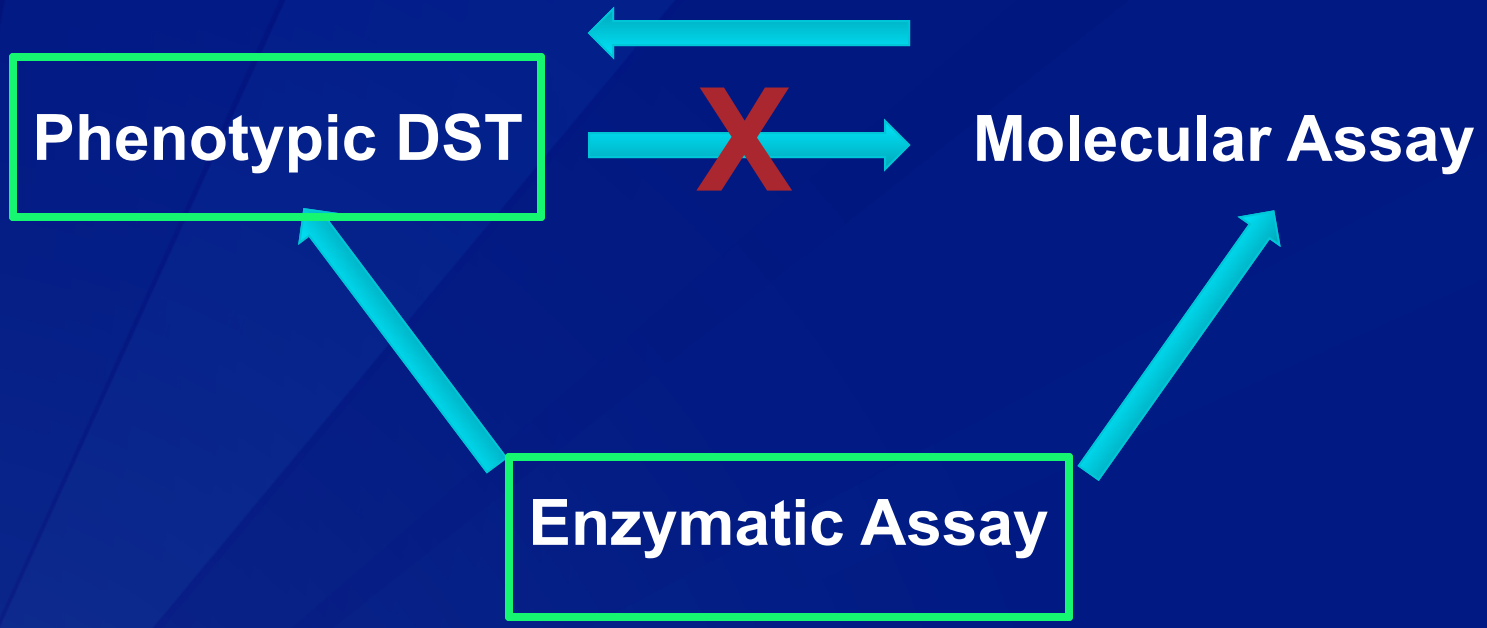
National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention
Division of Tuberculosis Elimination

Strategy



PZA *in vitro* Activity

- **Requires acidic culture conditions**
  - Narrow pH range
    - Too alkaline = no PZA activity
    - Too acidic = inhibition of growth in drug-free media
    - pH 5.6 – 6.0 best
- **Active against *M. tuberculosis* complex**
  - Except *M. bovis*
    - Inherently PZA-resistant

Phenotypic Detection of PZA Resistance

- **Indirect**
  - Pzase activity[1]
    - PZase positive = PZA-susceptible
    - PZase negative = PZA-resistant
- **Direct**
  - Culture methods
    - Growth on or in media containing PZA
    - Problematic
      - Both resistant and susceptible bacilli inhibited by acid pH
      - Inoculum size
    - Poor reproducibility[2, 3, 4]
      - **False resistance** and susceptibility

1. Wayne(1974), Amer. Rev. Resp. Dis., **109**:147-151.
2. Chedore, et. al., (2010) J. Clin. Mirobiol,, **48:**300-301
3. Chang, Yew and Zhang, (2011), Antimicrob. Agents & Chemo., **55:**4499-4505.
4. Simons, et. al., (2012), J. Clin. Microbiol., **50**:428-434.

Factors Affecting *in vitro* PZA Activity

- **pH of the medium[1]**
  - Can inhibit growth (~ 10% of strains)
  - Theoretical calculations[1] (Henderson-Hasselbach equation)
    - If PZA MIC = 50 µg/ml @ pH 5.5
      - MIC @ pH 6.1 = 200 µg/ml
  - Experimental results correlate well[2]
- **Inoculum size[1]**
  - Large inoculum reduces PZA activity
    - Much more pronounced than other drugs
    - Neutralization of media
      - Ammonia produced by deamination of PZA

1. Zhang et. Al. (2002), J. Med. Microbiol. **51**(1):42-49.
2. Salfinger and Heifets (1988), Antimicrob. Agents Chemother. **32**:1002-1004.

Research Plan

- **Improve MGIT testing protocol**
  - Hypothesis = False resistance is caused by over inoculation
  - Experimental Approach
    - Test reduced inoculum sizes (i.e., # of bacilli)
    - Test Time Past Detection (TPD) of seed tube
  - Pilot Project (16 strains)
    - Evaluate new dilution scheme
    - Evaluated time post positive of seed tube
    - 10 strains (with no *pncA* mutation) repeatedly found resistant using standard inoculation protocol were susceptible using a reduced inoculum

Research Plan

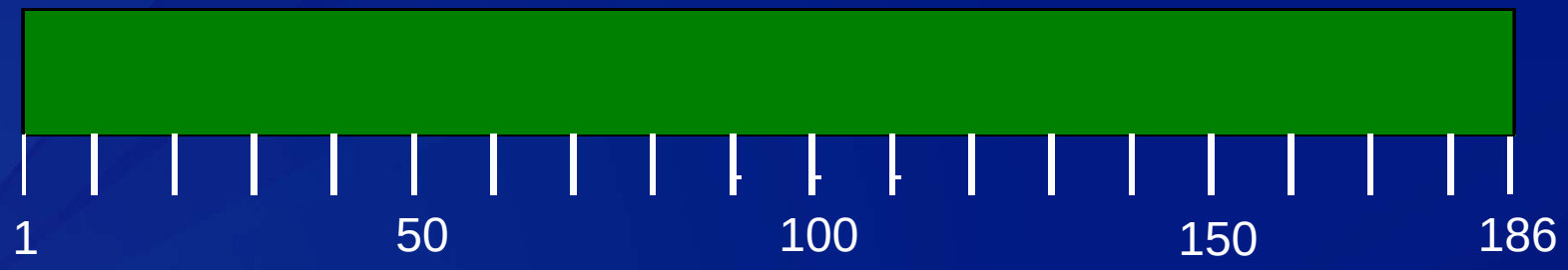
- **Impact of specific *pncA* mutations on PZA MIC**
  - Phase 1: High Concentration Range
    - PZA = 0, 300, 400, 600 & 800 µg/ml
    - NAm = 0, 1600, 1800, 2000, 2500 µg/ml
  - Phase 2: Low Concentration Range (when necessary)
    - PZA = 0, 50, 100, 200, 300 µg/ml
    - NAm = 0, 100, 300, 600, 1200 µg/ml
  - Phase 3: Susceptible Isolates (WT *pncA*)
    - Determine the best MIC range
    - Test new dilutions for 3-5 days post positive seed tubes

Molecular Diagnosis of PZA Resistance

- **What is the first requirement for development of a great molecular diagnostic?**
  - A mechanism of resistance
- **Mutations within *pncA* are associated with PZA resistance in *M. tuberculosis*.**

PncA

- 186 amino acids
- No “hotspot” for mutations
  - DTBE LB culture collection contains isolates with mutations at 70 different amino acids
  - A database of published mutations within PncA describes isolates with mutations in an additional 45 positions
- Multiple different substitutions can occur at each position
  - For example: the histidine at amino acid 57 has been changed to an arginine, aspartic acid, leucine, proline, or tyrosine in different isolates
- Some strains contain insertions or deletions in *pncA*
- Some strains contain mutations in the promoter region that would likely affect the amount of PncA protein made

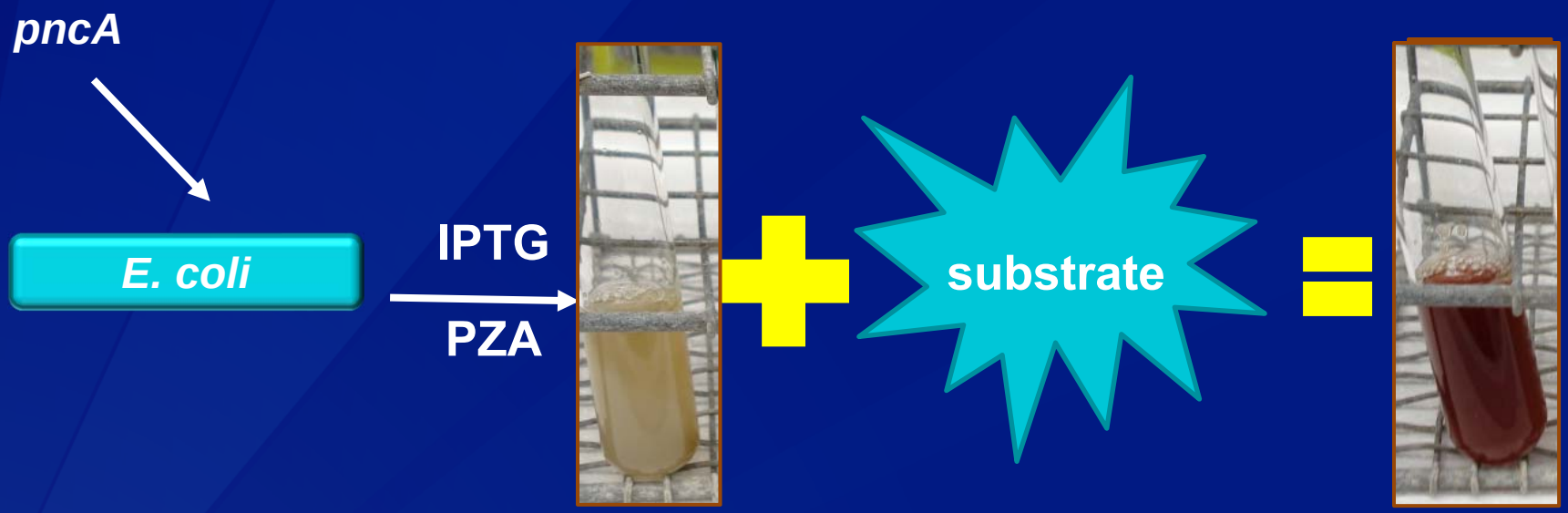


Goal:
Design a rapid, simple system to evaluate the effect of PncA mutations on PZase activity

PncA Enzymatic Assay

- Use *E. coli*
- Transfer mutant *M. tuberculosis pncA* alleles into an *E. coli* strain that does not produce its own PncA
- Make expression of the mutant PncA controllable
- Examine PZase activity of mutant *pncA* allele
  - Add substrate to *E. coli* culture
  - Observe color change

PncA Enzymatic Assay



Red color change = functional PZAase → susceptible

PncA Enzymatic Assay

Vector Only Control

1 2 3 4

WT PncA Control

1 2 3 4

Tube	IPTG	PZA	Expected Results
			Resistant
1	+	+	no color change
2	+	-	no color change
3	-	+	no color change
4	-	-	no color change

Correlate PZA MIC with Enzymatic Activity

pncA Mutation Collection

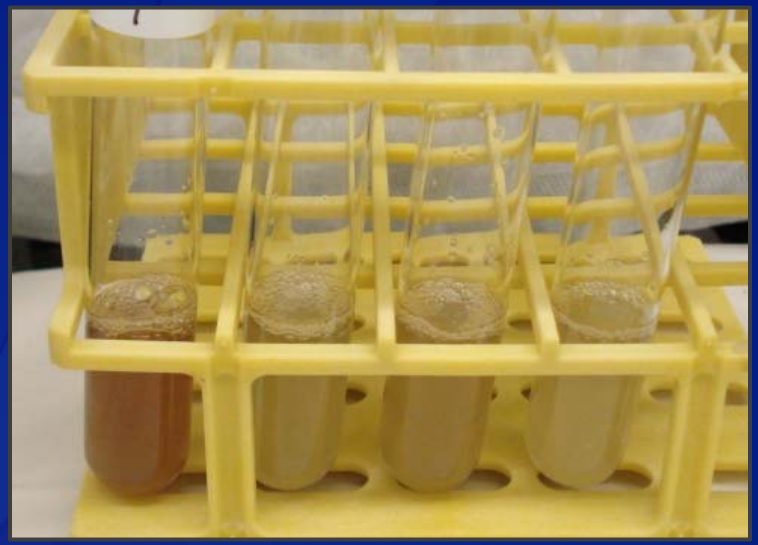
- **124 strains with a SNP**
  - 76 with MIC and enzymatic data
    - 50 unique SNPs
    - Occur at 39 different codons
- **32 strains with an indel or promoter mutation**
  - 22 with MIC and enzymatic data (indels only)
    - 14 unique indels
    - 4 unique promoter mutations
    - 100% exhibited MIC > 800 μg/ml for PZA

MIC and Enzymatic Data of 50 Unique *pncA* nSNPs

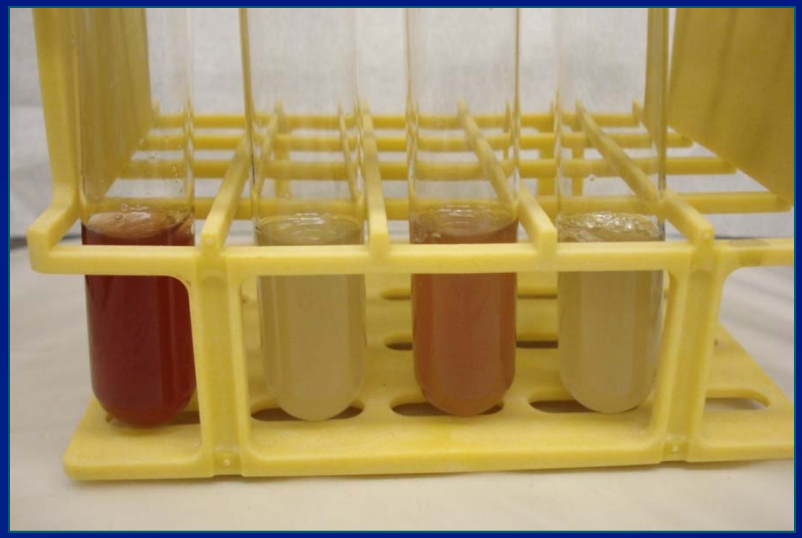
ENZYME ACTIVITY LEVEL	MIC LEVEL (μg/ml)	NUMBER OF STRAINS (%)
0 (not detected)		36 (Total)
	> 800	23 (64)
	400 - 800	8 (22)
	< 300	5 (14)
1-5 (weak to very strong)		14 (Total)
	> 800	1 (7)
	400 - 800	1 (7)
	< 300	12 (86)

Val139Gly MIC > 800 μg/ml

Asp110Gly MIC < 300 μg/ml



His137Arg MIC < 300 μg/ml



Glu37Val MIC < 300 μg/ml

Interesting Mutation

- **Codon 47**
  - Threonine to Proline (N = 2)
    - PZA MIC > 800 μg/ml
    - NAm MIC > 2500 μg/ml
  - Threonine to Alanine (N = 5)
    - PZA MIC < 300 μg/ml
    - NAm MIC < 1600 μg/ml

Summary

- **Improved the MGIT 960 PZA assay**
  - Reducing the size of the inoculum
- **Developed an PncA enzymatic assay**
- **Correlate a *pncA* mutation with PZA MIC and enzyme activity**
- **Useful for developing molecular assays**


Phenotypic DST
Molecular Assay
Enzymatic Assay

Future Work

- **Continue to perform MIC and enzymatic assays**
  - CDC collection
  - Collaborators
  - Susceptible strains (WT *pncA*)
- **Structural modeling of PncA mutants**
- **Generate antibody to PncA**
- **Develop a database**
  - Available to scientific community
  - MIC and enzymatic data for each mutation

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