Development of an Aspergillus Calibrator:
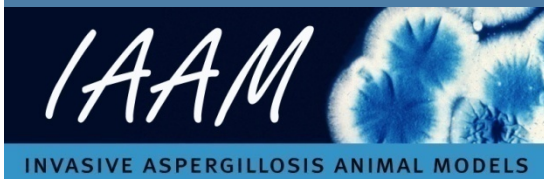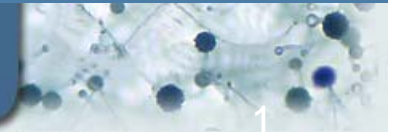
An ASTEC and IAAM Collaboration

Angela M Caliendo, MD, PhD
Emory University School of Medicine

Development of a Calibrator

- Why is a calibrator needed

- How are standards and calibrators developed
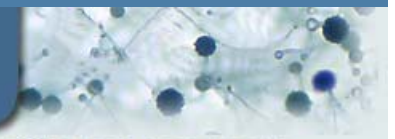
- Our plan for an Aspergillus calibrator

Why is a calibrator needed?

Experience with viral load testing
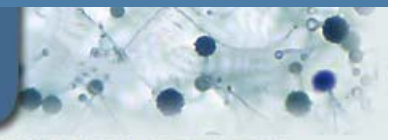
Multicenter Study: CMV Viral Load Tests

- 12 samples
  - 2 negatives (CMV seronegative plasma)
  - 7 samples - dilutions of purified nucleocapsid stock

- 3 clinical samples
  - UL54 mutation (not ganciclovir resistant)
  - UL97 mutation (ganciclovir resistant) and gB mutation
  - No mutation

- 33 laboratories in USA, Canada, Europe
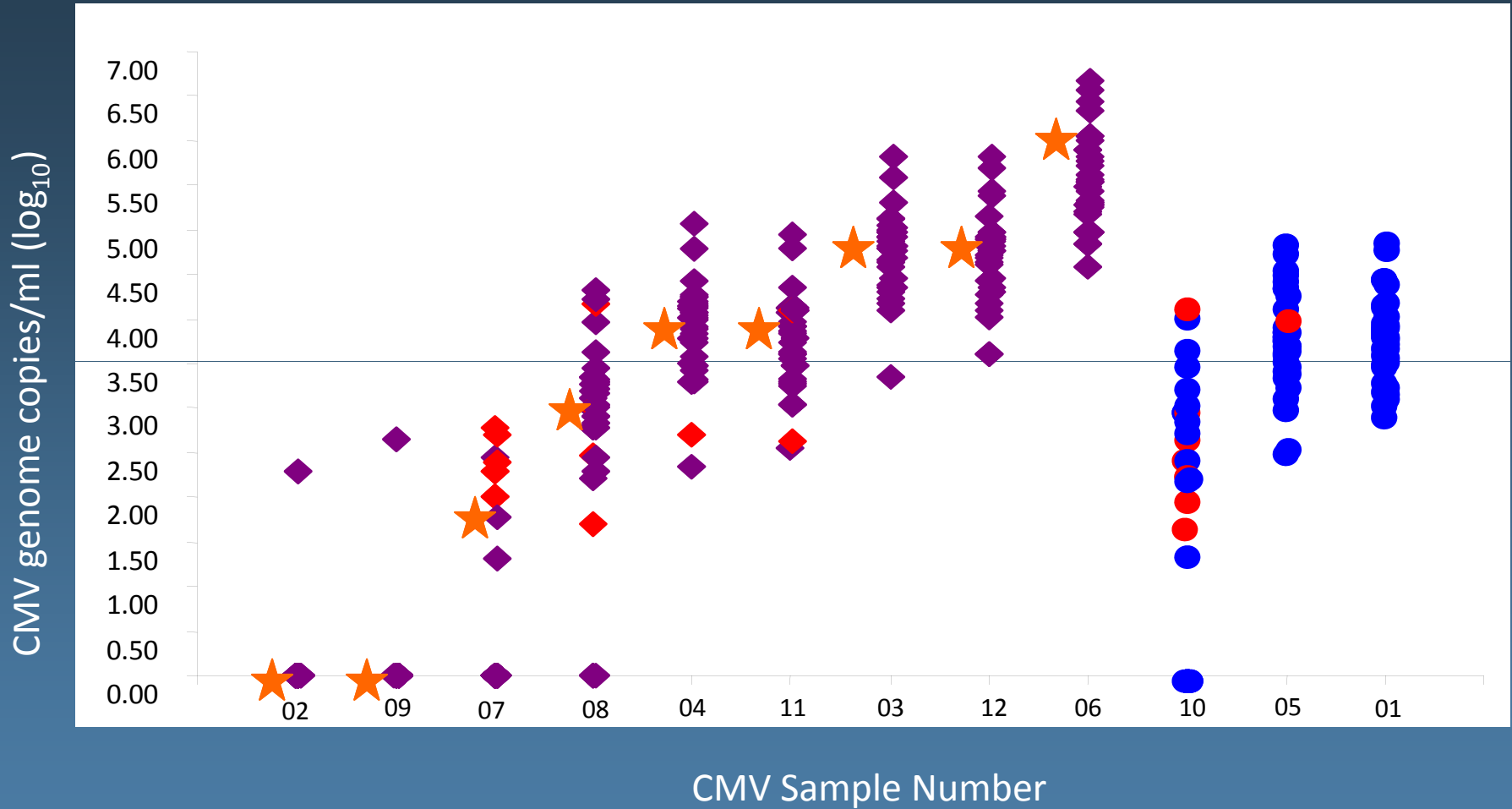
Pang et al. Am J Transplantation. 2009;9:258-268.

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Multi-Center CMV Study

Pang et al. Am J Transplantation. 2009;9:258-268

Why don't the values agree?

- Primers and probes may have different efficiencies of amplification

- Extraction method
  - Volume of specimen used (sensitivity)

- Use of different calibrators to determine VL
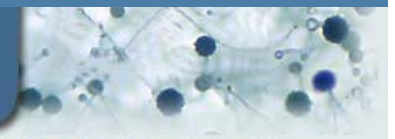 - International standards under development

Why is this important?

- Viral load values are used to determine when to begin therapy and monitor response to therapy

- Viral load values don't agree among labs
  - Determining clinical important cutoffs is difficult

- If you change tests/labs there can be a very big impact on viral load values and clinical decisions

Same test (same run) using the IAAM and Emory calibrators (standard curve)

Sample Number	IAAM (ng/ul)	Emory (ng/ul)	Fold Difference
1	7.7	117.3	15.2
2	1.1	17.7	16.1
3	8×10^{-2}	1.5	18.8
4	9×10^{-3}	1.8×10^{-1}	20
5	9.1×10^{-4}	1.9×10^{-2}	20.1
6	1.2×10^{-4}	3.0×10^{-3}	18.8
7	1.2×10^{-5}	2.9×10^{-4}	24.2
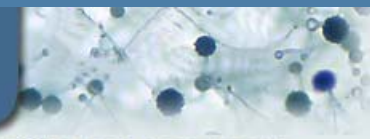
Fungal Calibrator

- **Variability between results from the two tests**

- **Likely more variability when more laboratories are included**

- **Develop a material that allows comparison of the performance of various tests**
  - LOD, LOQ, reproducibility, linearity

How are International Standards Developed

- **WHO International Standards are recognized as the highest order standards**

- **WHO approves these standards, do not make them**
  - NIBSC in the UK, CBER/FDA, Paul Ehrlich Inst

- **NIBSC**

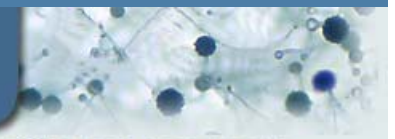 - Standardization of Genome Amplification Techniques (SoGAT)

The WHO Biological Standards Process

- Call for candidates

- The standard is produced

- Worldwide testing
  - Tests available at the time

- Establishment of a value in IU

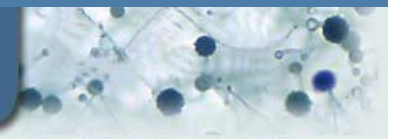- Replenishment is made by comparing with the previous standard.

The WHO Biological Standards Process

- SoGAT started with blood safety

- Developed the IS that are in global use
  - HIV-1, HCV, HBV

- Commercial tests are calibrated to the IS

- Various manufacturers provide calibrators that are traceable back to the IS
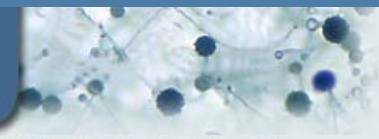 - Used in clinical laboratories

The WHO Biological Standards Process

- WHO standards are biological standards

- Consensus process for determining the concentration

- SoGAT expanded into clinical diagnostic testing

- International standards used to assess accuracy
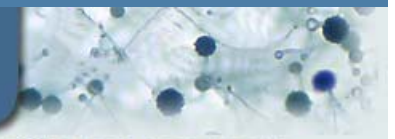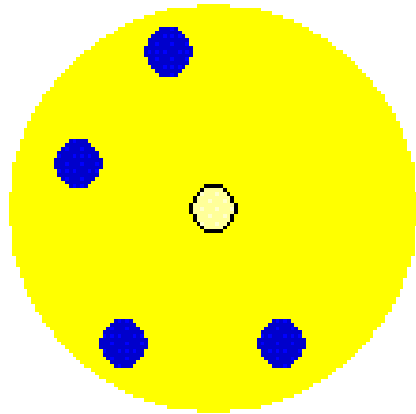 - Define the bulls eye on the target

Precision versus Accuracy

Not accurate,
Not precise
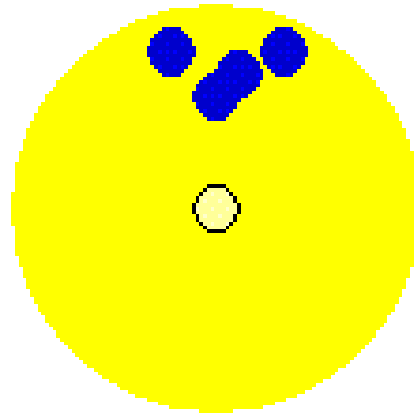
Not accurate,
Precise

Accurate,
Precise

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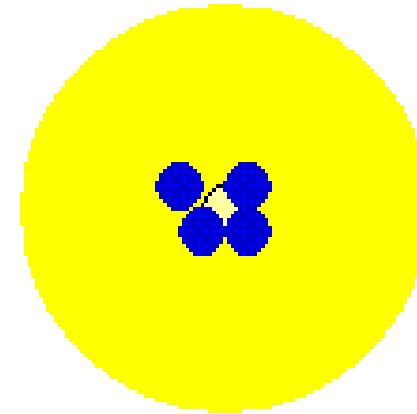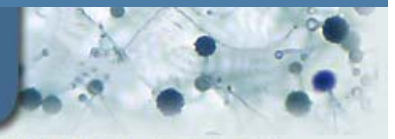
HCV International Standard

- **High titer HCV-positive donation (plasma, genotype 1)**
    - Diluted in cryosupernatant
    - Aliquots made and lyophilized

- **Tested in 22 laboratories**
    - Commercial tests available at the time
    - Laboratory developed tests

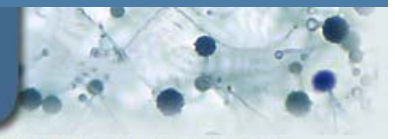- **4 vials per lab, tested 4 independent assays 1 week apart**

HCV International Standard

- Labs assayed ten-fold dilutions of the material
  - 10^{-1} to 10^{-7}

- In 3 subsequent runs eight 0.5 log10 dilutions on either side of the end point were tested

- Number of positive and negative results pooled for each laboratory (all 4 runs)

- Dilution giving 63% positive results has a concentration of 1 copy per reaction
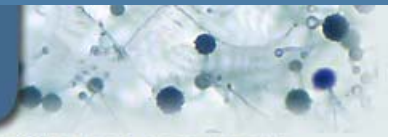 - Estimate copies/ml

WHO HCV Standard: The First Standard

Fig 1(a) – Sample AA

1ˢᵗ HCV International Standard

- **In addition to limiting dilution some labs provided quantitative results**
  - This brings in bias of their calibrator

- **Consensus copy number was determined**

Results are expressed as mean $\log_{10}$ equivs/ml

	Mean	Range	Number
Endpoint 1	5.01	3.06	25
Endpoint 2 (removing outliers)	5.26	1.16	20
Quantitative	5.82	1.09	8

Copy number established: 10^5 IU/ml

Vials contained 50,000 IU

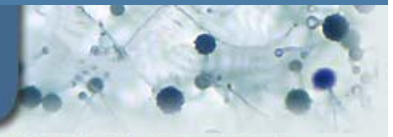**Saldanha J et al. Vox Sang 1999; 76: 149-158**

IAAM
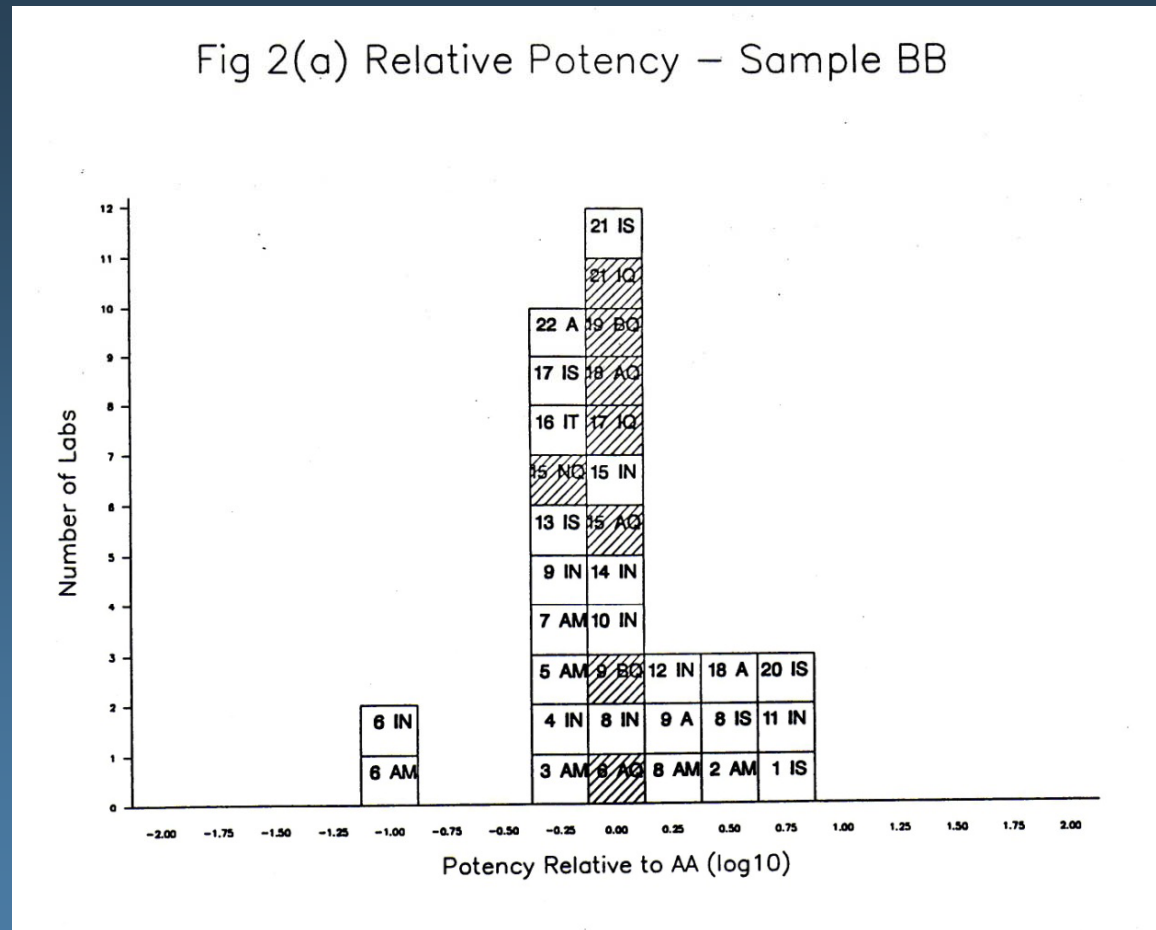INVASIVE ASPERGILLOSIS ANIMAL MODELS
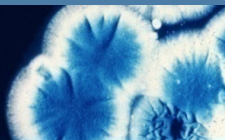
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WHO HCV Standard: Testing BB Relative to AA (BB became 2nd Int. STD)

Fig 2(a) Relative Potency – Sample BB

Saldanha J, Heath A., Lelie N, and the WHO collaborative study group: Establishment of the first international standard for nucleic acid amplification technology assays for HCV RNA. Vox Sang 1999; 76: 149-158
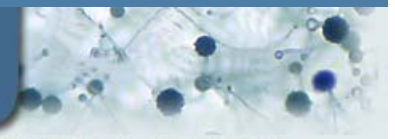
IAAM/AsTEC Plan for Aspergillus

- ## Calibrator material
  - Purified nucleic acid, rather than biological material
  - Ultimately a biological standard is needed

- ## Will not evaluate the extraction method

- ## IAAM purified nucleic acid from cultured *A. fumigatis*
  - Grinding, PK digestion, protein ppt, ETOH ppt, RNAase treat, ETOH ppt
  - DNA is further purified on a low melt agarose gel
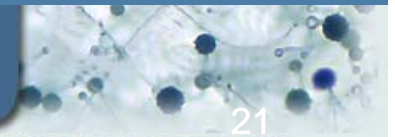 - Stored frozen in aliquots at -70°C

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Limiting Dilution Study

Dilution (x10^8)	Number Tested	% Positive Emory	% Positive IAAM
30	10	100	100
10	10	100	80
3	10	100	80
1	10	90	80
0.3	10	20	50
0.1	10	20	20

Limiting Dilution Study

- Probit analysis: 63% positivity rate = 1 c/rxn
  - Emory 0.51×10^{-8} and IAAM 0.90×10^{-8}

- 5ul/reaction for both, calculate concentration of starting material
  - 4.0×10^{10} copies/ml (Emory)
  - 2.2×10^{10} copies/ml (IAAM)
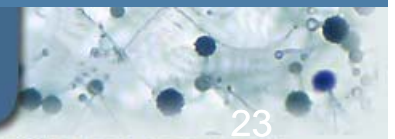
- 15-20 fold difference with calibrators

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IAAM/AsTeC Plan for Aspergillus

- **Start with a pilot study**

- **Limiting dilution study and quantification in a few laboratories**

- **Limiting dilutions studies require a high volume of testing and dilutions will vary for each lab**

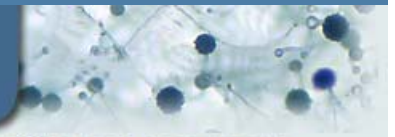- **Assess results and determine approach**
  - 15 to 20 laboratory study

Aspergillus Calibrator

- **Long term stability study**
  - Time 0, 1 month, 6 months, 1, 2, 5, years
  - Five replicates at each time point

- **Freeze-thaw stability study**
  - 0, 1, 2, 3, 5, 10 cycles
  - Five replicates per cycle
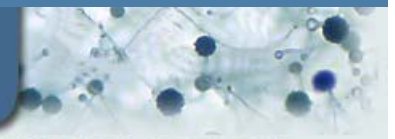 - Thaw at RT, maintain RT for 20 minutes, refreeze at -70°C for at least 1 hour

Summary

- Goal is to develop a calibrator that can be used to compare the performance characteristics (LOD, LOQ, reproducibility, linear range) of various molecular assays used to detect and quantify aspergillus DNA from clinical samples