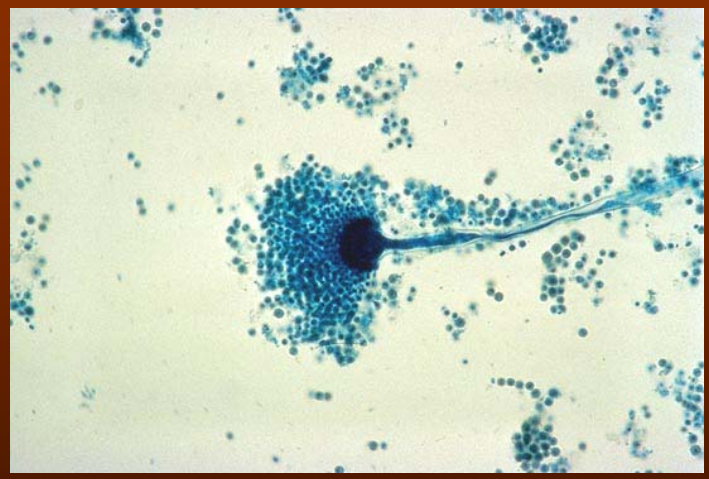
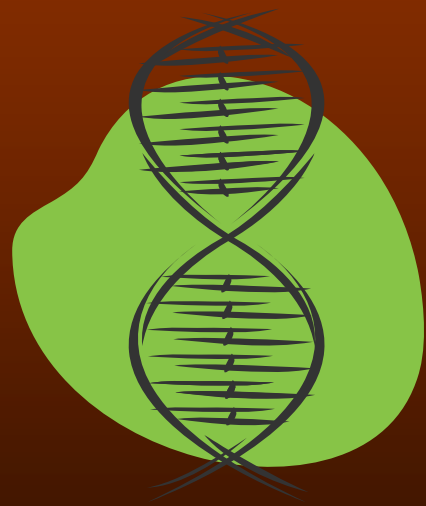
Conventional or molecular measurement of *Aspergillus* load





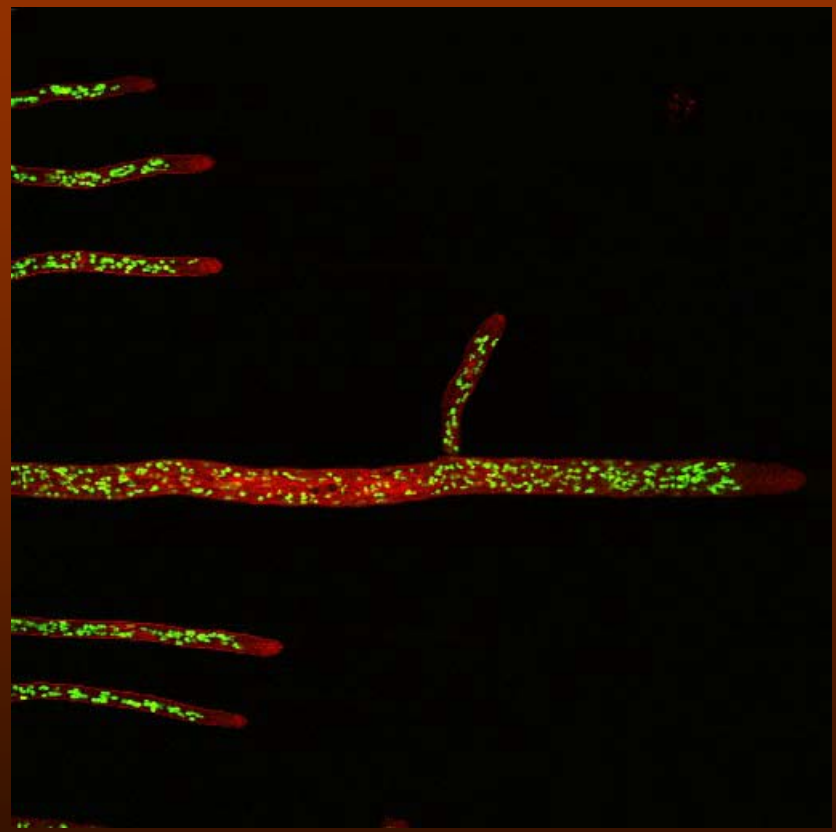
Dr Karl Clemons
California Institute for Medical Research

Everyone quantifies burdens or numbers of organisms, so what's the big deal??

Why does *Aspergillus* make it any different than doing bacteria or yeast?

Aspergillus growth

- Only conidia are single cells
- Growth is by hyphal tip extension; new growth at apical tip only
- Septa divides hypha into compartments
- Septa has a pore
- Compartments communicate show cytoplasmic and organelle streaming



Downloaded from: WWW.fungalcell.org
Prof. Nick Read, Univ. Edinburgh

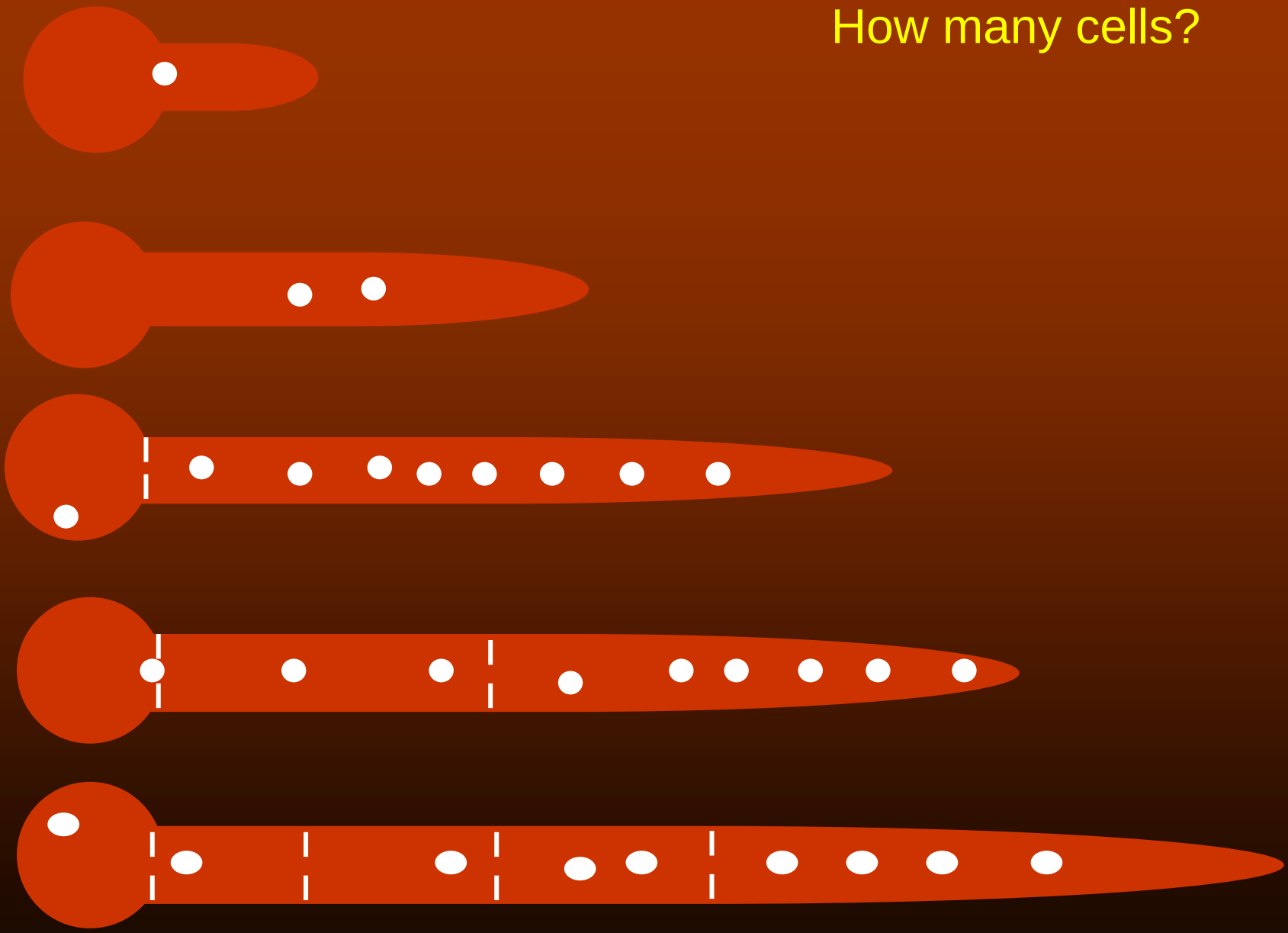
Aspergillus growth

The question:

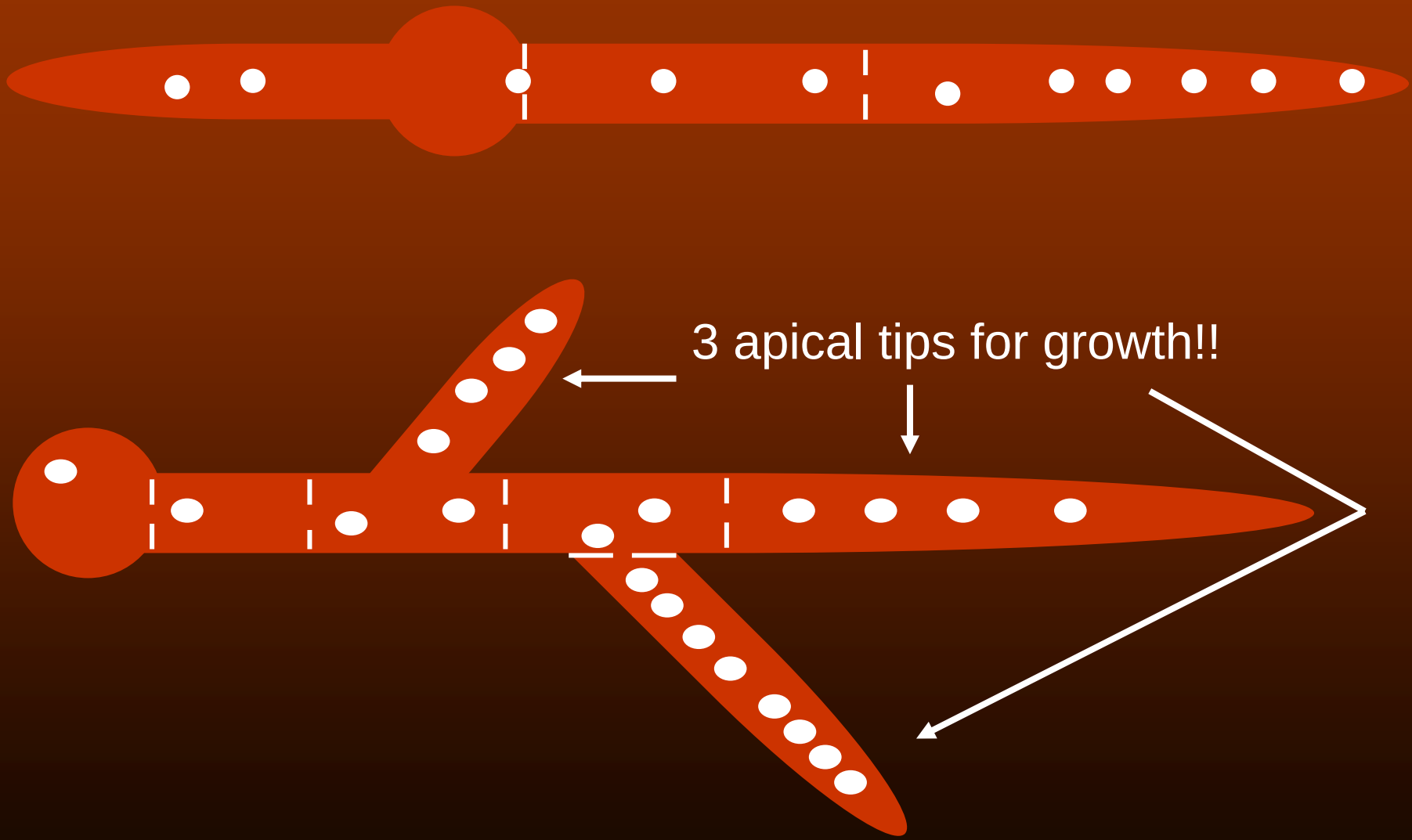
Define a single cell of a hyphal organism?

Conidial germination, nuclear division and hyphal extension

How many cells?

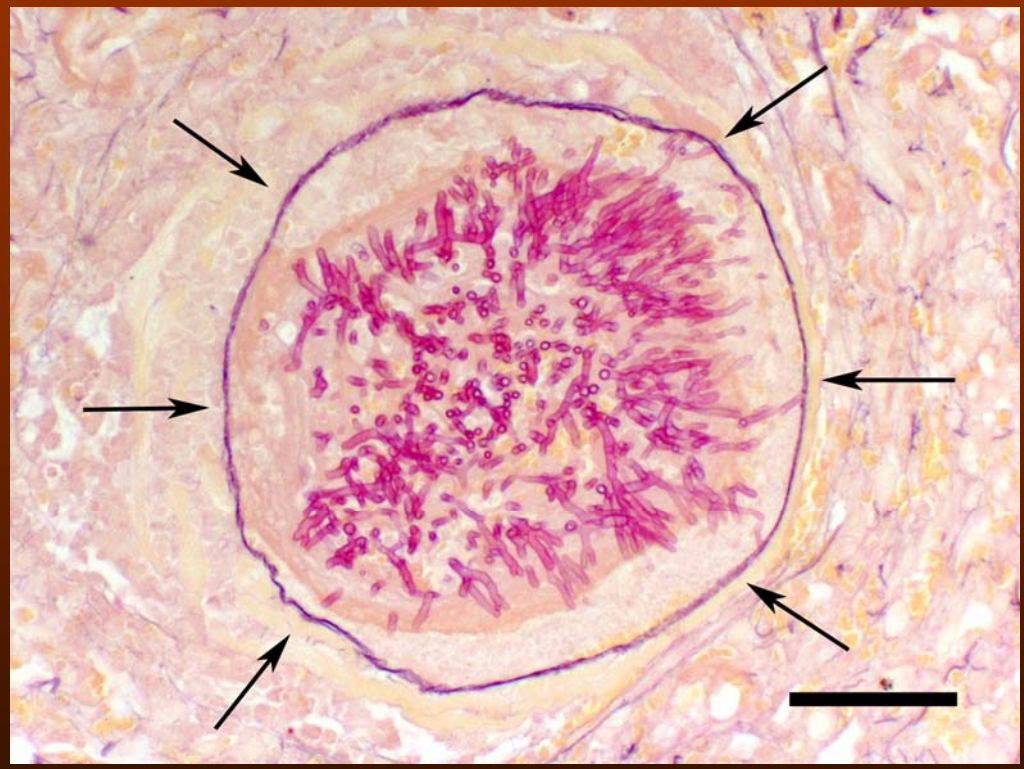


How many cells?



Aspergillus quantification

- Defining a “cell” for *Aspergillus* is not easy!
- Multiple sites of growth at apical tips.
- So, how is fungal load in tissues best determined?



PAS of micro-colony in murine lung

Methods

- Quantification a standard laboratory practice
- Methods
  - CFU quantitative plating – single cells
  - Microscopy hemacytometer counting – single cells
  - Chemical determination of cell wall, i.e., chitin – per what? needs a denominator
  - RT-PCR amplification of target genes – per nucleus
  - EIA of cellular components GM or β-glucan – per what?

Chemical Determination of Fungal Load

- Chitin assay (Lehmann and White, ’75, IAI)
  - Chitin not present in mammalian tissues and is fungal specific
  - Is a multi-step chemical extraction using KOH extraction and detection of an aldehyde derivative of chitosan by colorimetry
  - Lower limit is about 1 µg glucosamine
  - Usually reported as µg chitin per gram tissue

Chemical Determination of Fungal Load

Chitin assay

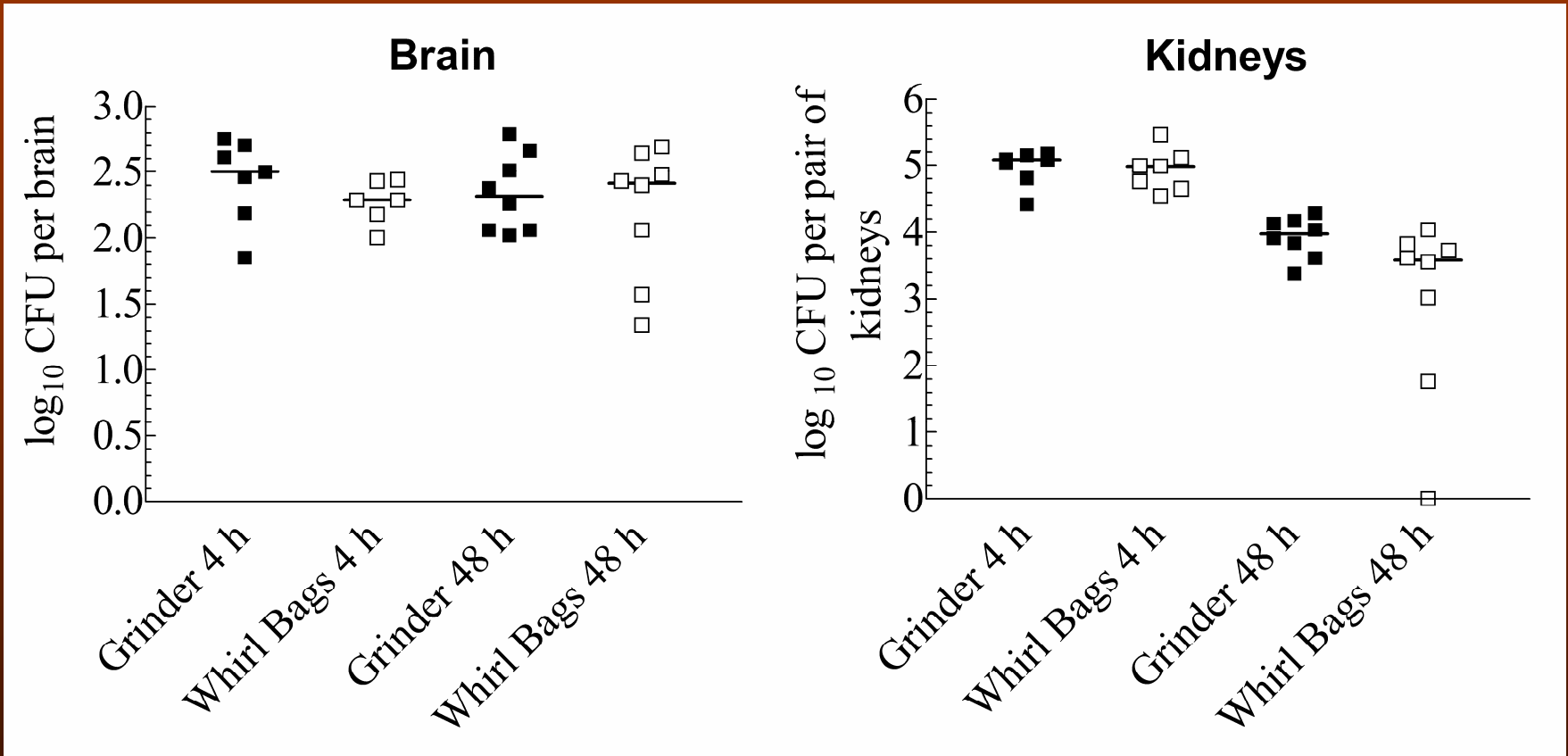
- Good
  - Specific for fungi
  - Assay doesn’t require special equipment
  - Shows increase in load through 7 days infection
- Bad
  - Labor intensive, multiple steps, harsh chemicals
  - Detects only hyphae, not conidia
  - Detects viable and nonviable hyphae
  - Really needs a better denominator, such as µg chitin per mg hyphae

Culture-based method

- Common easy method (good for conidia)
- Pluses
  - *Aspergillus* grows on about anything and FAST!
  - Can use 50C for *A. fumigatus*
  - Can be done by almost any lab with minimal equipment
  - Detects only VIABLE organisms

- Problems
  - How do CFU represent actual viable mycelial burden?
  - Does method of homogenization affect CFU by increase or decrease number due to fragmentation?
  - Is high speed mechanical or a gentler method better?

Homogenization method comparison

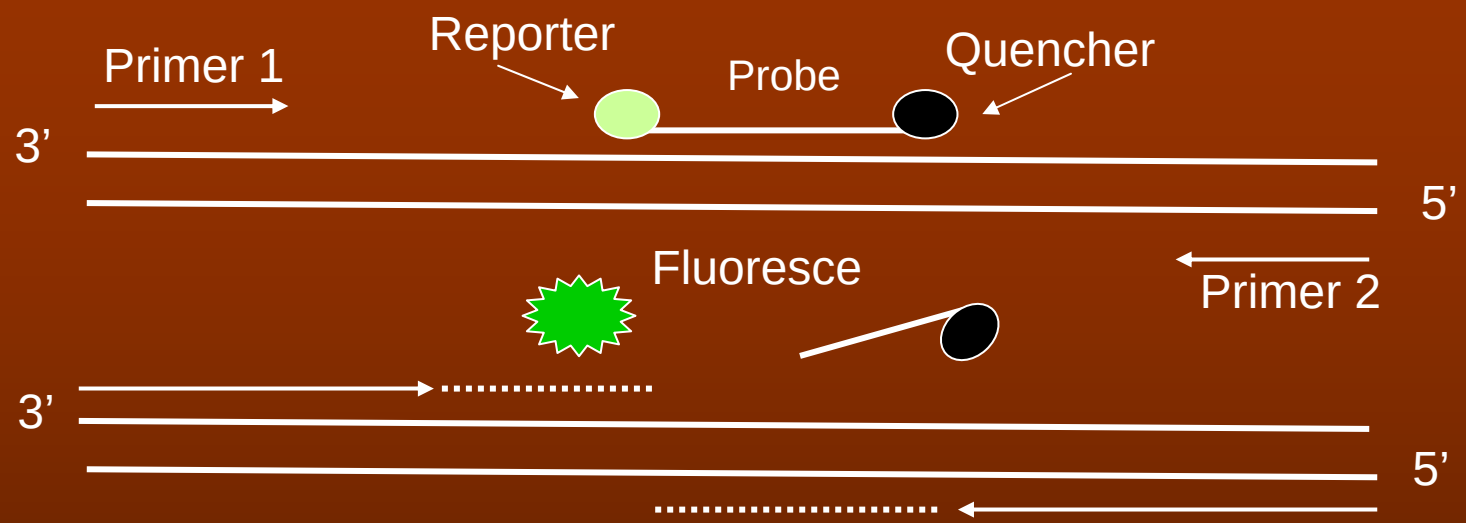


Overall, no significant difference in CFU recovered from Brain or Kidney based on homogenization method.
Whirl bags may not work as well for fibrous tissue (lung)

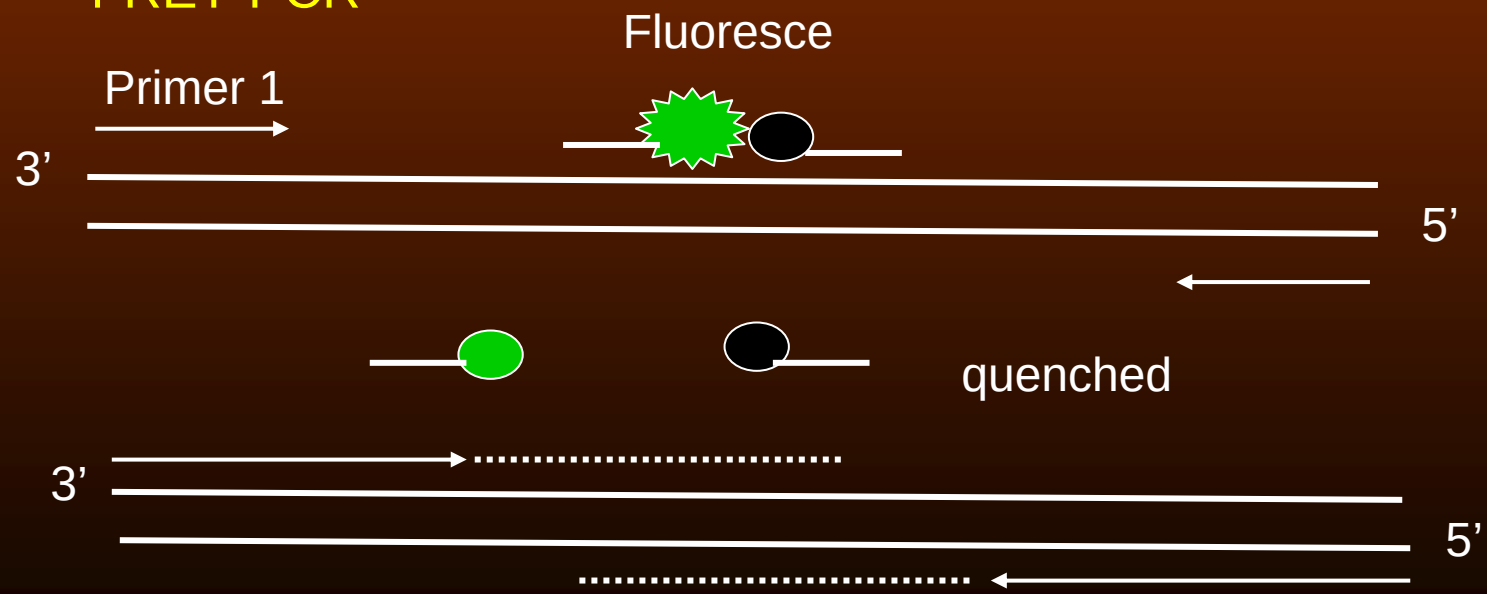
Molecular Methods

- qPCR (Taqman) – 18s rDNA or FKS1
- FRET-PCR – 18s rDNA
- EIA
  - Galactomannan
  - β-glucan

Real Time PCR

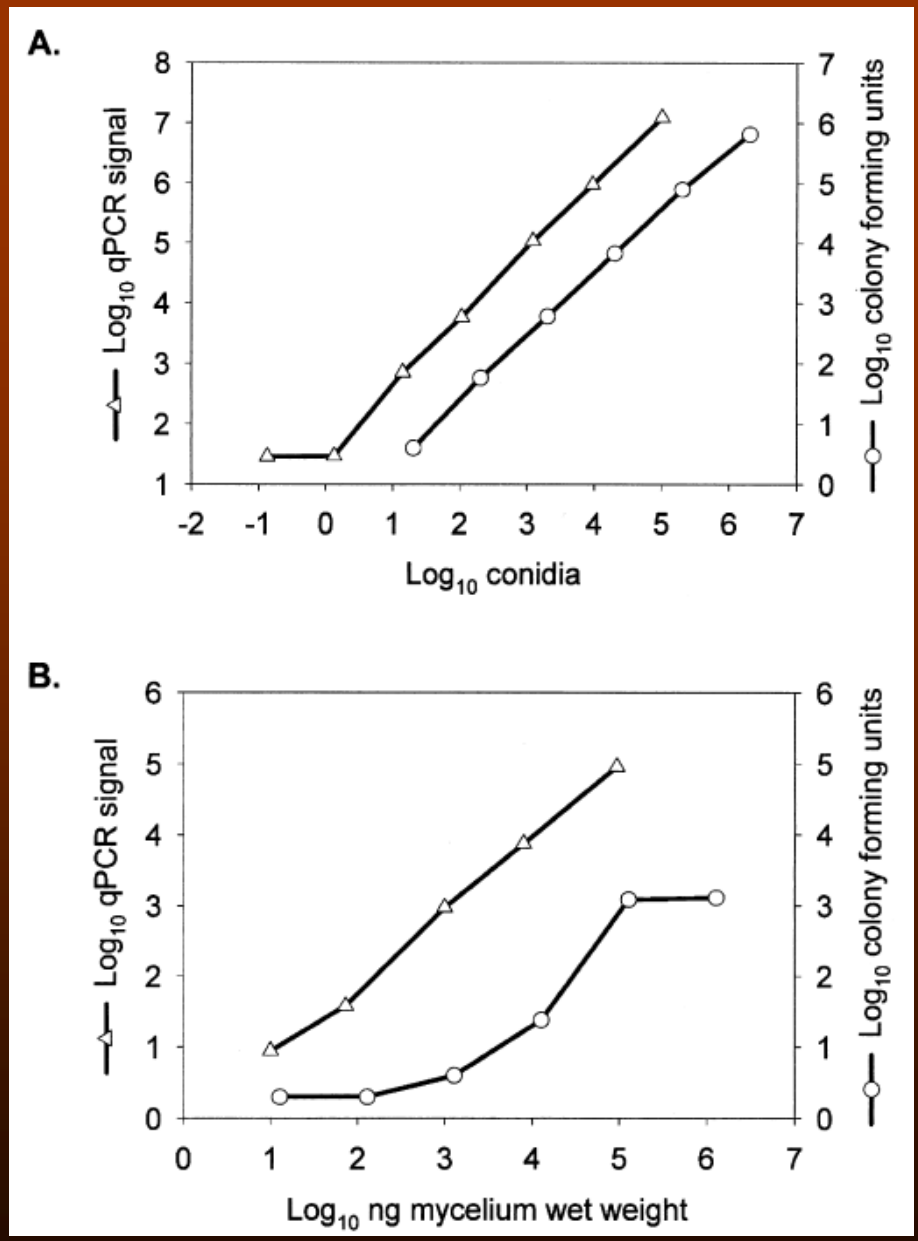


FRET-PCR



qPCR

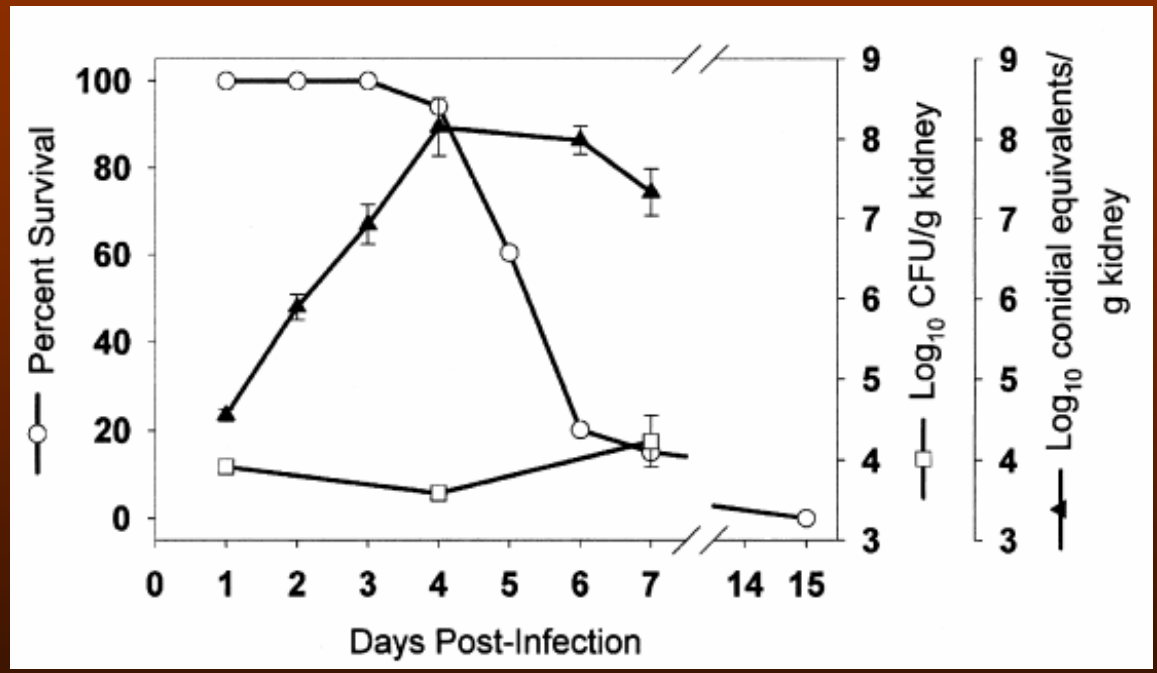
- Quantifies 18s rDNA
- ca. 100 copies/nucleus
- Data expressed as $\log_{10}$ conidial equivalents (CE)
- Lower limit of detection ca. $\log_{10}$ 1.79 CE



Bowman, et al (AAC, 2001)

qPCR

- Decrease survival with time
- Increasing log CE with time
- CFU remain flat to slight increase

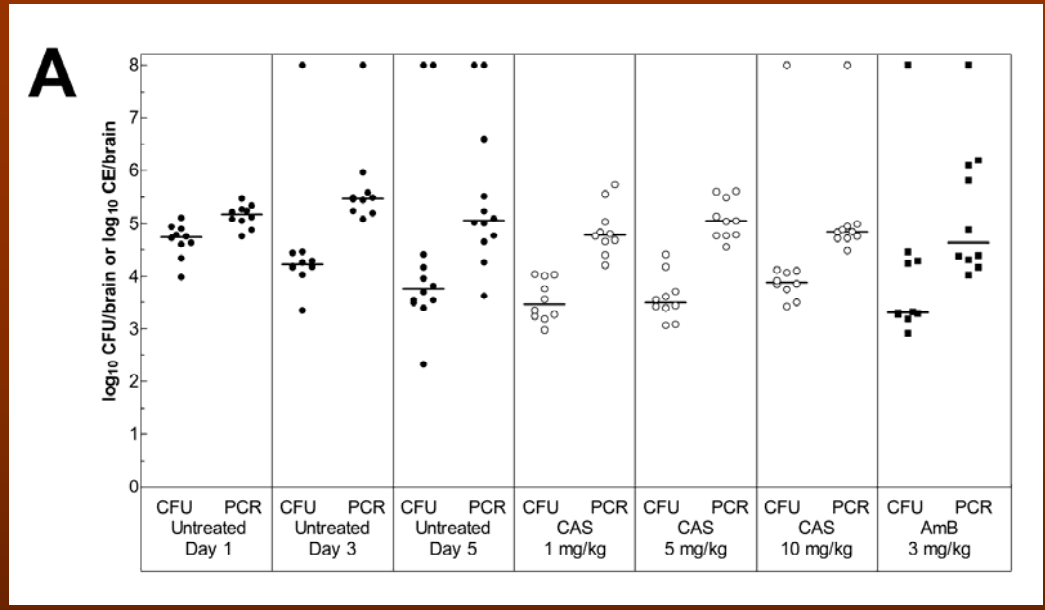


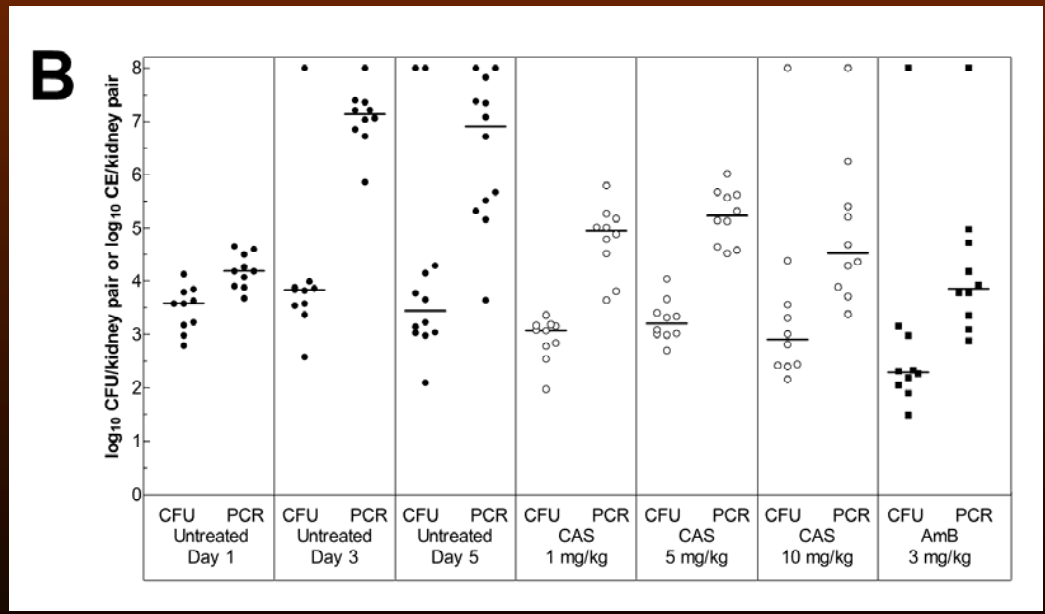
Bowman, et al (AAC, 2001)

qPCR vs. CFU

1. Direct comparison qPCR vs. CFU in homogenates
2. qPCR flat in brain increased only in kidney
3. CFU flat or decrease with day
4. No correlation between qPCR and CFU for progressive growth.
5. Both reflect CAS efficacy only in kidney; AmB in both

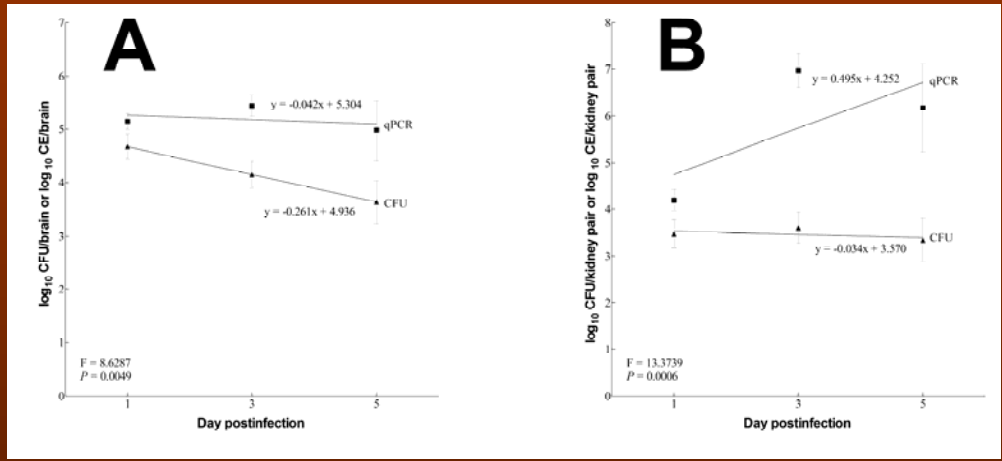
Singh, et al. AAC 2005

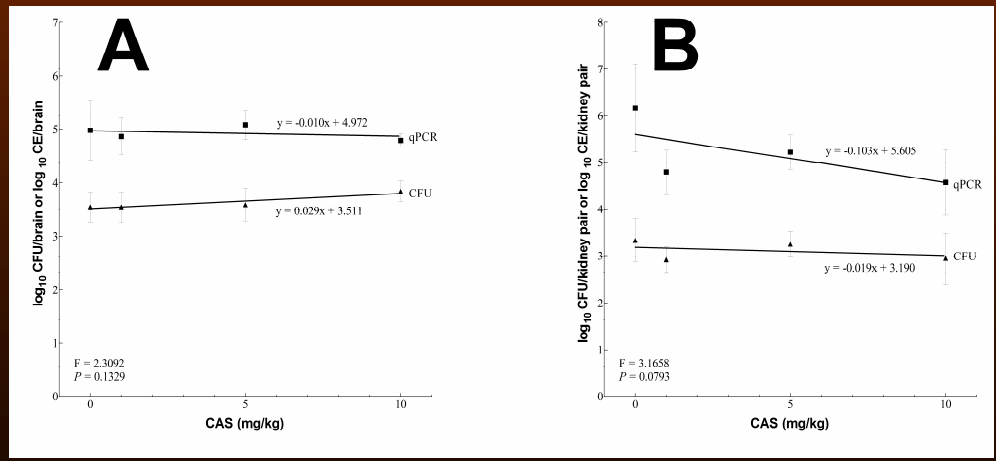




qPCR vs. CFU

- Significant correlation for qPCR & CFU was found in kidney only after treatment with CAS or AmB.
- qPCR better reflected progressive infection; greater dynamic range
- qPCR and CFU were same in efficacy evaluation

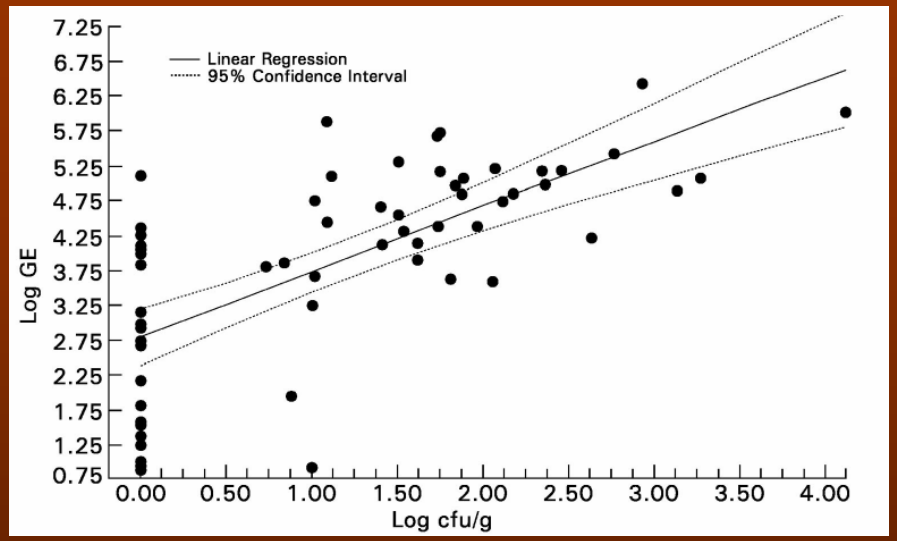


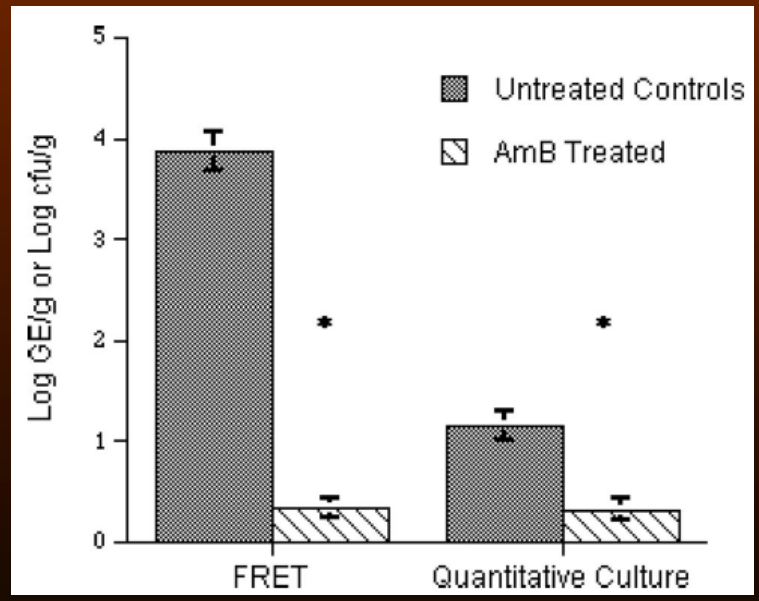


Singh, et al. AAC 2005

FRET-PCR

- FRET > sensitive than CFU in rabbit lung tissue
- 100% by PCR vs. 63% sensitivity by CFU
- FRET good correlation with CFU r = 0.72, P < 0.0001
- Both showed same type of result for AmB treatment



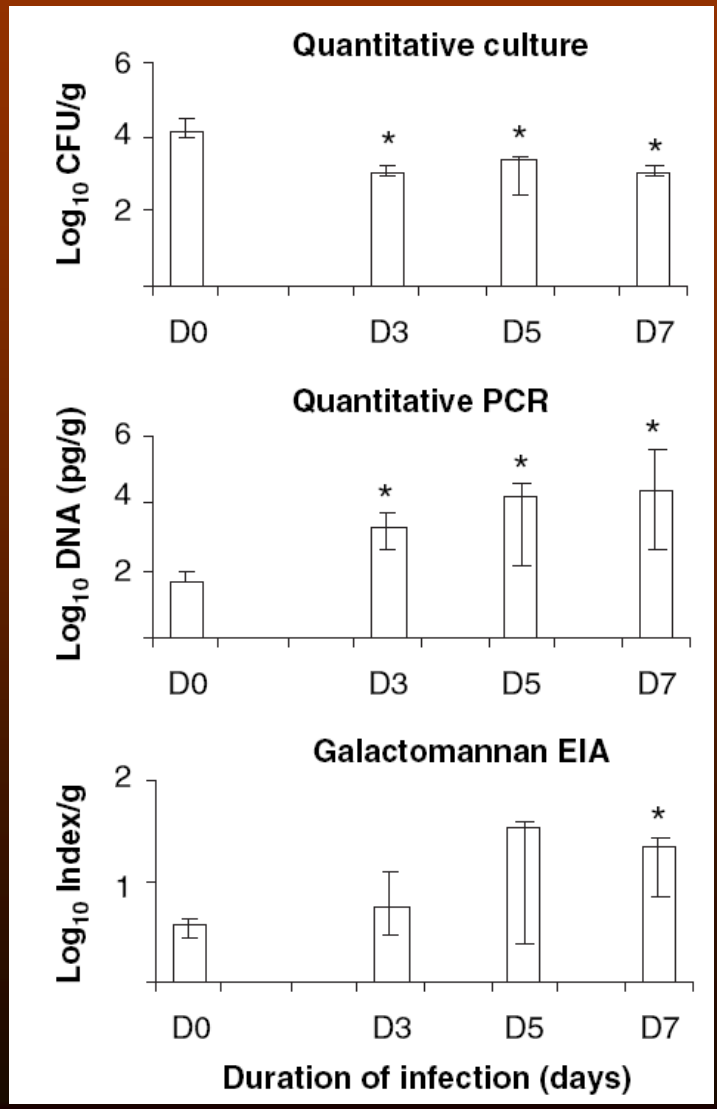


O'Sullivan, et al. JCM 2003

qPCR vs. GM vs. CFU

Progressive pulmonary infection in mice

- CFU flat and decrease
- qPCR significant increase
- GM – trended to increase but only significant at day 7



Sheppard, et al. Clin Microbiol Infect, 2006

qPCR vs. GM vs. CFU

- Poor correlations between methods when tissues contained only conidia
- Strong correlations between the methods when tissues contained hypha
- Significant CFU & qPCR or GM and strongest for GM & qPCR
- GM was most variable and showed some neg –could be due to cut-off value of > 0.5.

Sheppard, et al. Clin Microbiol Infect, 2006

PCR methods

Pluses

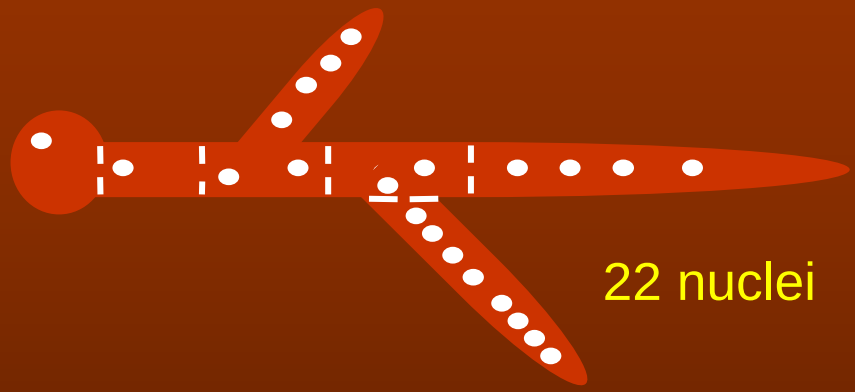
- Reflects progressive increases in load during progressive disease
- Detect both conidia and hypha
- Wide dynamic range, may lack low end sensitivity
- Reproducible and can be specific
- Denominator in conidial or nuclear equivalents

PCR methods

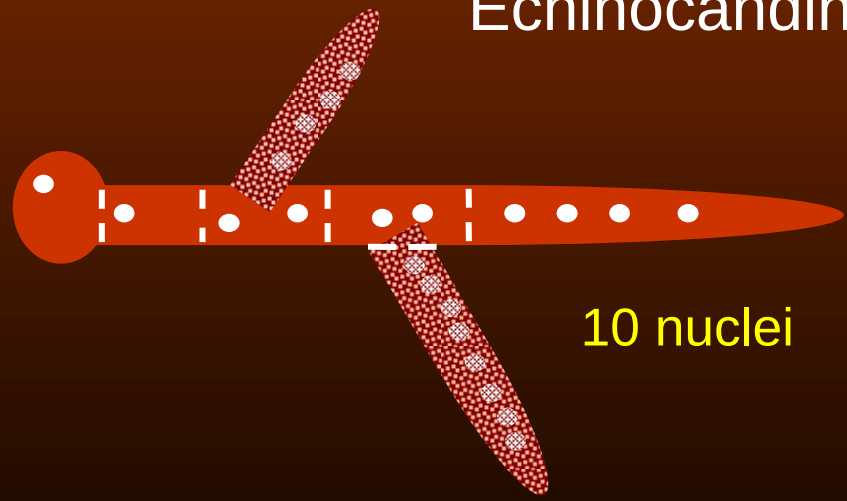
Problems

- Multistep DNA extractions
- Specialized equipment & reagents
- Cost
- Viable & nonviable?
- False negatives
- Detects number of nuclei and may over estimate load
- Possibly magnifies effect of drug efficacy that are not real

Untreated



Echinocandin



Still one viable unit present

EIA galactomannan

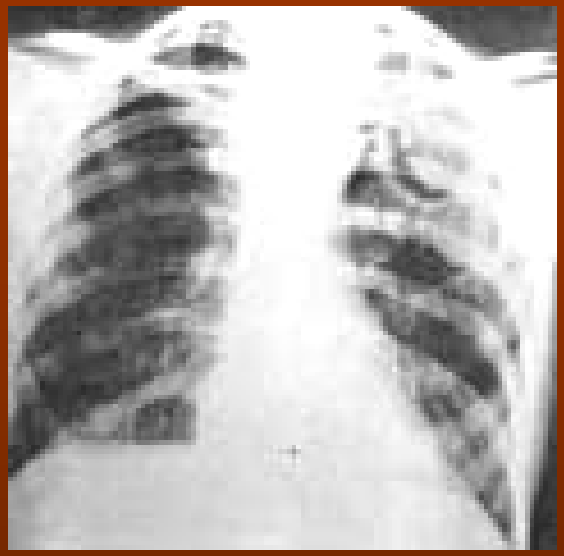
- Pluses
  - Relatively easy to perform
  - Specific for fungi
  - Likely reflects progressive increase in fungal load
- Problems
  - Seems more variable than PCR or CFU
  - Appropriate cut-off value for tissues not determined – sensitivity? False negatives with confirmed infection
  - Viable or dead? How long is the GM present?
  - What does the number represent?
  - Interpretation for drug efficacy studies?

Fungal Load Conclusions

- No one method as yet for a standard
- Tissue homogenization by Whirl-Pak may not work as well for lung as it does for kidney or brain
- If need to determine temporal progression non-culture based are likely better, having a more dynamic range than CFU
- If need a single time point only (i.e., a drug efficacy study) then any of the methods appear useful
- Use of a combination of methods may be best for fungal load

Conclusions cont.

- Histopathology and imaging of lesions for severity or measurement also useful but do not address actual fungal load
- What's in the Future
  - Has room for and need for improvements
  - in situ?
  - Metabolic?
  - Other, as yet, undefined surrogates?



Thanks!