Fungal Contamination and Stability Testing

David W. Denning
Director, National Aspergillosis Centre
Wythenshawe Hospital
The University of Manchester
Rates of contamination

- 8% of PCR assays were contaminated:
  - 5 DNA extractions (3.3%)
  - 7 PCR mixtures (4.7%)
- *A. fumigatus* or *S. cerevisiae* by sequencing
- Zymolase responsible for some

Loeffler et al, J Clin Microbiol 1999;37:1200
Contamination: *Aspergillus* PCR

*Aspergillus* PCR: 19% positivity among blood donors

<table>
<thead>
<tr>
<th>Source</th>
<th>No pos/total</th>
<th>False pos. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR reagents</td>
<td>24/992</td>
<td>2.4</td>
</tr>
<tr>
<td>Qiagen</td>
<td>17/82</td>
<td>12.5</td>
</tr>
<tr>
<td>Magnetic beads</td>
<td>6/104</td>
<td>5</td>
</tr>
<tr>
<td>Magnetic beads with blood</td>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

27 positive normal bloods ⇒ 12 different *Aspergillus* sequences

Palmer J. NIH & Roche. ICCAC 2001
# Sample tubes (1)

<table>
<thead>
<tr>
<th>Type of vessel</th>
<th>Additive</th>
<th>Manufacturer</th>
<th># of lots tested</th>
<th># tested per lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood collection tube 6mL</td>
<td>$K_2$EDTA (Spray Dried)</td>
<td>BD Vacutainer</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Whole blood collection tube 2mL</td>
<td>$K_2$EDTA (Spray Dried)</td>
<td>BD Vacutainer</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Whole blood Collection tube 6mL</td>
<td>$K_3$EDTA (Liquid)</td>
<td>BD Vacutainer</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Serum blood collection tube 6mL</td>
<td>Clot Activator (Spray Dried)</td>
<td>BD Vacutainer</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Serum blood collection tube 3mL</td>
<td>Clot Activator (Spray Dried)</td>
<td>BD Vacutainer</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Serum blood collection tube (10 ml)</td>
<td>None</td>
<td>BD Vacutainer</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Cell preparation tube (CPT)</td>
<td>Sodium citrate and Ficoll™ (a polysaccharide)</td>
<td>BD Vacutainer</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>PaxGeneRNA</td>
<td>Tetradecyltrimethylammonium oxalate solution</td>
<td>PreAnalytiX GmbH</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>RNA later®</td>
<td>RNA stabilization reagent</td>
<td>Ambion</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>
Sample tubes (2)

<table>
<thead>
<tr>
<th>Type of vessel</th>
<th>Additive</th>
<th>Manufacturer</th>
<th># of lots tested</th>
<th># tested per lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen collection and storage containers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL collection container, 40cc</td>
<td>None</td>
<td>Busse</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Urine collection container (Sterile cup only)</td>
<td>None</td>
<td>Medline</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Urine collection container, (Sterile mid stream collection kit)</td>
<td>None</td>
<td>Medline</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Cryovial container, 2mL</td>
<td>None</td>
<td>Simport</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Cryovial container, 3mL</td>
<td>None</td>
<td>Simport</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Pipette tips, 1000µL</td>
<td>None</td>
<td>Associates of Cape Cod/ Eppendorf</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Harrison et al, ICAAC 2008 – Abstr. D1095
Cultures

All tubes/containers tested for fungal sterility

1mL of sterile PDS/Tween vortexed in each

100uL plated on Sabouraud Dextrose agar

All (100%) negative
DNA Extraction

All tubes/containers tested for fungal DNA

1mL of sterile PDS/Tween vortexed in each

1mL used for DNA extraction using the MycXtra kit (Myconostica)
MycXtra™ Fungal DNA Extraction kit (Fungal DNA free)
Real Time PCR

All tested with Aspergillus real-time PCR kit (Myconostica)

SmartCycler platform (Cepheid)

18S target for all Aspergilli and Penicillia

LoD ~50 target copies = ~1 genome
Real Time PCR results

Figure 1: Results from each type of container shown as percentages
Real Time PCR results

Figure 3: Real time PCR Ct values and corresponding contaminating genome copy number in different types of collection vessel.

Amount of *Aspergillus* detected in different containers

- Whole blood 6mL (dry EDTA)
- Serum 6mL (dry clot activator)
- Urine collection
- RNA later

**Figure 3**: Real time PCR Ct values and corresponding contaminating genome copy number in different types of collection vessel.
## Additional testing in 2009

<table>
<thead>
<tr>
<th>Collection container</th>
<th>LOT #</th>
<th># tested</th>
<th>A. fumigatus positive</th>
<th>Inhibited</th>
<th>Positive Ct range</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA liquid 7ml</td>
<td>8219849</td>
<td>25</td>
<td>7</td>
<td>1</td>
<td>34.2 - 37.2</td>
</tr>
<tr>
<td>EDTA liquid 7ml</td>
<td>8339059</td>
<td>25</td>
<td>1</td>
<td>1</td>
<td>37.9</td>
</tr>
<tr>
<td>EDTA liquid 7ml</td>
<td>9007142</td>
<td>25</td>
<td>11</td>
<td>2</td>
<td>32.7 - 36.5</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>75</strong></td>
<td></td>
<td><strong>15%</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red top no additive 6ml</td>
<td>8331028</td>
<td>25</td>
<td>2</td>
<td>0</td>
<td>34.4 - 36.4</td>
</tr>
<tr>
<td>Red top no additive 6ml</td>
<td>9037525</td>
<td>25</td>
<td>9</td>
<td>0</td>
<td>35.4 - 37.9</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>50</strong></td>
<td></td>
<td><strong>18%</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Confirmation of positives

Positives also tested with an *A. fumigatus* specific Taqman real-time assay.

This showed 96% (48/50) agreement with samples that were MB positive.

Suggests the results are real, and most contamination is *A. fumigatus*.

Challier S et al, J Clin Microbiol 2004;42:844
Conclusions

- Sample collection containers were investigated for fungal DNA contamination with two real-time PCR assays and culture.
- All cultures were negative.
- 17% of 185 whole blood collection tubes contaminated.
- 10% of 160 serum blood collection tubes contaminated.
- Other tubes and containers less frequently contaminated.
- Most probably non-viable *A. fumigatus*.
Diagnostics of Invasive Aspergillosis: From Experimental Models to Clinical Evaluation

SAMPLE STABILITY
The Effect of Repeated Freeze-Thaw Conditions on DNA Yield in Infected Lung Tissue

n=40
500µl aliquots of homogenate was immediately processed for DNA extraction and the rest was subsequently frozen in 1ml aliquots at -70°C.
The Effect of Repeated Freeze-Thaw Conditions on DNA Yield In Serum

- n= 10
- DNA extraction from a 500µl sample of serum was immediately performed. The rest of the serum was subsequently frozen in 1.8 ml cryovials in 1ml aliquots at -70°C.
The Effect of Time (21 months) and Freeze-Thaw Cycling on DNA quantitation

- N=24
- A small aliquot (15µl) of the DNA sample was used for quantitative PCR analysis. All samples were then placed into the -70°C freezer to be frozen again for the next cycle.
Result and Conclusions

- There was a statistically significant decrease in the mean quantity of CE/ml of AF 293 detected from lung samples after repeated freeze thaw cycles (initial: mean log10: 5.4 ± 0.4 vs. cycle 10: mean log10 of 1.16 ± 0.5; p<0.0001).

- Similar results were obtained when assessing the CE/ml in serum samples (initial: mean log10 4.8 ± 0.62 vs. cycle 5: mean log10 of 0.49 ± 0.49; p<0.0003).

- In comparison, no significant change was detected in the quantity of AF 293 from DNA samples stored over 21 months or after 10 repeated freeze thaw cycles.

- Assessment of AF293 DNA extracted from frozen samples was stable and consistent after prolonged storage at -70°C and repeated freeze thaw cycles.

- Repeated freeze - thaw cycles of lung and serum samples prior to extraction of AF293 DNA led to a steady decrease in DNA yield over time.
Future Studies

- Stability of DNA in fresh vs frozen whole blood
- Stability of DNA in fresh serum vs frozen sera
  - Intralaboratory
  - Interlaboratory (2-3)
- Stability of calibrator DNA in buffer, serum or blood, fresh vs frozen
  - Intralaboratory
  - Interlaboratory