New diagnostic approaches to detect *Salmonella* spp. in blood

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• Blood culture, the standard diagnostic, is often unavailable in the places where it is needed most

• The median S. Typhi count in blood is ~1 CFU/ml and ~63% are intracellular (mononuclear cells)

• Sensitivity of buffy coat culture = blood culture
New *Salmonella* Diagnostic Assays

- Antibody-in-lymphocyte-supernatant (ALS) assay - F. Qadri

- Loop-mediated isothermal amplification (LAMP)- A. Pollard

- Microwave-accelerated metal-enhanced fluorescence
Microwave-Accelerated Metal-Enhanced Fluorescence (MAMEF) incorporates 2 technologies

1) Metal-Enhanced Fluorescence (MEF): increases the sensitivity of fluorescence-based assays

2) Low-power microwave heating: kinetically accelerates the recognition events thereby reducing the run time
Detection of *Salmonella oriC*

Fluorescent Probe
19 nt

Anchor Probe
22 nt

6 nt

oriC target
40 nt

Silver
Glass

SH

Anchor Probe

TAMRA

Fluorescent Probe

Target Probe / sequence

\[ \text{SH} \quad \text{Anchor Probe} \quad \text{TAMRA} \quad \text{Fluorescent Probe} \]

\[ 5'\text{–GTTTTTCAACCTGTTTTTGCGCC–3'} \quad 5'\text{–CTTTCAAGTTCCGCTTCTAT–3'} \]

\[ 3'\text{–GTTGGACAAACCGCGGTCGCGGAAAGTCAAGGCGAAGATA–5'} \]
Microwave accelerated metal-enhanced fluorescence (MAMEF)

Lysis & release of DNA using gold bow-tie slide

Hybridization in microwave

Detection

- We have shown that we can detect 1 CFU of *Salmonella* in 1 ml of bacteriological media using MAMEF

General Procedure for *Salmonella* Lysis and Detection

**Whole Blood**

- Remove RBCs/clotting factors

**Lyse and release *Salmonella* DNA**

**Detection**
Overview of Separation Methods

Whole blood:
- NH₄Cl only
- RBC lysis buffer
- Water

Buffy Coat:
- BD Ficol tube
- Dextran
- LSM

8.26 g ammonium chloride (NH₄Cl)
1 g potassium bicarbonate (KHCO₃)
0.037 g EDTA
1 L water
Salmonella Whole Blood Separation protocol

1. Anti-coagulated whole blood
2. Add 10X volume lysis buffer
   - Invert 10 times. Leave at RT for 2 mins
3. Spin at 400 x g for 5 mins
4. Resuspend pellet in lysis buffer
   - Immediately spin again 400 x g for 5 mins
5. Resuspend pellet in X ml
## Pros & Cons of best blood treatment methods

<table>
<thead>
<tr>
<th></th>
<th>Pros</th>
<th>Cons</th>
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</table>
| **RBC Lysis Buffer** | ✓ Cheap  
✓ Fast  
✓ Easy  
✓ Intracellular & some extracellular bacteria  
✓ Fresh or older blood ok | ✓ Need swing bucket centrifuge |
| **Dextran**       | ✓ Cheap  
✓ Fast  
✓ Easy  
✓ Intracellular & some extracellular bacteria  
✓ Microcentrifuge | ✓ Fresh blood better than old blood |
Detection of *Salmonella* in blood by MAMEF

- Detected 0.4 CFU *Salmonella*
Real-time PCR detection of S. Typhi

• We are using a probe set described in Nga et al. (BMC Infect Dis 2010, 10:125)

• STY0201-putative fimbrial-adhesion protein

• Probes were specific and had a detection limit of ~100-200 organisms per ml of whole blood
Using RBC lysis buffer and a mini DNA extraction kit we can detect ~0.5 CFU in 2 ml blood by qPCR 60% of the time

<table>
<thead>
<tr>
<th>S. Typhi CFU per 2 ml blood</th>
<th># of positive assays/total # of assays</th>
<th>Range of CT values</th>
</tr>
</thead>
<tbody>
<tr>
<td>2400</td>
<td>1/1 (100%)</td>
<td>27-28</td>
</tr>
<tr>
<td>240</td>
<td>1/1 (100%)</td>
<td>29-30</td>
</tr>
<tr>
<td>56</td>
<td>5/5 (100%)</td>
<td>34-39</td>
</tr>
<tr>
<td>5.6</td>
<td>4/5 (80%)</td>
<td>36-39</td>
</tr>
<tr>
<td>0.56</td>
<td>3/5 (60%)</td>
<td>36-39</td>
</tr>
<tr>
<td>0.08</td>
<td>0/2 (0%)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1*/5 (20%)</td>
<td></td>
</tr>
</tbody>
</table>
Conclusion

• We have prevented congealing of blood during microwave heating in MAMEF by removing RBCs and clotting factors

• MAMEF needs further optimization of metal surfaces before it can be field tested

• We have adapted our blood preparation method to improve sensitivity of qPCR

• Further optimizations need to be done to ensure reproducible detection of low concentrations of *Salmonella* by qPCR
Acknowledgements

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Method to Improve Sensitivity

2 ml Whole Blood

White Blood Cell (WBC) Pellet

WBC+Bacteria in 200 ul

50 ul purified DNA

RBC Lysis

Resuspend pellet in 200 ul buffer w/bacteria

Extract total DNA with Qiagen Blood Mini kit and Elute in 50 ul

Run 9.2 ul of sample in 20 ul qPCR total volume (triplicate)

Each reaction is now run with ~1/5th of the sample (compared to 1/60th)
Using RBC lysis buffer and a mini DNA extraction kit is cheaper and faster than using a midi DNA extraction kit

<table>
<thead>
<tr>
<th></th>
<th>Regular DNA extraction method</th>
<th>New DNA extraction method (CVD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-processing</td>
<td>None</td>
<td>RBC lysis</td>
</tr>
<tr>
<td>DNA extraction kit</td>
<td>QIAamp DNA Blood midi kit (Qiagen)</td>
<td>QIAamp DNA Blood mini kit (Qiagen)</td>
</tr>
<tr>
<td>Processing time</td>
<td>~5 h</td>
<td>&lt;2 h</td>
</tr>
<tr>
<td>Cost (USD)</td>
<td>$8.23 per sample</td>
<td>$2.37 per sample (plus &lt;$1 for RBC lysis)</td>
</tr>
<tr>
<td>Equipment required</td>
<td>Waterbath, centrifuge that can attain 4500 x g</td>
<td>Microcentrifuge, waterbath, benchtop centrifuge that can attain 300-400 x g (for RBC lysis)</td>
</tr>
<tr>
<td>Elution volume</td>
<td>300 ul</td>
<td>30-50 ul</td>
</tr>
</tbody>
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