Using Bacteriophage for the rapid detection of MAP: rapid detection of disease to food quality assurance

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Bacteriophage

- Bacteriophage are viruses that specifically infect bacteria
- Like all viruses they have a limited Host Range
  - determines the type of cell infected
- Have evolved to recognise structure on the surface of correct cell type

- Tail structures help virus inject DNA into host
Bacteriophage growth

• Viruses replicate inside the host cell and produce 50+ phage per infection
• Produces enzymes to break open the host once the new viruses are made
• Phage growth can be monitored as an indicator of the presence of viable host cells
History of Phage Amplification test

• Initially developed by UoN spin-out company for the detection of TB in human sputum samples
  • Low cost test as sensitive as culture methods
  • Results gained in 48 h
  • Licensed kit manufacture established
  • PCR superceded use of biological assay in human medicine

• Over last 10 years we have been investigating applications in area of livestock mycobacterial infections
  • Specifically detection of mycobacteria in blood and milk
Phage Amplification Assay

Target cell

INFECTION

PHAGE DESTRUCTION BY VIRUCIDE

BACTERIOPHAGE PARTICLES

NEUTRALISATION & ADDITION OF HELPER CELLS TO FORM LAWN

PLAQUES FORM ON AGAR PLATE

1 PLAQUE = 1 VIABLE CELL DETECTED

10 Oct 2017
DNA assay to identify cell

Plating out → Incubation → Plaques form

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>bTB</td>
<td>MAP</td>
<td>bTB + MAP</td>
</tr>
</tbody>
</table>

PCR Amplification of genomic “signature sequences”

DNA extraction and PCR for genotype determination

Initial target cell DNA
Development of blood assay

- Components of blood found to inhibit phage infection
- Method required to separate mycobacterial from blood
- Original approach used well characterised PMMS

- Isolates MAP from phage-inhibitors in sample
- Complex reagents needed (beads, peptides, magnetic separator)
- Multiple manipulations required
Summary of phage blood test

- False-positives unlikely
- False-negatives depend upon efficiency/specificity of PCR
MAP Blood test

• Blood samples taken from herd with known Johne’s disease problem and high status control herd with no known history of disease
  • Cattle selected on the basis of 3 consecutive positive milk ELISA results
  • Blood samples taken for MAP blood ELISA and direct PCR (Tetracore) assays
  • MAP recovered from lysed blood using peptide-coated magnetic bead capture
## MAP Blood test results

<table>
<thead>
<tr>
<th>Cow Number</th>
<th>Milk ELISA Status (3 tests)</th>
<th>Blood ELISA Status</th>
<th>Plaque Number</th>
<th>Plaque PCR IS900</th>
<th>Q-RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
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<td>35</td>
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<td>14</td>
<td>-</td>
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<td>0</td>
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</tr>
</tbody>
</table>

Reproducibility

- Comparison of duplicate blood samples showed that PMMS did not give good reproducibility
- More marked with higher cell numbers – suggested inefficient capture by magnetic beads

![Graph showing reproducibility data](image)

Swift et al. (2016)
BMC Vet Res 12:115
Predicted to give more reproducible and more sensitive results
Results of Blood Assay for MAP

<table>
<thead>
<tr>
<th>Cow Number</th>
<th>Milk ELISA Status (most recent a)</th>
<th>Blood ELISA Status</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Red</td>
<td>-</td>
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<tr>
<td>16</td>
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<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Red</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Amber</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Green</td>
<td>-</td>
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<tr>
<td>23</td>
<td>Green</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>Green</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Red - denotes a strong positive Milk ELISA reading.
Amber - denotes an inconclusive Milk ELISA reading.
Green - denotes a negative Milk ELISA reading.

a Represents the most recent Milk-ELISA status.
b Values show the numbers of plaques obtained in two independently tested sam

• Blood ELISA did NOT identify all Red cows
• All samples were culture-negative

Results of Blood Assay for MAP

- All Red animals were phage-PCR positive
- Presence of viable MAP in blood does not always generate a detectable antibody response

Results of Blood Assay for MAP

<table>
<thead>
<tr>
<th>Cow Number</th>
<th>Milk ELISA Status (most recent a)</th>
<th>Blood ELISA Status</th>
<th>Plaque Number</th>
<th>Plaque PCR</th>
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<tbody>
<tr>
<td></td>
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<td>Whole Blood b</td>
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<tr>
<td>15</td>
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<td>−</td>
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<tr>
<td>24</td>
<td>Green</td>
<td>−</td>
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</tbody>
</table>

Red denotes a strong positive Milk ELISA reading.
Amber denotes an inconclusive Milk ELISA reading.
Green denotes a negative Milk ELISA reading.

* Represents the most recent Milk-ELISA status.

b Values show the numbers of plaques obtained in two independently tested samples.

- 2 Green animals were phage-PCR negative;
- 1 Green and 1 Amber were phage-PCR positive; both blood ELISA negative

Reproducibility

- Comparison of duplicate blood samples showed marked improvement in reproducibility using buffy coat
  - Av. No plaques = 14.8 PMMS, 22.1 Buffy coat
- Demonstrated that MAP in blood is intracellular

Swift *et al.* (2016)
BMC Vet Res 12:115
MAP Blood test results

• All of these animals had detectable but **LOW** levels of viable MAP in blood using the Phage-PCR test
  • All 4 samples gave positive phage-PCR results
  • No samples were culture-positive
• No samples were positive using the commercial QPCR assay
  • Demonstrates sensitivity of phage-based method
• Buffy coat method improved reproducibility but multistage process not appropriate for high throughput analysis of samples
  • Method further developed and reformatted into 1 day Actiphage™ assay
Summary of New 1 Day Actiphage™ test

Sample arrives

Layers after Ficoll spin

- Plasma
- PBMCs (interphase)
- Ficoll
- Granulocytes
- RBCs

20 min

One tube assay format

3 h

5-8 h

Simpler & Faster

- predicted to improve sensitivity
Comparative results – MAP blood test

- Small study of experimentally infected cattle
- The original phage-PCR assay detected MAP in 40 % (6/15) of the blood samples
- Highest number detected 15 cells per 2 ml blood
- No MAP cultured
  - Below expected LOD

MAP detected in 87 % (13/15) samples using the Actiphage™ method

<table>
<thead>
<tr>
<th>Assigned Number</th>
<th>Number of Plaques</th>
<th>PCR (+/-)</th>
<th>One Day Test</th>
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<tbody>
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<tr>
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<tr>
<td>15</td>
<td>9</td>
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<td>+</td>
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</tbody>
</table>
Comparative results – MAP blood test

- Small set of blood samples from experimentally infected calves
  - Five animals experimentally infected with a cattle strain of MAP.
  - Six months post-infection, blood samples were obtained for parallel testing with the phage assay
    - 3 monthly samples obtained
  - After nine months the animals were culled to detect signs of infection post-mortem
  - Samples tested using both 2 day and Actiphage™ one day assay
Comparative results – MAP blood test

• At necropsy only animal 7 showed any sign of infection
• General pattern of progression of infection seen
• Average number of plaques detected = 14 pfu ml⁻¹
  • Animal No. 7 had 63 pfu ml⁻¹
  • Consistent with pattern seen in bovine TB infection study with increasing plaque number associated with increasing pathology
    • Swift et al., 2016 Virulence 7:779-88
• 9/33 samples both –ve; 13/33 samples both +ve
  • Concordance = 67 %
  • 1 = -ve only by 2 day assay; 1 = +ve only by Actiphage™ test
Summary of Phage tests

• Phage-based method detects the bacteria rather than an immune response
  • Useful to compare disease progression with immune response and bacterial clearance
  • DIVA test to support vaccination?

• Blood test could be used to identify earlier infection in animals
  • Better control of infection in eradication programs

• Actiphage™ method found to be more sensitive than plate-based phage method
  • One tube format means sample loss does not occur

• Actiphage™ test is simpler for high throughput testing
  • Could be performed in routine testing labs
Detecting MAP in milk using Phage-PCR assay
MAP in pasteurised milk

- UK study showed that 1.8% of retail pasteurised milk contained viable MAP
  - Grant et al., 2002 Appl. Env. Micro. 68, 2428-2435.
- US study found 2.8% of retail whole milk from 3 states
  - Ellingson et al., 2005 J. Food Prot. 68, 966-972.
- Czech Republic study isolated MAP from (1.6% samples pasteurised retail milk)
  - Ayele et al., 2005 Appl. Env. Micro. 71, 1210-1214
- Argentina isolated MAP from 2.8% of samples
  - Paolicchi et al., 2012 Brazil. J Microbiol. 43, 1034-37

Very good evidence that MAP is present in retail milk
Detection of MAP in milk by Phage-PCR

- Centrifugation used to separate MAP from milk
  - Cells pelleted along with somatic cells
  - Pellet washed using media to remove milk

- Method used to survey milk from multiple commercial retailers
  - Included commercial retailers (14) and doorstep providers (5)

- 368 retail semi-skimmed milk (1.7 % fat) samples provided by volunteers
Pasteurised Milk Survey Results

- Overall viable MAP was detected in 10.3% of samples by Phage-PCR
  - 1.1% potentially detectable by culture
  - 3.5% potentially detectable by PCR
  - 6.8% not detectable by other methods
The pasteurisation conundrum

- Many, MANY, studies show that MAP is inactivated by commercial HTST conditions
  - Even 15s shown to produced a 5 log$_{10}$ drop

<table>
<thead>
<tr>
<th>Country</th>
<th>Volume Tested (ml)</th>
<th>Map Survival Observed</th>
<th>Decontamination</th>
<th>Reference</th>
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<tbody>
<tr>
<td>UK</td>
<td>50</td>
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<td>None (2002)</td>
<td>Grant et al, 2002, Grant et al, 2005</td>
</tr>
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<td>McDonald et al, 2005</td>
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<td>NL</td>
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<td>No</td>
<td>HPC</td>
<td>Rademaker et al, 2007</td>
</tr>
</tbody>
</table>

- All pasteurised milk SHOULD be negative
The pasteurisation conundrum

Grant et al. 2002, pasteurised milk study

TABLE 1. IMS-PCR and culture results for 244 bulk raw and 567 commercially pasteurized cows’ milk samples from 241 United Kingdom dairy processing establishments

<table>
<thead>
<tr>
<th>Type of milk</th>
<th>IMS-PCR</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Raw</td>
<td>19 (7.8)</td>
<td>211 (86.5)</td>
</tr>
<tr>
<td>Pasteurized</td>
<td>67 (11.8)</td>
<td>480 (84.7)</td>
</tr>
</tbody>
</table>

* +, positive; −, negative; no result, insufficient sample was received, no clear result was obtained for IMS-PCR, or HEYM slopes and BACTEC medium were overgrown by contaminants for culture.

- Why didn’t % samples detected by culture decrease after pasteurisation?
- Suggests a long tail of low level survivors
Protection rather than process failure?

- MAP shown to grow to high numbers inside macrophage
- Can these provide a heat sink and allow survival?
Detection in Infant Formula

• Same sample preparation used to survey Powdered Infant Formula
  • 13 % (4/32) of PIF samples MAP positive by phage-PCR method
  • 9 % (3/32) PIF samples culture positive
  • All culture-positive samples also phage-PCR positive
Actiphage™ method for milk

- Artificially spiked milk samples
  - Processing by centrifugation and washing of pellet to remove milk
- One Day method performed using a nested IS900 PCR as the endpoint detection method
  - MAP detectable down to $10^{0}$ pfu.ml$^{-1}$
- Intensity of the band reflects reduction in the number of cells
  - Combination with qPCR needed for quantifiable enumeration

<table>
<thead>
<tr>
<th>M</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>$10^{2}$</td>
<td>$10^{1}$</td>
<td>$10^{0}$</td>
<td>+ve</td>
<td>-ve</td>
<td></td>
</tr>
</tbody>
</table>
New company established

- Commercial testing of blood and milk samples and sale of research kits will be available soon

www.pbdbio.com

PBD Biotech specialises in novel phage-based diagnostic technology in the field of veterinary diagnostics. The company has proprietary technology which can be used to detect the presence of mycobacteria such as Mycobacterium bovis (bovine TB) and Mycobacterium avium subsp. paratuberculosis (MAP; Johne’s disease), which are significant causes of morbidity and loss of production efficiency in dairy herds.

Latest news

13 Sep 2017

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Pasadena, CA
626 394 9771

info@pbdbio.com
Acknowledgements

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