Advances in pathogen diagnostics
Dr Rick Mumford

Over the last 15 years, Fera has established a world-class reputation in the development of plant diagnostics. Key achievements include establishing real-time PCR (polymerase chain reaction) as a routine tool for plant diagnosticians and taking lateral flow devices (LFDs) through from concept to a product; manufactured in the UK and used by inspectors and growers around the world. More recently, research has focused in two key areas: the development of sensitive DNA diagnostics for field use and the use of cutting-edge genomics to identify unknown pathogens associated with new diseases.

While LFDs are the ultimate simple-to-use field diagnostic, they have limitations, for example, they sometimes lack the required sensitivity to detect low concentration pathogens. To overcome these disadvantages, Fera has developed DNA-based field detection methods. This has included the field deployment of portable real-time PCR with the Plant Health & Seeds Inspectorate (PHSI), as part of the Phytophthora ramorum eradication campaign. More recently, the focus has shifted towards even simpler and faster technology, using a different DNA amplification technology: ‘Loop-mediated isothermal amplification’ (LAMP). While this newer technology is as specific and sensitive as existing methods like PCR, it does not require multiple cycles of cooling and heating to amplify a specific target gene sequence (ie it is ‘isothermal’). It is also less influenced by inhibitors found in plant material that can stop PCR from working unless removed by DNA clean-up methods. As a result, LAMP assays can be performed on simpler equipment and often without the need for multi-step sample processing. After extensive trials, this device is now being deployed; both with PHSI at Heathrow airport, to support import inspections, and with tree health inspectors, to aid field monitoring for tree diseases, including ash dieback. It allows results to be obtained in minutes, rather than waiting days for lab diagnosis.

The application of genomics for the rapid identification of new or unusual pathogens is another area of significant progress. This approach uses next generation sequencing (NGS), an incredibly powerful tool that combines advances in sequencing technology (hardware and chemistry) and DNA data analysis (‘bioinformatics’), to allow the generation of massive amounts of sequence information in days, where previously it took months. Now researchers can compare whole genomes of organisms, rather than just a handful of genes or study whole populations of organisms such as soil microbes (‘metagenomics’). Fera has applied NGS to diagnose new diseases. By taking samples from diseased and healthy plants, sequencing and comparing the DNA in them, it is possible to identify viruses and other pathogens that are the potential cause of the disease. As this approach is non-targeted (ie not looking for specific organisms), it is an excellent way of identifying ‘unknown’ pathogens, which are difficult to identify using traditional diagnostics. This new approach has proved critical in identifying the viruses causing a wide range of new crop diseases in the UK and abroad.
Advances in Plant Diagnostics

Rick Mumford
Head of Plant Science
The Food & Environment Research Agency (Fera)

Three major challenges for diagnostics

- Enhancing national biosecurity
- Identifying new pathogens
- Supporting sustainable intensification
Tree health: our greatest current biosecurity challenge
Detection in the field is not easy

Detecting pathogens in the lab

- HTP real-time PCR
- DNA bar-coding
- Diagnostic arrays
Rapid testing e.g. Lateral Flow Devices

- Antibody-based
- Single step
- Rapid (2-3 minutes)
- Genuine field testing

PCR methods in the field
LAMP: Loop-mediated isothermal AMPlification

- 3 pairs of primers (internal, external, loop)
- Bst DNA polymerase with strand-displacing activity
- Internal primers generate product containing single-stranded loops

Next generation portable DNA detection e.g. Guignardia citricarpa LAMP

Results in 10-15 mins
Trial of methods with inspectors

2013 – complete deployment for identification of 5 priority quarantine targets at Heathrow and Zurich airports

“...not much more involved than using a LFD kit...”

“The instrument itself also seemed very simple and easy to use.”

“It was impressive how quickly you could get a result...”

Ash Dieback (Chalara fraxinea)
LAMP for detection of *Chalara fraxinea*

- Primer design and initial test end of October
- Development of field extraction method for ash November
- Improved assay design mid-November
- Finalised protocol for validation

150 previously-tested samples tested by LAMP end November: 34% infected, 66% *C. fraxinea*-negative

![Graph showing fluorescence over time for NTC, *H. albidus*, *H. pseudoalbidus*, and *C. f*-infected ash.]

<table>
<thead>
<tr>
<th>TaqMan</th>
<th>LAMP</th>
</tr>
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<tbody>
<tr>
<td>+ 46</td>
<td>2</td>
</tr>
<tr>
<td>- 5</td>
<td>97</td>
</tr>
</tbody>
</table>

- positive predictive value = 96%
- negative predictive value = 95%

- sensitivity = 90%
- specificity = 98%

LAMP for detection of *Chalara fraxinea*

**Approx. 30 minutes hands-on time to test 14 samples (mostly sampling)**

- Take sample from leading edge of lesion
- Place in tube with PEG buffer and shake for 1 minute
- Transfer approx. 10 µl into tube containing 90 µl water
- Transfer approx. 1 µl per LAMP reaction
- Run LAMP on Genie II instrument

- 2-5 minutes per sample
- 1 minute up to 8 samples
- 2 minutes up to 14 samples
- 2 minutes up to 14 samples
- 35 minutes up to 14 samples

![Image of LAMP protocol steps and timing chart.]

20/09/2013
LAMP for detection of *Chalara fraxinea*

LAMP is now being performed routinely by FC inspectors for *Ash die back pathogen*

http://www.bbc.co.uk/news/uk-20217453

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New virus first findings in the UK: 1980-2013

Total = 55
High-risk hosts e.g. petunia

More than 150 potential viral pathogens identified

New virus discovery: the impact of new technology

Mean = 1.3 new viruses per year

Routine Molecular diagnostics

Fera invests in NGS
Next-generation DNA sequencing technology at Fera

<table>
<thead>
<tr>
<th>MiSeq (Illumina)</th>
<th>GS-FLX-Titanium + (Roche)</th>
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<tbody>
<tr>
<td>250bp x 2 x 18 million</td>
<td>750bp x 1 million</td>
</tr>
<tr>
<td>Hands on time to run : 20 min</td>
<td>Hands on time to run : 3.5 days</td>
</tr>
<tr>
<td>Cost per run: hundreds of pounds</td>
<td>Cost per run: thousands of pounds</td>
</tr>
<tr>
<td>High error rate at end of reads</td>
<td>Problems with homopolymers</td>
</tr>
<tr>
<td>Microbial genome sequencing, virus sequencing</td>
<td>Amplicons</td>
</tr>
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</table>

The use of NGS technologies for diagnostics


Case Study: Internal browning of carrot (HDC-FV382a)

• Long standing industry issue (10 years +) of unidentified cause
• Carrots showing internal necrosis symptoms linked to viral infection
• Whole crop rejection on processing line due to >5% affected carrots

Conventional approaches

• Assessed 3300 carrots for browning symptoms
• 3% showed symptoms
• 100 affected and 100 unaffected screened for virus
• Conventional diagnostics by RT-PCR:
  – Parsnip yellow fleck virus
  – Carrot Motley Dwarf (Carrot red leaf virus, Carrot mottle virus, Carrot red leaf associated viral RNA)
• No association between infection and necrosis was found
MiSeq Sequencing

- Deep sequencing included in experimental plan
- 12 necrotic carrots & 12 healthy
- 2 MiSeq runs: 16 million reads
- Detailed bioinformatics analysis

Viruses found by NGS
Summary of NGS

Complete genomes of:

- Carrot yellow leaf virus (most prevalent in affected)
- Carrot mottle virus
- Carrot red leaf virus
- Carrot red leaf associated virus
- Beet western yellows associated (unexpected)
- a novel virus in the same genus as Carrot yellow leaf virus
- 2 novel Betaflexiviruses
- a novel carrot Torradovirus

Summary of Real-time PCR results

“Carrot yellow leaf virus (CYLV) is strongly associated with necrosis”
‘Diagnosis is not the end, but the beginning of practice’ Martin H. Fischer

Crop losses from pests, diseases and weeds

- Global pre-harvest losses are estimated to be between 25-40% (depending on crop)

<table>
<thead>
<tr>
<th>2001-03 Figures</th>
<th>Weeds</th>
<th>Pests</th>
<th>Diseases</th>
<th>Total (Global Mean)</th>
<th>Total (NW Europe)</th>
<th>Total (Central Africa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>7.7</td>
<td>7.9</td>
<td>12.6</td>
<td>28.2</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>Potato</td>
<td>8.3</td>
<td>10.9</td>
<td>21.1</td>
<td>40.3</td>
<td>24</td>
<td>50</td>
</tr>
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</table>

After Oerke, 2006
**Sustainable intensification**

‘producing more output from the same area of land while reducing the negative environmental impacts………..’

<table>
<thead>
<tr>
<th>Fungicide treatments on cereals</th>
<th>1990</th>
<th>2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area treated (Ha)</td>
<td>16.6M</td>
<td>27.6M</td>
</tr>
<tr>
<td>No. of applications:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>65%</td>
<td>36%</td>
</tr>
<tr>
<td>3 or more</td>
<td>25%</td>
<td>51%</td>
</tr>
</tbody>
</table>

**Wheat yields**

**Fungicide usage**

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**Common scab of potato (R448)**

*Using molecular diagnostics to improve crop management*

- Potato scab disease is controlled by (often excessive) irrigation
- Molecular diagnostics used in field experiments to inform recommendations to farmers for better targeted irrigation
NGS analysis of microbial communities

Glasshouse experiments show that, in absence of soil microbial communities, irrigation has no effect on scab level

NGS analysis of bacterial and fungal communities in soil to establish links between water-soil microbes-disease suppression

The future of diagnostics?

Researchers  Diagnosticians  Inspectors  Industry & general public  Remote & real-time monitoring
Generic surveillance networks

- e.g. Integrated biosensor disease risk management system

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Earlier detection

- Use existing monitoring & surveillance systems
- Smart traps
- Remote sensing
- Better detection tools
- Acoustics
- Citizen science
The further use of Omic technologies

Identification of non-DNA markers of disease or resistance

Innovation in platforms will drive future applications
Acknowledgements

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  - Field diagnostics: Neil Boonham, Jenny Tomlinson, Sioban Ostoja-Starzewski, Paul Beales
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Thank you