nak7tuinbouw
Validation of a RT Taqman PCR for detection of pospiviroids in tomato seeds

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Overview

- Development pospiviroid assay
- Validation study, EPPO Guideline PM 7/98 (2)
  - Analytical sensitivity
  - Analytical specificity
  - Repeatability and reproducibility
  - Trueness
- Retrospective analysis
- Conclusions
Pospiviroids

- Infectious, small, circular RNA
- Can cause severe damage in tomato and potato
- Easily transmitted
- Often symptomless in ornamentals
- PSTVd has a quarantine status in many countries
- Several countries have broadened their phytosanitary regulations to include several other pospiviroids
Current practice PSTVd at Naktuinbouw

- ISO 17025 accreditation for tomato/PSTVd
- Several seed lots positive for PSTVd: all subs positive!
- All blind samples with PSTVd detected
- Bakker et al. 2015 “Detection of PSTVd and TCDVd in seeds of tomato using real-time RT-PCR” EPPO Bull. 45: 14-21

Project:
Develop and validate new broad pospiviroid assay for matrix tomato (and pepper) seeds

Scope:
Detection of seven pospiviroids in tomato seeds
## Protocol pospiviroids

### Pospiviroids in new assay

<table>
<thead>
<tr>
<th>Viroid Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrus exocortis viroid</td>
<td>CEVd</td>
</tr>
<tr>
<td>Columnnea latent viroid</td>
<td>CLVd</td>
</tr>
<tr>
<td>Pepper chat fruit viroid</td>
<td>PCFVd</td>
</tr>
<tr>
<td>Potato spindle tuber viroid</td>
<td>PSTVd</td>
</tr>
<tr>
<td>Tomato apical stunt viroid</td>
<td>TASVd</td>
</tr>
<tr>
<td>Tomato chlorotic dwarf viroid</td>
<td>TCDVd</td>
</tr>
<tr>
<td>Tomato planta macho viroid</td>
<td>TPMVd*</td>
</tr>
</tbody>
</table>

* Including former MPVd
### Protocol pospiviroids

- **3 x 1000 seeds**
- **Improved soaking of tomato seeds**
  - GH+ buffer (PN1)
  - 30-60 min. at RT (overnight soak)
  - Spike: viroid DLVd (endogenous nad5)
- **RNA isolation**
  - 90 seconds in minimixer
  - Improved Sbeadex RNA extraction with Kingfisher

#### "Multiplex" Taqman

<table>
<thead>
<tr>
<th>&quot;Multiplex&quot; Taqman</th>
<th>Pospiviroid targets</th>
<th>Internal amplification control (IAC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix A</td>
<td>PSTVd, TCDVd and MPVd*</td>
<td>PCFVd**</td>
</tr>
<tr>
<td>Mix B</td>
<td>CEVd*, CLVd*</td>
<td>DLVd</td>
</tr>
<tr>
<td>Mix C</td>
<td>TPMVd**</td>
<td>DLVd</td>
</tr>
<tr>
<td>Mix D</td>
<td>TASVd*</td>
<td>Nad5 RNA</td>
</tr>
</tbody>
</table>

* FERA
** Naktuinbouw
Validation within EU-TESTA

1. Analytical sensitivity
   - dilution series

2. Analytical specificity of primer sets
   - 18 pospiviroids
   - 29 non-target viroids and viruses

3. Repeatability and reproducibility
   - Same conditions
   - Varying conditions

4. Trueness
   - Comparison with previously validated PSTVd/TCDVd-assay

EPPO Guideline PM7/98 (2)
Analytical sensitivity

- ‘the lowest value, in a laboratory sample, of the target pathogen, which can still be determined with a certain degree of reliability’

- Requirement: 100x dilution detected

- 4 samples composed of:
  - Positive seeds for PSTVd, TCDVd, TASVd, CLVd
  - Diluted seed extract for PCFVd
  - Spike in negative seed lot CEVd and TPMVd

- 3 dilutions measured in triplicate
Analytical sensitivity

Pospiviroid

- PSTVd
- CLVd

- 100 seeds
- 10 seeds
- 1 seed

Pospiviroid

- TCDVd
- TASVd

- 100 seeds
- 10 seeds
- 1 seed

Pospiviroid

- PCFVd
- TPMVd

- 10 x
- 100 x
- 1000 x

Pospiviroid

- CEVd

- 10 x
- 100 x
- 1000 x
Analytical specificity

- ‘The ability of a method to distinguish the target organism (pathogen) from other organisms, whether related or not, and the extent to which the analysis can distinguish (known) variants of the organism’

- Requirements:
  - False-negatives unacceptable
  - Cross-reaction between pospiviroids acceptable

- Samples:
  - 18 pospiviroids
  - 14 non-target viroids, 15 viruses
## Analytical specificity

<table>
<thead>
<tr>
<th></th>
<th>Target viroids</th>
<th>Non-target</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSTVd/TCDVd Taqman positive</td>
<td>Real Pos 7</td>
<td>False pos 0</td>
</tr>
<tr>
<td>PSTVd/TCDVd Taqman negative</td>
<td>False neg 0</td>
<td>Real neg 40</td>
</tr>
<tr>
<td>PCFVd Taqman positive</td>
<td>Real Pos 1</td>
<td>False pos 0</td>
</tr>
<tr>
<td>PCFVd Taqman negative</td>
<td>False neg 0</td>
<td>Real neg 46</td>
</tr>
<tr>
<td>CEVd/CLVd Taqman positive</td>
<td>Real Pos 6</td>
<td>False pos 3</td>
</tr>
<tr>
<td>CEVd/CLVd Taqman negative</td>
<td>False neg 0</td>
<td>Real neg 38</td>
</tr>
<tr>
<td>TPMVd Taqman positive</td>
<td>Real Pos 1</td>
<td>False pos 0</td>
</tr>
<tr>
<td>TPMVd Taqman negative</td>
<td>False neg 0</td>
<td>Real neg 46</td>
</tr>
<tr>
<td>TASVd Taqman positive</td>
<td>Real Pos 3</td>
<td>False pos 0</td>
</tr>
<tr>
<td>TASVd Taqman negative</td>
<td>False neg 0</td>
<td>Real neg 44</td>
</tr>
</tbody>
</table>
Repeatability and reproducibility

- ‘The degree of correspondence between the results of successive measurements of the same sample performed under equal or varying conditions’

- Requirement: >95%

- 4 samples, 6 replicates
  - 3 on 1 day in 1 lab
  - 3 on different days by different operators

1. PSTVd, CEVd
2. PCFVd, CLVd, TASVd
3. TCDVd, TPMVd
4. Negative seeds
Repeatability and reproducibility

- Conclusion: repeatability and reproducibility 100%
Trueness

• ‘The ability of a method to do what it ‘says’ (i.e. detection of pospiviroids in the matrix tomato seeds). In other words, the ability to detect the target organism in the matrix assessed with a second method’

• Requirement: 100 % match between results

• Comparison between new assay and previously validated PSTVd/TCDVd assay

• 3 samples, 8 replicates:
  • 1 PSTVd-positive seed in 999 negative seeds
  • 1000 seeds of PSTVd-positive lot
  • 1 TCDVd-positive seed in 999 negative seeds
Trueness

- Comparison of detection of PSTVd and TCDVd
- Significantly lower Ct-values with new assay

![Bar chart showing trueness comparison between PSTVd and TCDVd samples SPN-V043 V2.0 and SPN-V003. The chart displays Ct-values for samples 1, 2, and 3, with PSTVd and TCDVd samples shown separately.]
Conclusions

- Validation requirements are met for:
  - Sensitivity
    - >100x dilution detected
  - Specificity
    - No false-negatives
    - Cross-reaction between target pospiviroids
  - Repeatability and reproducibility
    - 100%
  - Trueness
    - New assay more sensitive
- Assay is ‘fit for purpose’
Findings at Naktuinbouw

Detection pospiviroids in tomato seeds

<table>
<thead>
<tr>
<th>Year</th>
<th>not detected</th>
<th>suspect</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>294</td>
<td>2</td>
</tr>
<tr>
<td>2012</td>
<td>446</td>
<td>4</td>
</tr>
<tr>
<td>2013</td>
<td>206</td>
<td>3</td>
</tr>
<tr>
<td>2014</td>
<td>186</td>
<td>13</td>
</tr>
</tbody>
</table>
Retrospective analysis of data

- In the beginning only Boonham RT Taqman for PSTVd/TCDVd/TPMVd
- Few positive seed lots
- Tomato: low viroid load and high incidence
Seed contamination with pospiviroids new?

Naktuinbouw vegetable seed collection (ZZB)

15 tomato lots tested:
- 9 negatives
- 6 positives* (6 with CLVd & 3 double with PCFVd)

*Produced in 1992-1994!
In summary

- Pospiviroids seed Taqman assay has been developed:
  - Assay for 7 viroids
  - Improved RNA isolation
  - New IAC DLVd to monitor validity of test result
  - Successfully validated

- Several pospiviroids contaminated seed lots were detected
  - Contaminated seed lots have been around for considerable time

- Next steps:
  - EPPO Protocol
  - Broaden scope to pepper seeds
Acknowledgements

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