nak tuinbouw

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

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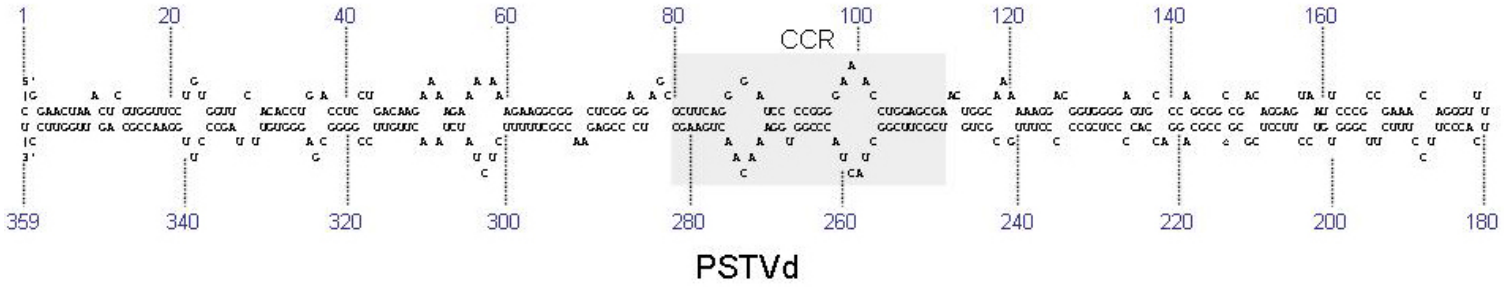
Overview

- 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



PSTVd

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	
Citrus exocortis viroid	CEVd
Columnea latent viroid	CLVd
Pepper chat fruit viroid	PCFVd
Potato spindle tuber viroid	PSTVd
Tomato apical stunt viroid	TASVd
Tomato chlorotic dwarf viroid	TCDVd
Tomato planta macho viroid	TPMVd*

* 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	Pospiviroid targets		Internal amplification control (IAC)
	FAM	VIC	TR
Mix A	PSTVd, TCDVd and MPVd*	PCFVd**	DLVd
Mix B	CEVd*, CLVd*		DLVd
Mix C	TPMVd**		Nad5 RNA
Mix D	TASVd*		No IAC
* 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

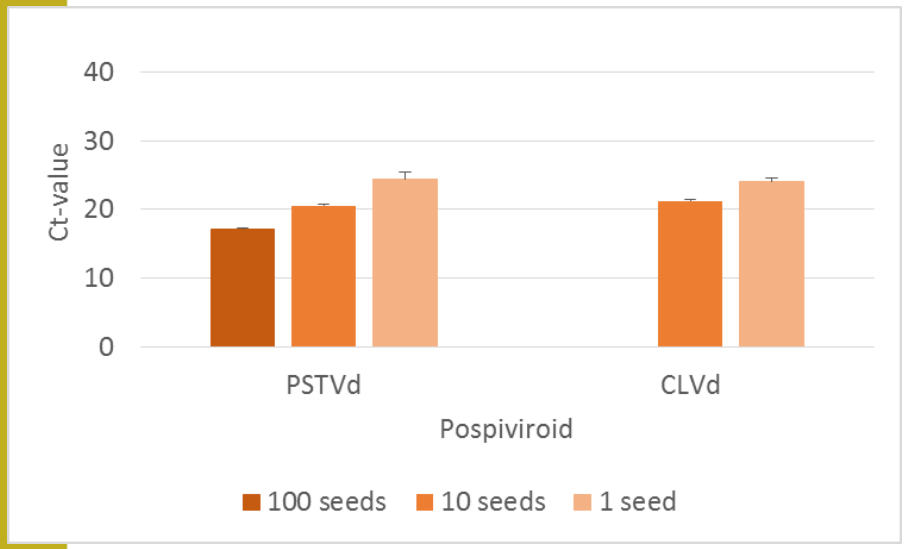
Analytical sensitivity

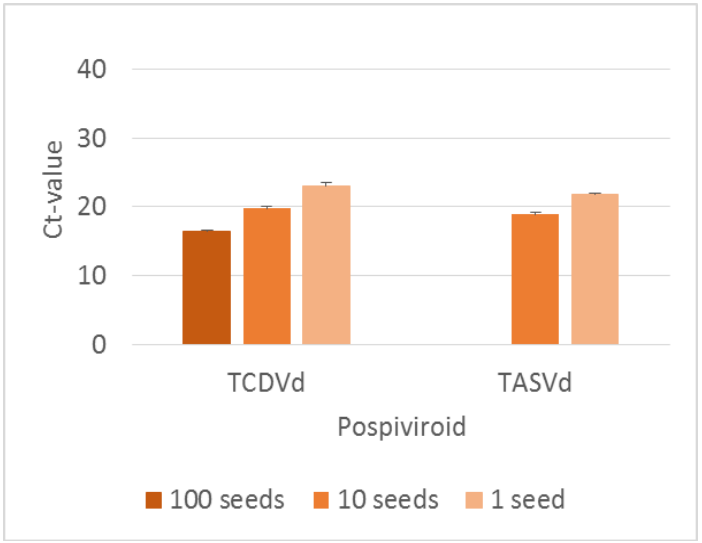
- ‘the lowest value, in a laboratory sample, of the target pathogen, which can still be determined with a certain degree of reliability’

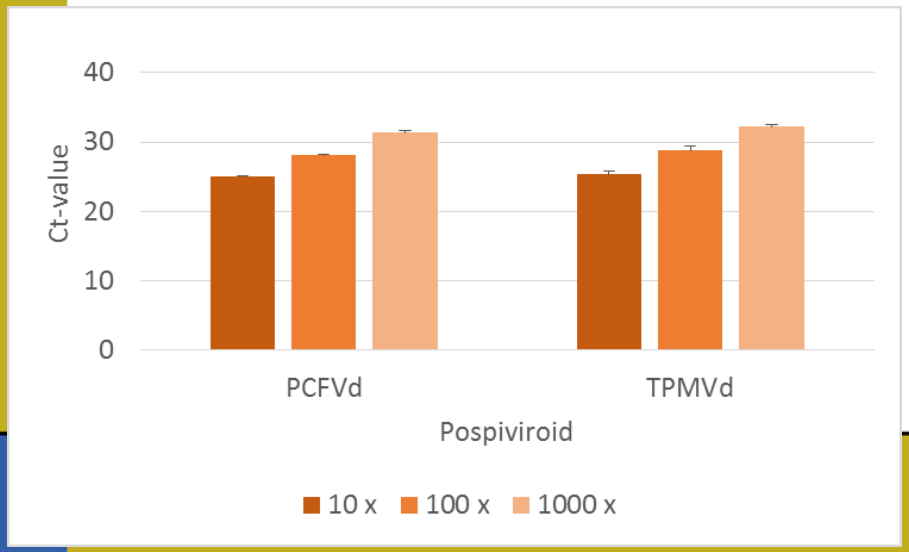
- Requirement: 100x dilution detected

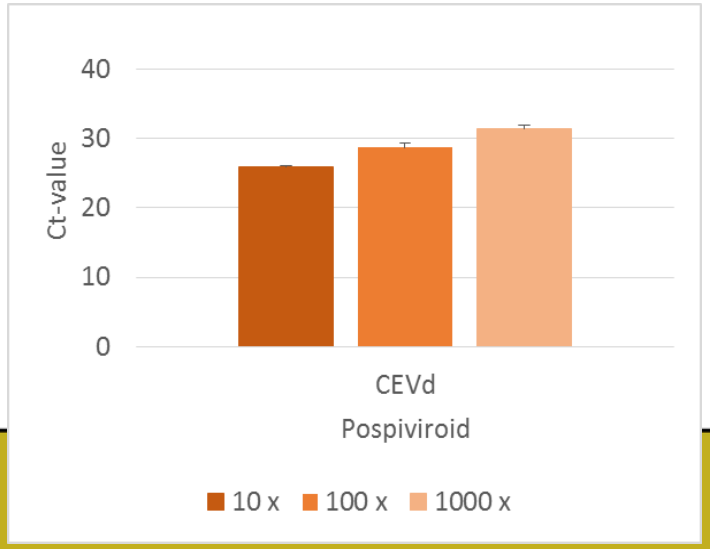
- 4 samples composed of:
  - Positive seeds for PSTVd, TCDVd, TASVd, CLVd
  - Diluted seed extract for PCFVd
  - Spike in negative seed lot CEVd and TPMVd
- 3 dilutions measured in triplicate

Analytical sensitivity









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

	Target viroids	Non-target
PSTVd/TCDVd Taqman positive	Real Pos 7	False pos 0
PSTVd/TCDVd Taqman negative	False neg 0	Real neg 40
PCFVd Taqman positive	Real Pos 1	False pos 0
PCFVd Taqman negative	False neg 0	Real neg 46
CEVd/CLVd Taqman positive	Real Pos 6	False pos 3
CEVd/CLVd Taqman negative	False neg 0	Real neg 38
TPMVd Taqman positive	Real Pos 1	False pos 0
TPMVd Taqman negative	False neg 0	Real neg 46
TASVd Taqman positive	Real Pos 3	False pos 0
TASVd Taqman negative	False neg 0	Real neg 44

Repeatability and reproducibility

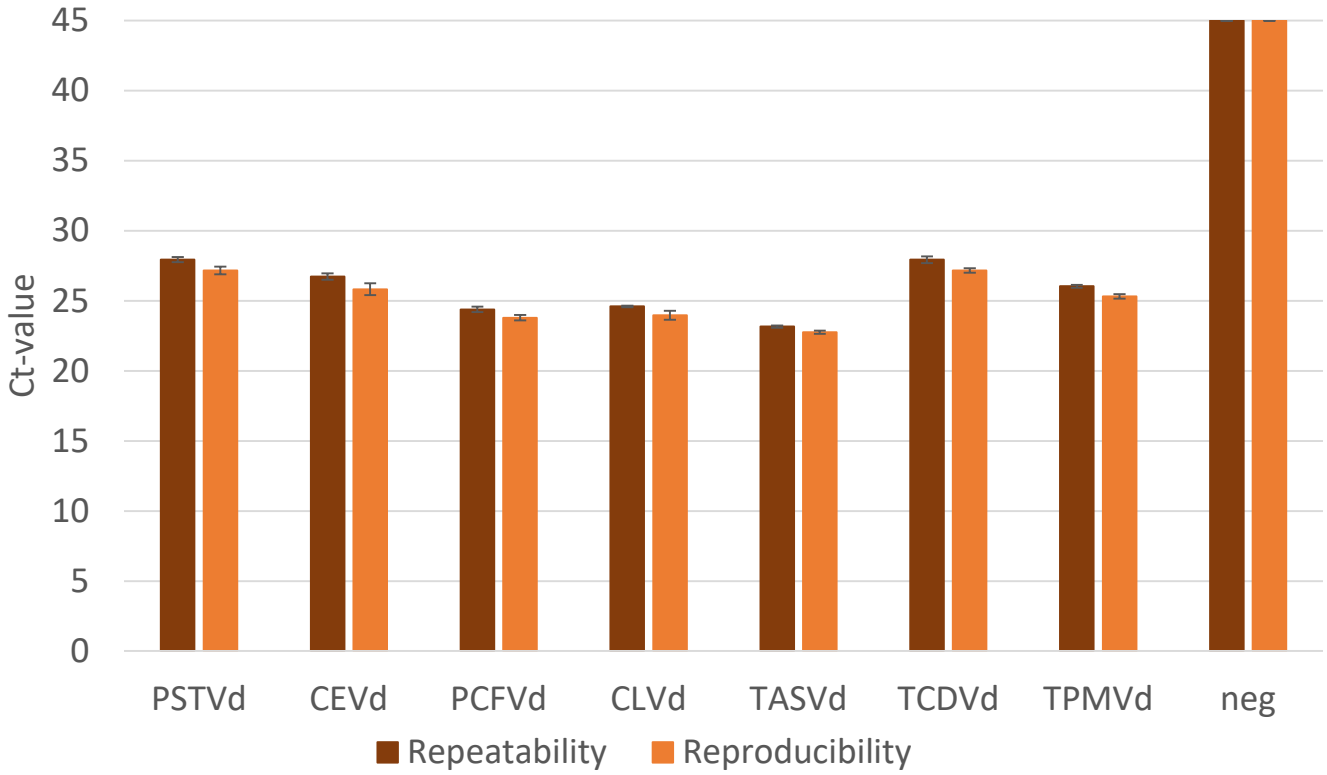
- ‘The degree of correspondence between the results of successive measurements of the same sample performed under equal or varying conditions’

- 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%

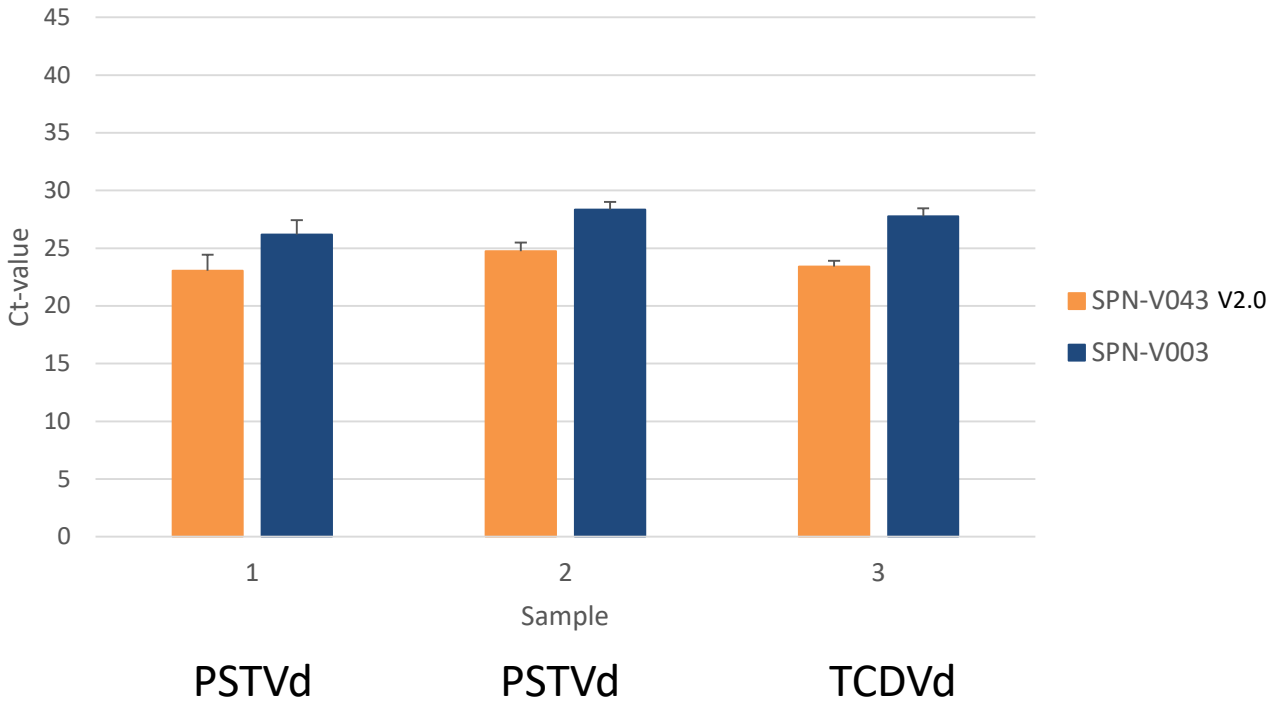
Trueness

- ‘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

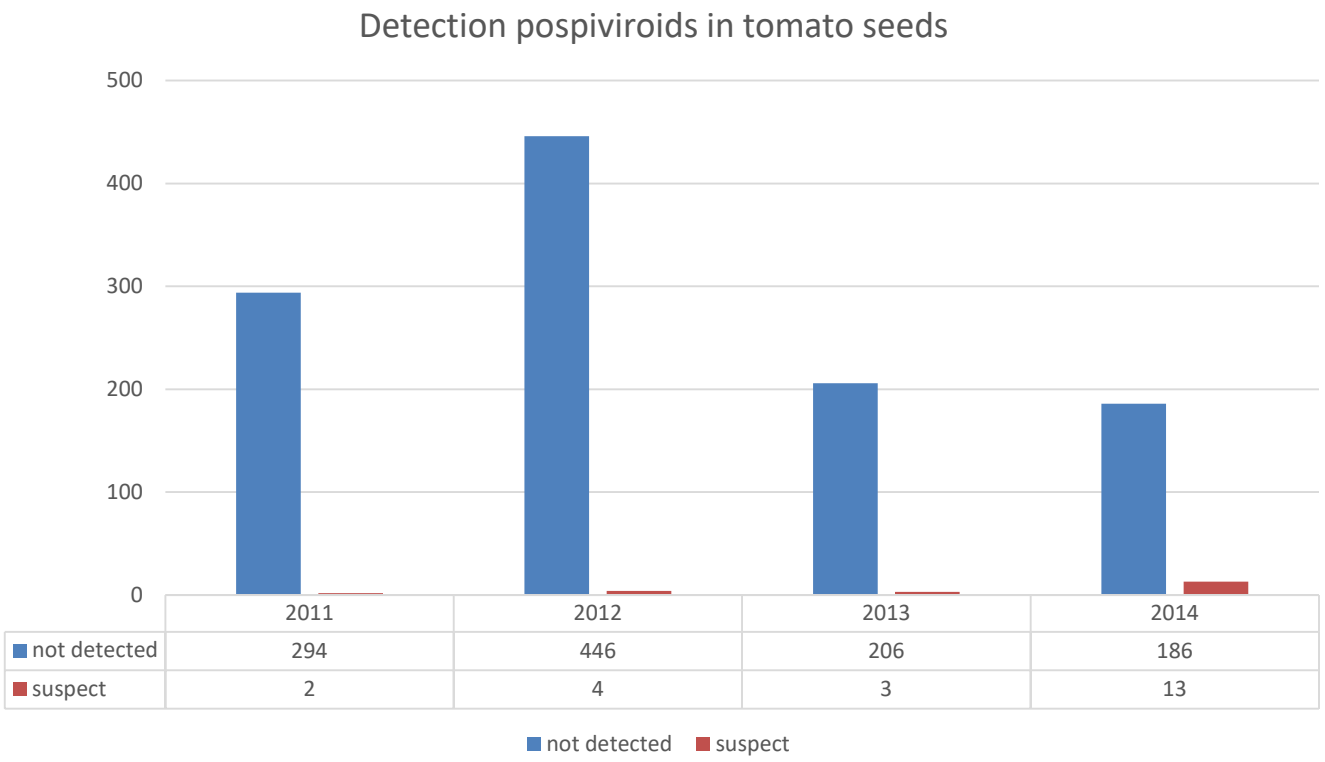
- Comparison of detection of PSTVd and TCDVd
- Significantly lower Ct-values with new assay



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



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
  - Succesfully 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

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