Chemiluminescent detection of potato spindle tuber viroid (PSTVd) using a digoxigenin-labeled RNA probe

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Abstract

The potential use of a simple, sensitive and nonradioactive method for detecting potato spindle tuber viroid (PSTVd) in potato leaves, based on nucleic acid hybridization with a PSTVd-specific RNA probe labeled with digoxigenin was investigated.

Four simple procedures for the clarification of plant extracts suitable for a nonradioactive detection system were also investigated. The technique described here could detect the viroid from 0.0155 mg of tissue that was estimated to contain about 17pg PSTVd. In samples extracted with CTAB buffer the DIG-probe detected down to 300pg of TNA and from crude sap, DIG-probe detection limit was 1:288 sap dilution in 5X SSC buffer. The benefits of this nonradioactive detection system are discussed.

Key words: Potato spindle tuber viroid (PSTVd), nonradioactive NASH, Digoxigenin-labeled RNA probe, anti-Dig AP-conjugate.

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INTRODUCTION

Viroids are the smallest and structurally best-characterized infectious molecules. They are unencapsidated; single stranded covalently closed RNA molecules with a chain length of 240-380 nucleotides (Singh and Boucher, 1987). These naked RNA molecules cause serious diseases among many crop plants, fruit trees and ornamentals, including potato, tomato, cucumber, chrysanthemum, avocado, and coconut palms, to name only a few. The viroid induces diseases that lead to dramatic economic losses in agriculture and horticulture worldwide.

The potato spindle tuber viroid (PSTVd) was the first plant viroid to be discovered (Diener, 1971). It consists of the existence of 359 nucleotides (Gross et al., 1978). Various PSTVd strains produce disease in tomato (*Lycopersicon sculentum* Mill.) characterized by mild, intermediate or severe symptoms (Fernow, 1987; Sanger, 1984).

In potato, natural spread of PSTVd has been repeatedly shown to occur either by foliar contact or botanical seed (Querci et al., 1997) and is also known to be highly pollen-transmissible in several host plants (Puchta, 1990). However, currently only PSTVd has been found infecting cultivated potato naturally, although Mexican papita viroid has been found infecting the wild potato species *Solanum cardiophyllum* (papita guera, cimantli) (Martinez-Soriano et al., 1996). Early reports (Gross, 1930) suggested that chewing insects might transmit PSTVd, but these reports have not been confirmed. De Bokx and Piron (1981) reported a low rate of transmission by the aphids *Macrosiphum euphorbiae* (Thomas) but no transmission was obtained with *Aulacorthum solani* (Kaltenbach). In 1995, Salazar et al. reported that *Myzus persicae* (Sulz.) could transmit the viroid to potato and other test plants only when the source plant is doubly infected with PLRV and PSTVd.

The importance of the potato spindle tuber viroid in seed potato production, germplasm collections and potato breeding programs has been widely recognized (Diener, 1979). CIP currently uses nucleic acid spot hybridization (NASH) using radioactively labeled probes as a routine screening tool for the presence of PSTVd in germplasm material. This screening is particularly important for material that is to be exported outside of Peru. Radioactive probes give very clear and unambiguous results (signals) and require minimal sample preparation. However, due to the nature of radioactive decay, the probe does not last and samples have to be tested in

batches, reducing flexibility. Working with radioactivity also necessitates additional safe working procedures as well as generating waste disposal issues. During the last decade, the diagnosis of plant diseases and pests has undergone remarkable improvement as a result of the development of recombinant DNA technology. The detection method must fulfill the following practical requirements: be simple to perform, have a high sensitivity, be suitable for testing many samples and preferably not involve potentially dangerous procedures such as hybridization with radioactively labeled probes. This last requirement is especially important if the method is to be implemented in developing countries (Borkhardt et al., 1994).

Several nonradioactive labeling systems are currently available. Thus digoxigenin (DIG), the steroid hapten synthesized exclusively in foxglove plants and therefore far less likely to contribute to false positive results, has been used for labeling molecular probes for PSTVd detection (Borkhardt, 1994).

Here we report the results of the standardization and validation of a sensitive method of detecting PSTVd in leaf tissue based on non-phenolic clarification of plants extracts combined with dot spot hybridization using a specific DIG-labeled RNA probe.

Theory

The rapid and reliable detection of viroids is the only way to control the spread of viroids by eradication of all infested material at a very early stage of infection. Although PSTVd can be eliminated from infected plants by cold treatment and meristem culture, most control measures are based on prevention rather than cure (Salazar et al., 1983). Both bioassay on tomato and electrophoresis (PAGE) have played an important role in PSTVd detection in the past, but these methods were too laborious and not suitable for large-scale testing (Salazar et al., 1988). The development of recombinant DNA technology in the last decade made possible the construction of PSTVd specific nucleic acid probes. Owens and Diener (1981) developed a sensitive method of detection based on nucleic acid hybridization utilizing double-stranded DNA probes. Although these probes have been successfully utilized (Salazar et al., 1983), their preparation was highly dependent on the quality and storage of the reagents for nick translation (Salazar et al., 1988).

At present, molecular hybridization techniques, which include those using radioactively and nonradioactively labeled DNA/RNA probes and PCR (RT-PCR, Taq-Man PCR) techniques are most frequently used in practice.

From these available molecular hybridization techniques, nonradioactive detection methods offer several advantages, and all the reagents needed to perform the labeling of RNA/DNA and detection of hybrids are commercially accessible. No special protective equipment is required during the hybridization and detection steps, and nonradioactively labeled probes are stable.

Thus the hybridization solution containing the labeled probe can be saved and reused several times. These properties should make nonradioactive detection systems very attractive, especially for laboratories where handling or disposal of radioactive material presents special problems (Borkhardt et al., 1994).

MATERIALS AND METHODS

Plant materials

All PSTVd-infected materials used in this study were obtained from research materials (cv. Maria Tambeña) and/or from PSTVd-infected tomato cv. Rutgers inoculated with a severe strain of PSTVd maintained at the International potato Center in Lima, Peru. Healthy controls from potato and tomato of the same cultivars when available were also used.

Dig-Protocol Standardization

Preparation and extraction of samples

Samples of potato and tomato (1gram each) leaves were homogenized in either (i) 5X SSC buffer (Salazar and Jayasinghe, 1997), (ii) 5X SSC / 18.5% HCHO (formaldehyde) buffer (Salazar and Jayasinghe, 1997), (iii) AMES buffer (Palukaitis et al., 1985) or in CTAB (Doyle and Doyle modified, 1987)

Dot blotting of nucleic acid samples

Extracted samples were tested in two steps:

First step, 3µl of each sample extracted by the four methods described above were spotted directly onto nylon and nitrocellulose membranes positively charged; air dried for 15-30 minutes and fixed by exposure to UV-light using a UV-crosslinker (Stratagene) for analysis comparison using Digoxigenin-labeled and ^{32}P-labeled probes.

Second step, extracted samples were serially diluted in two-fold dilutions in 5X SSC and CTAB buffers to test the sensitivity of the probe in crude sap extracts and total nucleic acid extractions.

In addition, about 0.1g of samples were extracted with the Plant RNA purification reagent (Invitrogen) and serial dilutions of this total RNA were assayed to determine detection limit of the PSTVd probe.

As for set 1, three micro liters (3ul) of each sample (extracted with CTAB and Invitrogen kit) were spotted onto a nylon and nitrocellulose membranes positively charged (Sigma-Aldrich), air dried for 15-30 minutes and fixed by exposure to UV light using a UV-Crosslinker (Stratagene).

Probe labeling

To prepare the DIG-RNA labeled probe, pST65-B2 clone containing the PSTVd insert in the form of tandem dimmer was digested with the restriction enzyme *Pst* 1 and labeled with Digoxigenin by RNA transcription according to manufacturer's (ROCHE) instructions with minor modifications. For comparison purposes, a PSTVd ^{32}P-RNA labeled probe was also prepared using the same recombinant plasmid (pST65-B2) as for DIG-probe and following CIP's standard protocol for RNA transcription described by Salazar and Jayasinghe (1997).

DIG-hybridization (Adgia Inc.) modified protocol

The protocol used throughout this study for PSTVd detection by nonradioactive NASH was that provided by the ring test (Dr. C. Jeffries personal communication Appendix 1) with minor modifications.

Protocol

- Grind the tissue sample in 5X SSC extraction buffer to a concentration ratio of 1:2 (sample weight: buffer volume), e.g., 0.5g of tissue per 1 ml of 5X SSC Buffer.
- Transfer 0.5 ml to a 1.5 ml micro centrifuge tube.
- Wearing gloves, add an equal volume of research grade phenol/chloroform (1:1, v/v) to each tube. Close the tube caps tightly, then mix the contents thoroughly by shaking, vortexing, or inverting until an emulsion has formed.
- Centrifuge the tubes for 5 minutes at 12,000 g to separate the contents into aqueous (top) and chloroform (bottom) layers.

Spotting samples

- Pipette 4 µl from the aqueous layer onto the nylon N+ membrane and air-dry the membrane at room temperature.
- UV crosslink the air-dry membrane

Hybridization/Detection

- Place the membrane containing fixed samples in a hybridization bag.
- Add the required volume of DIG Easy Hyb Buffer (0.15 ml/cm^2), and the required amount (approx. 2µl/cm^2) of thawed digoxigenin-labeled probe.

- Hybridize in a hybridization incubator (or shaking water bath or shaking incubator) overnight at 55°C.
- The next morning, wash the membrane for 5 min at room temperature in 200 ml of Wash Buffer 1 followed by washing for 15 min at room temperature in 200ml of Wash Buffer 1 containing 1 µg/ml RNase A.
- Wash the membrane twice, for 15 min per wash, at 65°C in 200 ml of pre-heated Wash Buffer 2.

NOTE: Preheat the wash buffer to 65°C.

- Rinse the membrane in 50 ml 1X Maleic Acid Buffer (100mM maleic acid, pH 7.5; 150mM NaCl) for 1 min at room temperature.
- Then block for 1h at room temperature in 25 ml 1X Blocking Solution prepared just before use, (i.e., 2.5 ml of 10x Blocking Solution + 22.5 ml 1x Maleic Acid Buffer) using a shaker at 100 to 150 rpm.

NOTE: 25 ml of 1x Blocking Solution is for 1 membrane. If more membranes are processed increase the amount.

- Spin centrifuges the anti-DIG-alkaline phosphatase solution at maximum rpm (c.10, 000-12,000 rpm) but briefly before use to prevent "speckling".
- Add the anti-DIG-alkaline phosphatase directly to the Blocking Solution used in the previous blocking step at a dilution of 1:10,000 taking care not to add the anti-DIG-alkaline phosphatase directly onto the membrane.
- Incubate the membrane for 30 min at room temperature on the shaker.
- Pour off the solution and wash the membrane twice, for 15 min per wash, at room temperature in approximately 150 ml 1x Maleic Acid Buffer. Use a shaker at 50 to 80 rpm.
- Wash the membrane once for 5 min at room temperature in 50-100 ml Detection Buffer (100mM tris-HCl, pH 9.5; 100mM NaCl).
- Place the wet membrane, sample side up, on an acetate sheet (or Cling Film) and dribble CSPD substrate all over the membrane using a pipette.
- Carefully pick up the sheet and membrane and gently move around to disperse the substrate.
- Carefully place another acetate sheet (or Cling Film) over the membrane and using the side of fingers, gently remove air bubbles and further disperse the substrate. Use a paper towel to wipe up any substrate that leaks out. Do not rub the paper towel across

the acetate sheets or static might develop that can create "lightning-like marks" on the film when developed.

- When the air bubbles have gone and the excess substrate removed, place the membrane in an autoradiography cassette.
- In a dark room place a film in the cassette. Firmly close the cassette and turn on the light.
- Expose membranes to film for 1.0–2.0 h at room temperature and then develop the film.

Solutions

20X SSC

10% SDS

Washing Buffer 1: 2X SSC, 0.1% SDS

Washing buffer 2: 0.1X SSC, 0.1% SDS

Additional facilities, equipment and reagents

Cling film or acetate sheets

Container for blocking, anti-dig washes, detection buffer

Incubator

Heat resistant dishes for stringency washes

Hybridization incubator or shaking benchtop incubator (55°C) or shaking water bath (55°C).

Micro centrifuge

Micro centrifuge tubes 1.5ml

Orbital shaker or tilting table

Pipettes

RNase A Sigma cat no r-4875, 100mg

UV Crosslinker, UV transilluminator or oven (80°C)

Water bath (65°C) also needed for stringency washes

Dark room

X-Ray cassette

X-ray film OMAT 13x18cm

Developer and Fixer

Trays for developing (x 3)

Additional experiments were conducted with the aim of optimizing the use of reagents and materials by preparing our own solutions and buffers to reduce the use of commercial kits and costs by saving and reusing for instance, the hybridization buffer containing the

probe after the hybridization step, the anti-Dig AP-conjugate as well as some key consumables such as hybridization bags and cling films or acetate sheets.

Thus, the performance of buffers and solutions prepared in the Molecular Virology Laboratory at CIP (except the blocking solution for which there is no information accessible) was evaluated against that of the ROCHE commercial kit that provides its clients with ready-to-use reactives and solutions. Healthy and infected samples from greenhouse as well as field samples from CIP internal and external users were tested.

In addition and due the urgent need of good alternatives to replace commercial cling films/acetate sheets and hybridization bags that are becoming difficult to obtain or were discontinued, a common polyvinyl chloride cover lamination (known as Vinifan) was assayed in comparison with the commercial acetate sheet (Amersham), while in the case of the hybridization bags, a commercial brand of plastic bags (Pharmacia) that use to supply us with hybridization bags was compared with using simple sterilization bags. A three times replicated experiment was conducted in order to determine how many times a good Dig-RNA labeled probe can be reused.

Finally, probe stability was tested by storing a Dig-RNA labeled probe prior to and after using it for as long as 6 months. This test was repeated twice with two Dig-labeled probes coming from different batches.

PSTVd DIG-RNA labeled probe validation

From May 2005 through the first semester of 2006, more than 1000 samples (from field and greenhouses) received for routinely testing by conventional Nucleic Acid Spot Hybridization (NASH) using ^{32}P-RNA probe were spotted in duplicate onto nylon and nitrocellulose membranes in order to analyze them by using the DIG-RNA labeled probe and by our routine radioactive NASH method.

RESULTS AND DISCUSSION

The usefulness of the digoxigenin-RNA labeled probes was examined for the detection of PSTVd (severe strain only) in potato, tomato and eventually avocado materials.

Four sample extraction buffers (5X SSC, 5X SSC/18.5% HCHO, AMES and CTAB), were compared in order to determine the most suitable extraction procedure to be used as a routine method for PSTVd detection by DIG-NASH as part of the standardization process. Of these buffers, 5X SSC and CTAB buffers performed better, based on their higher sensitivity (fig. 1).

Although, 5X SSC is recommended as the more suitable buffer also because of its simplicity. This relies on the fact that the detection method has to be as simple as possible, maintaining a balance between simplicity and sensitivity.

Tissue printing method for PSTVd detection was also evaluated in potato and tomato leaf samples from greenhouse. Preliminary results were promising but the method needs further evaluation, particularly including field samples.

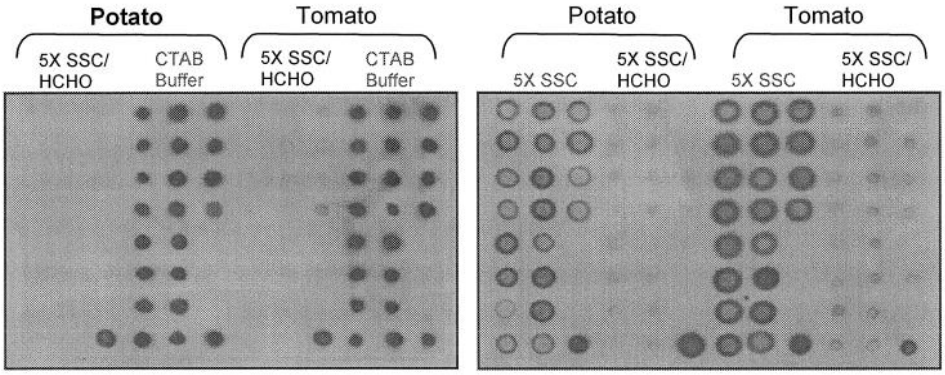

Figure 1. Evaluation of buffer extraction method suitability. 5X SSC and CTAB buffers performed better, based on their higher sensitivity.

CIP's DIG-RNA labeled probe detection limit was 300pg from infected samples extracted by CTAB TNA extraction method and could detect up to 1:288 dilution of leaf samples extracted with 5X SSC (fig. 2). The detection of PSTVd in total RNA extracts (Invitrogen's kit), confirmed the results obtained with Agdia's DIG probe (Ring test report, 2003).

Our data demonstrate that CIP's 32P- labeled RNA probe can reliably detect PSTVd strains in crude sap and nucleic acid extracts (Ring test report, 2003 and current study). In the same way, Borkhardt's (1994) statement indicating that the DIG-based hybridization system in combination with chemiluminescent detection of hybrids had the same detection level for PSTVd as the 32-P based-detection system (autoradiographed with an intensifying screen for 16 hours at minus 70ªC) was confirmed.

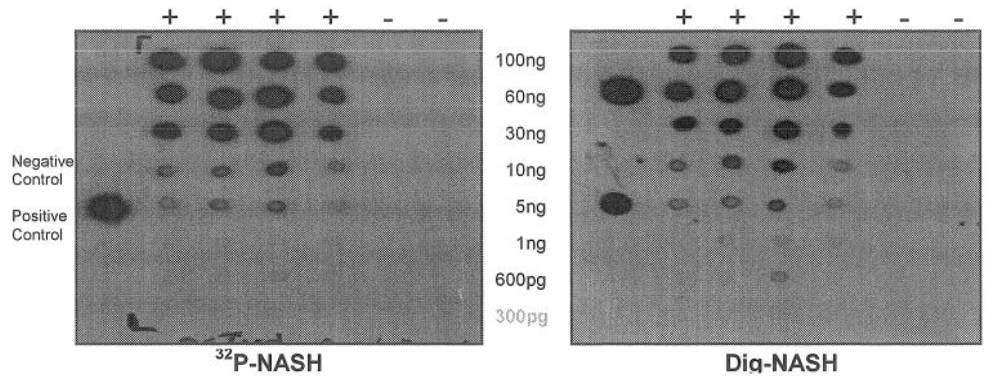

Figure 2. Dig-labeled and 32P-labeled RNA probes performance comparison. CIP's DIG-labeled RNA probe detected up to 300pg from infected samples extracted by CTAB TNA extraction method.

The first attempts to detect PSTVd in avocado samples spotted onto nylon membranes extracted with 5X SSC buffer gave good results showing that it is possible to extend the use of the nonradioactive method for testing avocado samples (fig. 3).

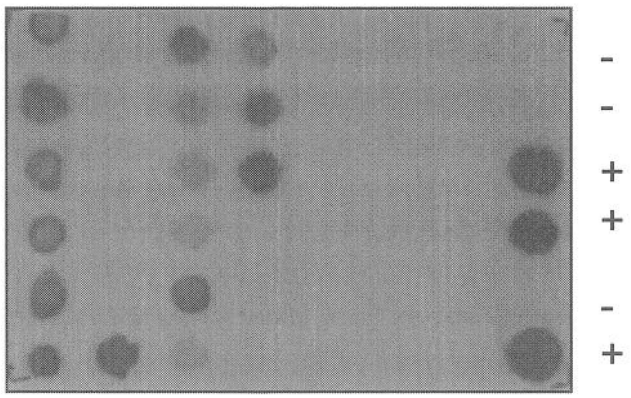

Figure 3. PSTVd Dig- labeled RNA probe validation. PSTVd detection in avocado field samples using Dig-labeled probe.

The Dig-RNA labeled probe is specific—for instance we have no cross-reaction with Avocado sun blotch viroid (ASBVd)—and rarely, if ever, results in false positive indications.

Despite DIG- NASH having some disadvantages when compared with 32P- NASH in terms of background which is mainly caused by the serological component (AP-conjugate) of the method, the advantages of the nonradioactive technique easily outweigh these problems, making this

technique an excellent and cheaper alternative for PSTVd detection and diagnosis. Among these advantages, stability is an important one (Kazunori et al., 2003) facilitating repeated experiments. Results showed that Dig-RNA labeled probe was stable and kept its quality of detection after three months storage at –70°C without thawing and freezing. However we need to extend the storage period in order to establish the shelf live of a nonradioactive probe for kit preparation purposes. A three times replicated experiment was conducted in order to determine how many times a good Dig-RNA labeled probe can be reused. Results indicate that after hybridization, buffers containing the probe can be reused no more than three times and no longer than 1 month, when appropriately stored (fig. 4).

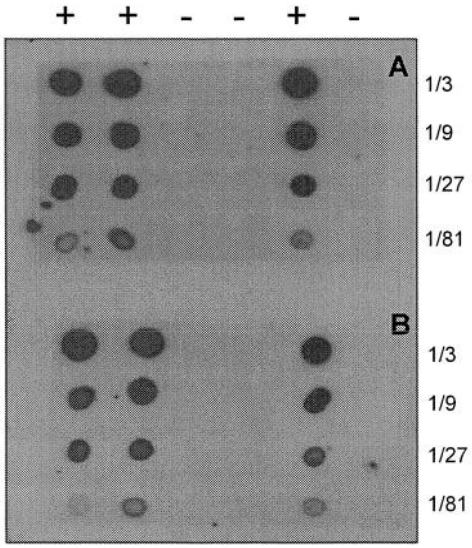

Figure 4. Re-use of Dig-Labeled RNA probes: The probe can be re-used three times and within one month, when appropriately stored. **A**, 2nd time use and **B**, 3st. time use. Samples extracted and diluted in 5X SSC buffer.

Buffers and solutions prepared in the Molecular Virology Laboratory at CIP performed well when compared with those supplied by ROCHE. Samples from greenhouse as well as from field coming from CIP internal and external users were successfully tested indicating that there is no difference at any level when using commercial or "home made" buffers.

Finally, after trying different alternatives for replacing consumables, we got the best results using the common pvc **cover** lamination Vinifan (fig. 5) and there were no significant differences between this material and acetate sheets.

When commercial hybridization bags were compared with sterilization bags available at CIP, there were no differences between using one or the other.

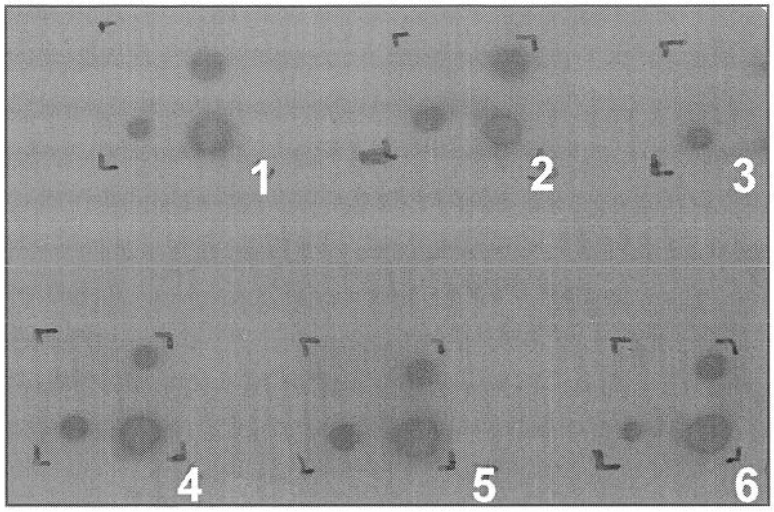

Figure 5. Alternatives for replacing consumables : Plastic bags of different materials tested for signal developing purposes: **1**, Vinifan; **2**, Polypropylene bag; **3**, Hybridization bag; **4**, Pharmacia development folder; **5**, over-head plastic and **6**, plastic bag.

During 2005 to the first semester of 2006, more than 1000 samples from different users in Peru (CIP's and external users) were tested by the PSTVd nonradioactive probe labeled with digoxigenin developed at CIP as part of the validation step but also tested by the routine detection method using the PSTVd ^{32}P-labeled probe. Results are encouraging after comparing both the ^{32}P-RNA labeled probe with the Dig-RNA labeled probe. We were able to detect PSTVd in field samples (data not shown) and the percentage of correspondence between both methods was 99%. Even when a high percentage of samples gave negative results by both techniques, this supports the fact that the possibility of unspecific reactions is rare. There are still some background problems when more than 4 membranes are assayed in a row. This would mean that even increasing the amount of washing buffer to wash the membranes is not enough to avoid static and attachment of membranes during washing steps.

Therefore, further research is also needed to continue to fit large-scale detection of PSTVd and validate the method through the series of surveys to be conducted in CIP regions as China, ESEAP and SWCA.

CONCLUSIONS

Based on the results of the ring test lead by SASA (Scottish Agricultural Research Agency, UK) and conducted with CIP from 2001-2003 the DIG RNA probe (Agdia Inc, USA), was confirmed as suitable for recommendation in the EU PSTVd diagnostic protocol (EU Ring test report, 2003). The results obtained from our own experience of 1 year of evaluating and standardizing this method confirm this statement. An important consideration in recommending methods for quarantine testing is the ability to detect all PSTVd isolates and possibly other viroids that may infect potato. Therefore, the DIG probe and R-PAGE were recommended as primary diagnostic methods by the Ring test group because they—the tests—and in particular R-PAGE, will detect all PSTVd isolates and other viroids that may possibly infect potato.

None of the detection methods will specifically identify PSTVd, since they will detect other viroids. For specific identification the viroid has to be sequenced and analyzed.

The hybridization with DIG-RNA labeled PSTVd probes, as proposed in this study, is a sensitive and broad-spectrum PSTVd detection method specially suited for use in quarantine services.

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Appendix 1

A nonradioactive kit that used a PSTVd-Fluorescein labeled probe was available by 2003 at CIP and was being successfully distributed to China and other regions when a systematic failure in the quality of the marker obliged us to discontinue the production of the kits until a new similar or better alternative was available. Nevertheless and due to the auspicious results obtained with Agdia's DIG-labeled RNA probe to which we had access through our participation in the "performance audit" lead by the Scottish Agricultural Science Agency (SASA) (2002–2003) that comprised two ring tests in order to select the most appropriate methods for multi-laboratory validation of some methods used within the European Union (EU) and elsewhere, by mid-2004 we initiated the standardization of this method but using our own PSTVd recombinant plasmid and were thinking of using this method at CIP as a routine method for PSTVd detection but also as an attempt to switch—in the near future—from radioactive to nonradioactive nucleic acid hybridization method that would considerably reduce the cost of NASH service and the intrinsic risk of using radioactive material.

Thirteen laboratories were involved in the ring tests of the EU methods and CIP was one of them. In addition, each laboratory used its own PSTVd detection method(s). CIP participated from the ring tests (2002–2003) for the DIG-NASH and RT-PCR methods, in order to compare the results obtained using these techniques with our routine PSTVd 32P- RNA labeled probe. Ring Test coordinators (SASA team) provided the ring tests participants with positive and negative controls, as well as with reagents and materials needed to perform the DIG-NASH and RT-PCR methods.