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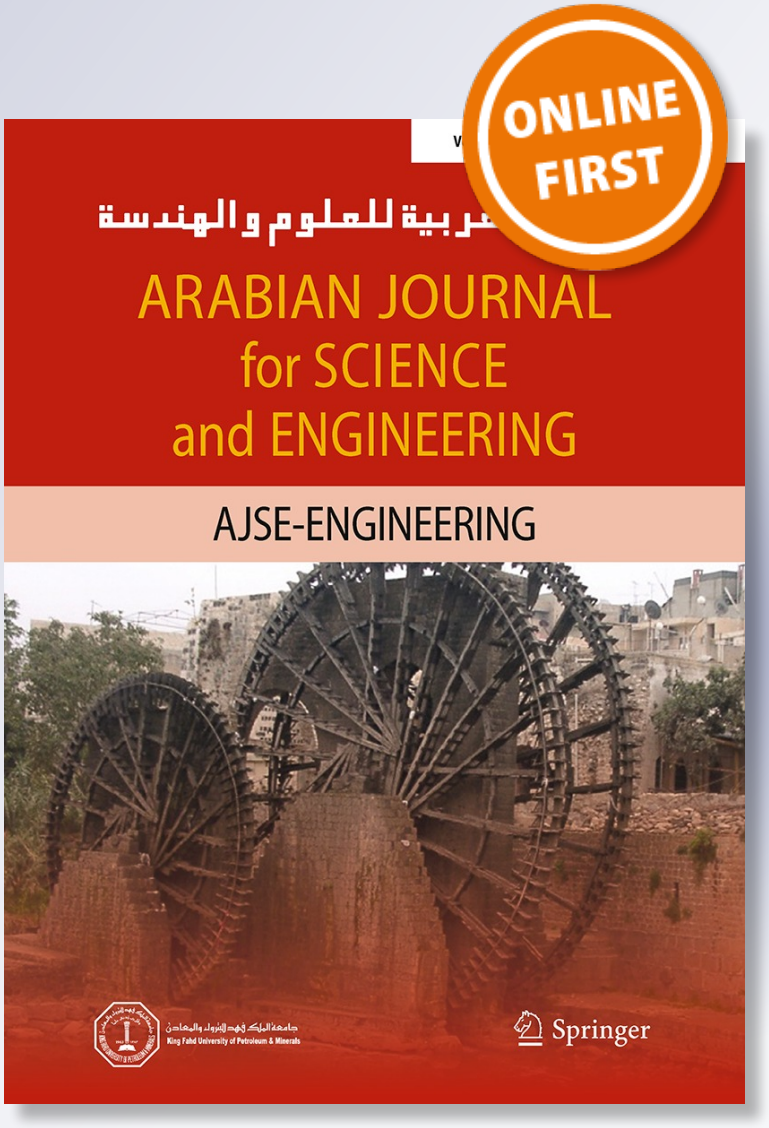
**Arabian Journal for Science and Engineering**

ISSN 1319-8025

Arab J Sci Eng
DOI 10.1007/s13369-014-1490-z




Springer

Arab J Sci Eng
DOI 10.1007/s13369-014-1490-z

RESEARCH ARTICLE - BIOLOGICAL SCIENCES

# Detection of *Citrus psorosis virus* Using an Improved One-Step RT-PCR

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Received: 4 March 2014 / Accepted: 30 May 2014


**Abstract** We report an improvement of a one-step reverse transcription polymerase chain reaction (RT-PCR) assay for the detection of *Citrus psorosis virus* (CPsV; genus *Ophiovirus*) in citrus trees. Two different sample preparation procedures were compared. The data showed that when using total RNA extracted by the Qiagen procedure more virus isolates could be detected compared with the Trizol procedure. Three pairs of primers reported in literature and selected within the coat protein (CP) region of the virus were used for a reliable detection of CPsV. Only one primer pair has been able to detect all CPsV isolates collected from different citrus regions of Morocco. In addition, this report showed that one-step RT-PCR was more sensitive and more consistent than ELISA test. Notably, 15 out of 22 samples tested positive by one-step RT-PCR could not be detected by Mab Ps29, thus providing a degree of differentiation among isolates. The detection protocol described in this study could be used in citrus certification programs and to test trees in nurseries and commercial orchards for CPsV infection.

**Keywords** Psorosis · CPsV detection · Biological indexing · ELISA · One-step RT-PCR

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## 1 Introduction

Citrus is one of the most important fruit crops in Mediterranean countries and also in several areas of the world. Plant viruses that are transmitted by grafting and insect vectors can cause economic damages to citrus industry. Most of the citrus cultivars are grown as grafted plants, which constitute a high source of viruses.

Psorosis is a widespread graft-transmissible viral disease in old citrus plantings, especially sweet orange. Disease development is slow; it may take several years to manifest symptoms, which include bark scaling of the trunk, chlorotic flecks and spots on young leaves. Gum may accumulate below the bark scales and may impregnate the xylem producing wood staining and vessel occlusion. These symptoms have been used for field diagnosis of psorosis [1,2].

*Citrus psorosis virus* (CPsV), the type species of the genus *Ophiovirus* [3], is the presumed causal agent of psorosis disease [4]. The virus is tripartite and its genome consists of three single-stranded RNAs of negative polarity. RNA1 carries two open reading frames (ORFs): a 24-kDa polypeptide of unknown function and separated by an intergenic region, the putative RNA-dependent RNA polymerase of 280 kDa [5]. RNA2 carries one gene coding for a polypeptide of 53.6 kDa of unknown function, while RNA3 codes for the coat protein (CP) of 48.6 kDa [6–8].

Detection, identification and differentiation of viruses and viroids infecting various crops constitute the basic steps for the development of effective crop disease management programs.

Several methods are available for the detection of psorosis [2,4]. Biological indexing is undertaken by graft-inoculating citrus indicator plants and then testing for cross-protection with a severe isolate [1,2,4]. The procedure is slow and costly, requires adequate facilities and trained personnel, and

Published online: 16 November 2014

cannot be used for large-scale indexing [4]. DAS-ELISA [9] and TAS-ELISA [10] are methods developed and applied for psorosis diagnosis in the field. The antisera produced against recombinant viral CP form an additional or alternative tool for manufacturing standardized kits for serological detection of CPsV [11]. However, it is sometimes difficult to use these techniques to detect certain citrus viruses due to their low titter or uneven distribution in infected trees. The antigenic properties of the pathogens to be detected, and variations in the sensitivity/accuracy of the tests also limit the use of these serological tests [1,12]. Considerable efforts have been done during the last 10 years to develop and apply other more reliable, less expensive and more sensitive molecular methods based on RT-PCR and dot blot or tissue-print hybridization [4,6,12–16], and more recently singleplex and multiplex one-step real-time RT-PCR settled [17].

After CPsV sequences became available, reliable systems were designed to detect CPsV by conventional RT-PCR. This research aims firstly to establish and compare different protocols for nucleic acid extraction from citrus tissues, to determine their effectiveness in relation to their sensitivity and costs, to find specific primers for Moroccan psorosis isolates between selected primers pairs, and finally, to compare the results with those of DAS-ELISA and biological assay.

## 2 Materials and Methods

### 2.1 Plants Materials and Reagents

Samples from 42 field trees of Maroc late, Salustiana and Washington navel sweet orange (*Citrus sinensis* (L.) Osb.), and Nules clementine (*Citrus clementina* Hort. ex Tan) from symptomless plants as well as from plants showing typical psorosis bark scaling symptoms in the trunk or limbs, were collected in three different citrus-growing regions of Morocco, located in the Gharb region (Northwest Morocco), Moulouya (Northeast) and Tadla region (Central Morocco). To reduce the effects of potential uneven virus distribution, four young shoots were collected from the four sides of each tree and leaves from these shoots were used for DAS-ELISA and to prepare RNA extracts for one-step RT-PCR analysis. Similar extracts from greenhouse-grown Madame Vinous seedlings, healthy and CPsV infected, were used as negative and positive controls, respectively.

Biological indexing was performed by graft-inoculating budsticks from each source tree onto Madame Vinous seedlings, using two plants per field source. These plants were grown in a temperature-controlled (18−26 °C) greenhouse and symptoms on the new flush (flecking, spots and shock) were recorded in five successive flushes post-inoculation. CPsV infection in these plants was tested by DAS-ELISA and one-step RT-PCR.

### 2.2 DAS-ELISA

DAS-ELISA was performed according to the protocol provided by the manufacturer (AGRITEST SRL, Valenzano, Italy), which based on the use of monoclonal antibody alkaline phosphatase conjugate (hybridoma line Ps 29, DPPMA University of Bari, Italy) [18,19].

Each sample was tested in two replicates, and two wells each of positive and negative controls were included in each plate. Readings were done after incubations of 2 and 4 h with the substrate *p*nitrophenyl phosphate at room temperature. Mean experimental readings at least three times the mean reading of the negative controls were considered positive. Optical density (OD) at 405 nm was measured using Lab-Systems Multiskan RC ELISA reader.

### 2.3 Sample Preparation

Two extraction protocols were followed to extract total RNA from 100 mg of field-collected samples for the use as template in one-step RT-PCR. In the first protocol, TRI Reagent (Sigma Aldrich, France) was used according to the manufacturer's instructions, while in the second protocol the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) was used.

### 2.4 One-Step RT-PCR

One-step RT-PCR was performed using the primer pairs CPV1/CPV2 [6], Ps66/Ps65 [4], and CPsV-forward/ CPsV-reverse [12] that amplify a fragment of CPsV coat protein gene. The expected sizes of the amplified product were 600, 430 and 390 bp, respectively.

One-step RT-PCR was carried out using the $QIAGEN^{®}$ One Step RT-PCR Kit (QIAGEN, Hilden, Germany) that allows reverse transcription and amplification to be performed sequentially in the same tube as described by Martin et al. [4] with minor modifications. In summary, the RT-PCR was conducted in a 25-µl mixture consisting of 1 µl of total RNA, 5 µl of 5× RT-PCR buffer, 1 µl of 10 mM of each dNTP, 5 µl of 5× Q-solution, 0.6 µM of each primer, 1 µl of enzyme mix (Omniscript and Sensiscript Reverse Transcriptases and HotStarTaq DNA Polymerase), and RNase-free water. The thermocycling conditions included 30 min at 50 °C for RT, 15 min at 95 °C as heating step of HotStarTaq DNA polymerase, 40 cycles consisting of 30 s at 94 °C, 1 min at 58 °C and 1min at 72 °C, and a final elongation step of 10 min at 72 °C. The PCR products were analyzed by electrophoresis on 2 % agarose gel using the 100 bp Plus DNA Ladder (Fermentas) for size estimation. Each RT-PCR analysis included negative, positive and no-template controls.

**Table 1** Diagnosis of psorosis in the Northeast and the Northwest citrus regions

| Sample | Origin | Symptoms in the field | Symptoms in indicators plants | DAS-ELISA results | One-step RT-PCR results (TRI Reagent procedure) | One-step RT-PCR results (Qiagen procedure) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Ps66/Ps65 | CPV1/CPV2 | CPsV-F/CPsV-R |
| 1 | Field (Northeast) | BS | LS | + | + | + | − | − |
| 2 | Field (Northeast) | BS | S, LS | + | + | + | + | + |
| 3 | Field (Northeast) | BS | S, LS, NE | + | + | + | + | + |
| 4 | Field (Northeast) | No symptoms | LS | − | + | + | − | − |
| 5 | Field (Northeast) | BS | S, LS | + | + | + | − | − |
| 6 | Field (Northeast) | BS | LS | − | + | + | − | − |
| 7 | Field (Northeast) | BS | S, LS, NE | − | + | + | + | + |
| 8 | Field (Northeast) | BS | S, LS | − | + | + | + | + |
| 9 | Field (Northeast) | BS | S, LS | − | + | + | + | + |
| 10 | Field (Northeast) | No symptoms | LS | − | − | − | − | − |
| 11 | Field (Northeast) | No symptoms | LS | − | − | − | − | − |
| 12 | Field (Northeast) | BS | S, LS | + | − | + | + | + |
| 13 | Field (Northeast) | No symptoms | LS | − | − | − | − | − |
| 14 | Field (Northeast) | BS | S, LS | − | − | + | + | + |
| 15 | Field (Northeast) | BS | S, LS, NE | + | + | + | − | + |
| 16 | Field (Northeast) | No symptoms | LS | − | − | + | + | + |
| 17 | Field (Northeast) | No symptoms | S, LS | − | − | − | − | − |
| 18 | Field (Northeast) | No symptoms | S, LS, NE | − | − | + | + | + |
| 19 | Field (Northeast) | No symptoms | LS | − | − | − | − | − |
| 20 | Field (Northeast) | No symptoms | S, LS | − | − | + | + | + |
| 21 | Field (Northeast) | No symptoms | S, LS | − | − | + | + | + |
| 22 | Field (Northeast) | No symptoms | S, LS | − | − | + | − | − |
| 23 | Field (Northwest) | No symptoms | LS | − | − | − | NT | NT |
| 24 | Field (Northwest) | BS | S, LS | + | − | + | NT | NT |
| 25 | Field (Northwest) | No symptoms | LS | − | − | − | NT | NT |
| 26 | Field (Northwest) | BS | S, LS | − | + | + | NT | NT |
| 27 | Field (Northwest) | BS | S, LS | − | − | + | NT | NT |
| 28 | Field (Northwest) | BS | S, LS, NE | − | − | + | NT | NT |
| 29 | Field (Northwest) | No symptoms | LS | − | − | + | NT | NT |
| 30 | Field (Northwest) | No symptoms | LS | − | − | − | NT | NT |
| 31 | Greenhouse (sample 4) | No symptoms | LS | − | + | + | − | − |
| 32 | Greenhouse (sample 9) | BS | S, LS | − | + | + | + | + |
| 33 | Greenhouse (sample 10) | No symptoms | LS | − | − | − | − | − |

**Table 1** continued

| Sample | Origin | Symptoms in the field | Symptoms in indicators plants | DAS-ELISA results | One-step RT-PCR results (TRI Reagent procedure) | One-step RT-PCR results (Qiagen procedure) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Ps66/Ps65 | CPV1/CPV2 | CPsV-F/CPsV-R |
| 34 | Greenhouse (sample 11) | No symptoms | LS | − | − | − | − | − |
| 35 | Greenhouse (sample 12) | BS | S, LS | + | − | + | + | + |
| 36 | Greenhouse (sample 13) | No symptoms | LS | − | − | − | − | − |
| 37 | Greenhouse (sample 16) | No symptoms | LS | − | − | + | + | + |
| 38 | Greenhouse (sample 17) | No symptoms | S, LS | − | − | − | − | − |
| 39 | Greenhouse (sample 18) | No symptoms | S, LS, NE | − | − | + | + | + |
| 40 | Greenhouse (sample 19) | No symptoms | LS | − | − | − | − | − |
| 41 | Greenhouse (sample 20) | No symptoms | S, LS | − | − | + | + | + |
| 42 | Greenhouse (sample 21) | No symptoms | S, LS | − | − | + | + | + |
| 43 | Greenhouse (sample 22) | No symptoms | S, LS | − | − | + | − | − |
| 44 | Greenhouse (sample 23) | No symptoms | LS | − | − | − | NT | NT |
| 45 | Greenhouse (sample 25) | No symptoms | LS | − | − | − | NT | NT |
| 46 | Greenhouse (sample 29) | No symptoms | LS | − | − | + | NT | NT |
| 47 | Greenhouse (sample 30) | No symptoms | LS | − | − | − | NT | NT |

Comparison of the indexing, DAS-ELISA, and one-step RT-PCR for the detection of CPsV in filed citrus. Samples 2–4, 9 were taken from Maroc late sweet orange trees, samples 10, 12–13 were taken from Salustiana sweet orange trees, and sample 15 was taken from Washington navel sweet orange trees, all from the Northwest region. Samples 1–18 were taken from Washington navel sweet orange trees, samples 19–22 from Nules clementine trees, all from the Northeast region. Samples 31–47 were taken from the graft-inoculated seedlings in the greenhouse

BS bark scaling, LS chlorotic flecking or spotting in young leaves, S shock reaction (leaf shedding and necrosis of young shoots), NE necrotic etching, NT not tested

+, positive reaction; −, negative reaction

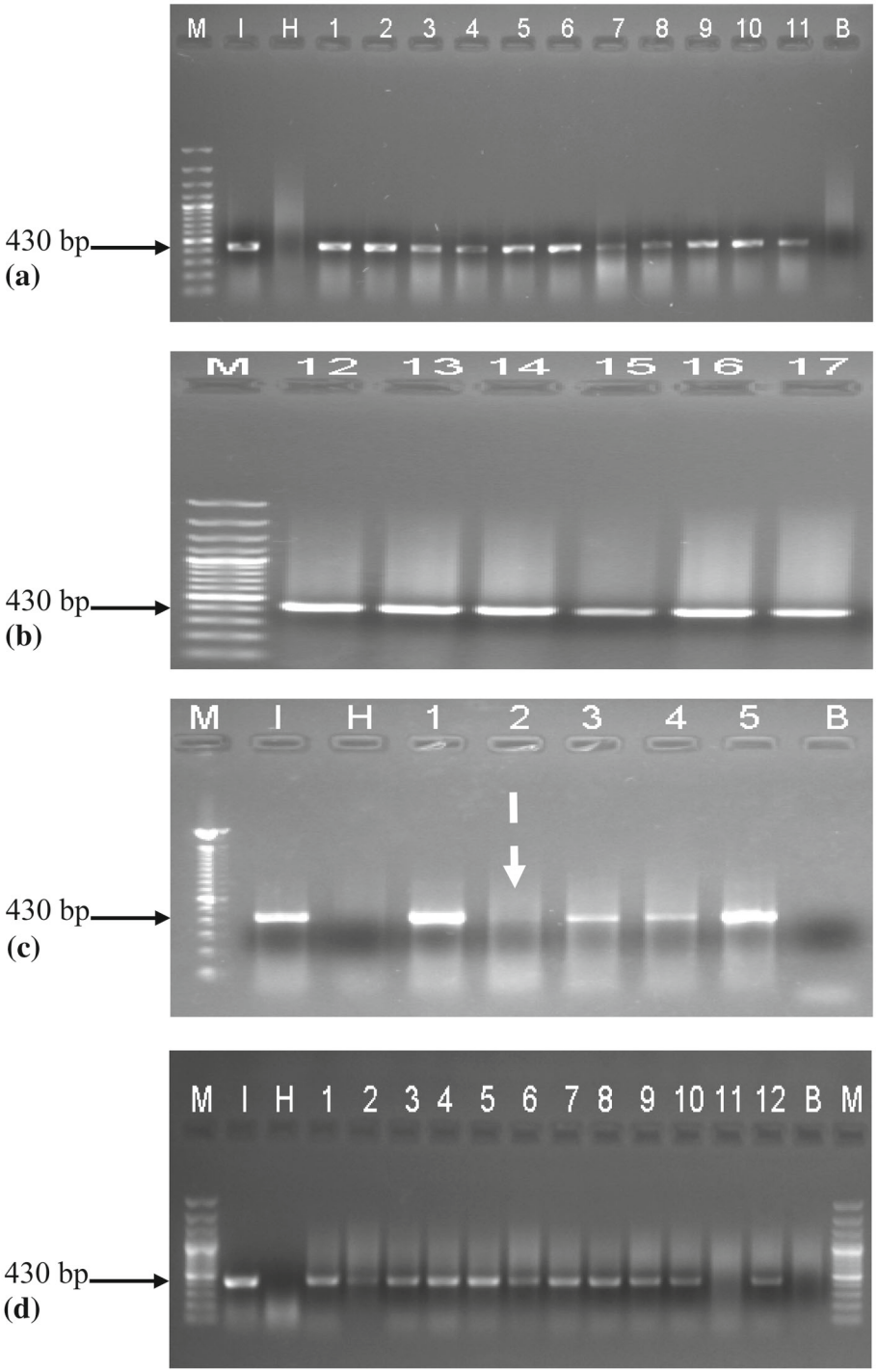


**Fig. 1** Detection of CPsV in citrus by one-step RT-PCR using Qiagen kit for RNA extraction and the primer pair Ps66/Ps65. Gels (**a**, **b**) show the one-step RT-PCR products obtained from 17 field samples from the Northeast region (*lanes 1–17*), gel (**c**), the one-step RT-PCR products from five field samples from the Northwest (*lanes 1–5*), sample two shows a faint band (e.g., *lane 2*) and is indicated by the *dashed arrow*, and gel (**d**) from 12 field samples from the Central region (*lanes 1–12*). *Lanes I* and *H* corresponds to an infected and a healthy control plant. *Lane B* in the gels corresponds to a control reaction with all the reagents but no template

## 3 Results and Discussion

The procedure of extraction may affect the overall results obtained using RT-PCR. In preliminary tests, we compared one-step RT-PCR results on field samples using total RNA extracted with TRI Reagent and Qiagen Kit procedure. The first was less cost-effective but required a second purification with phenol/chloroform/isoamyl alcohol (25:24:1) to improve the quality of template used in RT-PCR reactions and minimize the effects of PCR enzyme inhibitors present in the plant extract. The protocol using Qiagen kit yielded high-quality RNA allowing consistent results by RT-PCR. Preliminary data showed that 11 out of the 30 samples tested as negative using TRI Reagent tested positive when Qiagen kit was used for RNA extraction (Table 1). In the contrary to the RNA extracted using the TRI Reagent, RNA extracts

obtained using Qiagen kit had always the best quality and remained stable over long periods at −20 °C. Overall, detection of CPsV using Qiagen procedure was more reliable, rapid and reproducible; therefore, the advantage of increased sensitivity using the RT-PCR must be considered in spite of the high cost of sample extraction.

The expected size of CPsV coat protein fragment (430 bp) could be amplified from all CPsV-positive samples using the primer pair Ps66/Ps65 (Fig. 1a–d). Various parameters of one-step RT-PCR reaction were modified such as the concentration of forward and reverse primers, temperature and time for the RT reaction, time for the RT inactivation step, cycle times, annealing temperature and use of HotStarTaq DNA polymerase were directly compared with the parameters employed by Martin et al. [4] utilizing the same primers. 0.6 μM of each primer, a 50 °C of RT step for 30 min, 15 min for the RT inactivation step, 40 cycles consisting of 30 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C, and a final elongation step of 10 min at 72 °C were used to get the best amplification by comparison to the protocol previously described by Martin et al. [4]. According to the literature, the HotStarTaq DNA polymerase prevents the formation of misprimed products and primers dimers during the reaction and increased the possibility of amplifying virus target, which had very low copy numbers.

Genetic variation within targeted population of a virus can potentially affect the specificity and reliability of the RT-PCR test, leading to false negative results. Three sets of primers (CPV1/CPV2, Ps66/Ps65 and CPsV-F/CPsV-R) were selected within the coat protein (CP) region of the virus to ensure the detection of the majority, if not all, CPsV isolates.

The primer pair Ps66/Ps65 targeted the most conserved region of the CP gene could detect the virus in 22 out of the 30 samples tested (Fig. 1a–c) (Table 1), whereas the primer pairs CPV1/CPV2 and CPsV-F/CPsV-R could detect CPsV only in 11 and 12 samples out of 22 samples collected from Northeast region, respectively. Therefore, the primer pair Ps66/Ps65 were selected for further tests.

We report an improvement of the one-step RT-PCR assay, previously described by Martin et al. [4]. In addition, we compared different diagnostic tools to detect CPsV testing both field and greenhouse–grown plants. As shown in Table 1, one-step RT-PCR was more sensitive than ELISA test in detecting CPsV in field-collected samples. Thus, this may indicate that different CPsV isolates are present in Morocco.

The presence of the virus was also investigated in the indicator plants (31–47) of the biological indexing of isolates (4, 9–13, 16–23, 25, 29–30) (Table 1). The results obtained from these plants were the same as those obtained from original field trees.

In order to compare selected primers in the improved one-step RT-PCR protocol on a wide range of CPsV isolates, 12 field samples from Central region of Morocco were also analyzed. All tested samples were positive by one-step RT-PCR except one symptomless sample (Fig. 1d).

In light of the data obtained in this study, it can be concluded that one-step RT-PCR is more sensitive than commercially available ELISA tests for CPsV detection, and more rapid than the biological assay. This one-step RT-PCR assay may prove useful as a sensitive tool to test a large number of samples in programs of sanitary status assessment.

**Acknowledgments** This work was supported by the Belgium development agency, BTC (project FRAB II).

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