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# Concentration and detection of tobacco etch virus from irrigation water using real-time PCR

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**Abstract:** Irrigation water can be polluted by the tobacco etch virus (TEV), which causes serious economic loss in tobacco. However, it is difficult to monitor the sanitary status of irrigation water because TEV presents at extremely low concentrations. This study designed a procedure for concentrating TEV from a standard water sample using polyethylene glycol and detecting it using SYBR Green-based quantitative real-time polymerase chain reaction (qRT-PCR). The concentration factors were optimized through orthogonal tests and the highest recovery efficiency of the TEV genome from a standard water sample was 92.05%. The sensitivity was evaluated using nine 10-fold dilution series of TEV plasmids, and the detection limit of the qRT-PCR was about 10 viral copies/µL. In the infectivity test, TEV was first detected in the roots of NC89 at 7 days after treatment and in the upper leaves at 14 days after treatment. Field diagnosis results showed that 56 of the 180 samples tested positive for TEV, which indicated that this method may be suitable for concentrating and detecting TEV in irrigation water.

**Key words**: Tobacco etch virus, irrigation water, polyethylene glycol, quantitative real-time PC

## 1. Introduction

Water plays a very important role in the activities of plants and animals (Novo, 2012). However, more than hundreds of thousands of microorganisms exist in water, especially in drinking water and irrigation water, which causes serious problems for animals and plants (Toze, 2006; Boben et al., 2007; Wu and Liu, 2009; Radin and D'Souza, 2011). Therefore, it is necessary to monitor the sanitary conditions of water. Water-borne animal viruses (WBAVs) have attracted much attention from researchers, and many studies have been performed to prevent WBAVs from causing serious problems for animals (Sevik, 2011). However, water-borne plant viruses (WBPVs) have not drawn that much attention (Boben et al., 2007). At present, about 60 kinds of plant viruses, mainly belonging to the *Necro-*, *Potex-*, *Poty-*, *Tobamo-,* and *Tombusvirus* groups, have been found and isolated from water (Plese et al., 1996; Zheng et al., 2000; Rosner et al., 2006; Polischuk et al., 2007). WBPVs have become one of the most important factors in the spread of plant virus diseases (Zheng et al., 2000). Therefore, it is essential to develop effective strategies for controlling WBPVs.

Tobacco etch virus (TEV), with a positive-sense RNA surrounded by a coat protein, is a *Potyvirus* (Riechmann et al., 1992; Carrasco et al., 2007). This virus infects at least 120 species in 19 dicotyledonous families, among which tomato, tobacco, and other plants in the family Solanaceae are the most susceptible (Riechmann et al., 1992). More than 10 species of aphids can transmit TEV in a nonpersistent way, and mechanical inoculation is also an important transmissible factor (Zhang, 1993; Huang et al., 2012). TEV has been found in water and may be spread through water (Pocsai and Horvath, 1997; Zheng et al., 2000). Currently, TEV has become one of the most serious worldwide diseases, and some measures are needed to prevent TEV from spreading. However, it is difficult to detect TEV in water due to its low concentration. Consequently, a more sensitive method is needed to detect its route or behavior in water.

Recently, quantitative polymerase chain reaction (qPCR) methods have gained greater acceptance (Boben et al., 2007). Based on an internal control, qPCR can be divided into fluorescently labeled probes and fluorochromes (Lian et al., 2009; Luigi and Faggioli, 2011; Fedick et al., 2012; Gao et al., 2012). SYBR Green is one of most popular fluorochromes and SYBR Green-based qPCR assays have been applied to the diagnosis of many viral diseases (Riechmann et al., 1992; Varga and James, 2005; Radin and D'Souza, 2011; Balamurugan et al., 2012). Santhosh et al. (2007) reported a one-step SYBR Green I-based RT-PCR assay for the detection and quantification of the chikungunya virus. A SYBR Green I-based RT-PCR

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method was also used for the quantitative detection of the rice tungro bacilliform virus, the rice tungro spherical virus, the H5 subtype AI virus, porcine circovirus 2, the porcine parvovirus, and the pseudorabies virus, as well as torque teno sus virus species 1 and 2 (Perez et al., 2011; Perez et al., 2012; Sharma and Dasgupta, 2012). However, none of the described detection procedures can be applied to the detection of TEV in water.

Polyethylene glycol (PEG) is an attractive method to recover viruses from environmental water samples. PEG is a nontoxic water-soluble synthetic polymer and is widely used in chemical and biomedical industries (Atha and Ingham, 1981). It was first used to concentrate viruses in the 1960s and has been proven to be rapid, inexpensive, and nondestructive (Colombet et al., 2007). From 2000 to 2012, PEG had been demonstrated to be suitable for concentrating the hepatitis A virus, norovirus GII, and influenza A viruses from water (Huang et al., 2000; Guevremont et al., 2006; Deboosere et al., 2011). Since PEG has been used as precipitation for the concentration and purification of WBAVs (Jaykus et al., 1996; Schwab et al., 1996), it is worth investigating whether PEG precipitation is suitable for concentrating TEV from water samples.

The purpose of this study was to develop an efficient method for the detection of TEV in environmental water samples. Therefore, PEG6000 was used as a precipitate to concentrate TEV, and the SYBR Green-based qRT-PCR was used to detect TEV in the concentrates. The optimized method was used to detect TEV in irrigation water samples and the results confirmed that this new method was rapid, efficient, and highly sensitive for evaluating and monitoring irrigation water.

## 2. Materials and methods

### 2.1. Water samples

From July to August in 2011, 180 water samples were collected from Shaanxi Province, among which 41 were from Long County, 47 from Xunyang County, 49 from Fu County, and 43 from Nanzheng County. All the water samples were filtered by double gauze, stored in plastic bottles, put in an ice box, and delivered to the laboratory within 24 h.

### 2.2. Virus

The TEV was maintained in *Nicotiana tabacum* under greenhouse conditions at 25 °C with a 16-h light period. The purified TEV was obtained from the infected tobacco leaves according to the method described by Dougherty and Hiebert (1980). The purified TEV solution was used as a mother liquid for future work.

### 2.3. Design and selection of primers

Seven complete sequences of TEV isolates were obtained from NCBI and analyzed by DNAMAN software (Dai et al., 2012). Based on the 7 complete sequences of TEV isolates in the NCBI database, the conserved domains were used to design a set of primers that could detect all TEV strains. Four special primer pairs were designed according to the genome sequence of TEV using the program Primer Premier 5.0 (Dai et al., 2012). The primer sequences, the expected size of amplification products, and the target genes are listed in Table 1.

### 2.4. Reverse transcription and PCR

According to the manufacturer's instructions, RNA was extracted from the mother liquid using the Universal Plant Total RNA Extraction Kit (BioTeke, China) and cDNA was synthesized using the Prime Script RT Reagent Kit (TaKaRa, Japan). PCR was carried out in a 25-µL PCR mixture including 2 µL of cDNA template, 2.5 µL of 25 mmol/L $Mg^{2+}$ (Promega, USA), 2.5 µL of dNTP mixture with each dNTP at 5 mmol/L, 2.5 µL of 10X polymerase buffer (Promega), 0.5 µL of 5 U/µL Hot-start Taq polymerase (Promega), and 2 µL of sense and antisense primers (10 µmol/L each). The reaction process was as follows: first denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 1 min; and the final extension at 72 °C for 10 min. The size of the PCR products was examined by 2.5% Ago-Gel under UV light.

**Table 1.** Primers used for detection TEV.

| Primer | Sequence 5'-3' | Positions | Amplicon size (bp) |
|---|---|---|---|
| F1 | CTAGGTTATTTGGTCTTGATG | 9196–9216 | 104 |
| R1 | GACCCCTAATAGTGTGTG | 9282–9299 | |
| F2 | TGTCCTGGCAATAGTCTCC | 2526–2544 | 233 |
| R2 | TAACCCTAACACCGTCAATC | 2754–2773 | |
| F3 | TGATGGATGGTGAGGAG | 8893–8909 | 347 |
| R3 | GTGCCGTTCAGTGTCTT | 9239–9255 | |
| F4 | CAATGGCGATGACTTACTG | 7996–8016 | 124 |
| R4 | TGTCTTGTCCCTGGTGGT | 8122–8139 | |

### 2.5. Standard curves for real-time PCR

The PCR products of TEV were purified from Ago-Gel using a gel extraction kit (BioTeke), cloned into a pMD18-T simple vector (TaKaRa), and transformed into *Escherichia coli* JM109. The plasmids with the target gene were extracted from the positive clones and the purity was analyzed by measuring $OD_{260}$ with a spectrophotometer ND Drop-1000 (Thermo Fisher NanoDrop, USA). The copy of the plasmids was calculated using the formula:

$[X (g/\mu L) \times 6 \times 10^{23}] / [\text{plasmid length (bp)} \times 660] = Y$ viral copies/μL.

The starting amount of the plasmids was diluted to 0.31 ng/μL, equal to $1 \times 10^8$ viral copies/μL of the virus, and was then diluted from $1 \times 10^8$ to $1 \times 10^0$ viral copies/μL with a series of 10-fold dilutions for the standard curve of qRT-PCR. These plasmids were used to optimize the primer concentration, annealing, and extension temperature conditions. The standard curve and standard equation were established with the threshold cycle and the original copy number of every plasmid template from 6 standards.

### 2.6. Viral concentration

The standard water sample was obtained from 1 μL of mother liquid dissolved into 10 mL of distilled water. PEG6000 (Kerme, China) was added with a final concentration of 25% (w/v), vigorously mixed, incubated at room temperature for 40 min, and centrifuged at 6000 × *g* for 30 min at 4 °C. The precipitation was resuspended by 30 μL of 0.01 mol/L phosphate buffer (PBS; pH 7.4) and transferred into a 1.5-mL RNase- and DNase-free centrifuge tube.

Virus RNA was extracted from the 30 μL of 0.01 mol/L PBS (pH 7.4) solution and reverse transcribed in a 25-μL total reaction volume following the method of Section 2.4. The cDNA was used for qRT-PCR with the primer pair TEV F4/R4 (Table 1) for TEV detection and quantification. The mixture consisted of 1X SYBR Green PCR master mix, template cDNA, and 200 nmol/L of specific primers. Pyrocarbonic acid diethyl ester-treated water was added to achieve a final volume of 25 μL, and the reaction was carried out in the IQ5 qPCR Detection System (Bio-Rad, USA). The optimized procedure was as follows: 1 cycle at 95 °C for 10 min, and 45 cycles of 15 s at 95 °C and 30 s at 53 °C (annealing and extension).

For each sample, 3 technical replicates were performed in parallel. A no template control (NTC) was used as a negative control for determining the background fluorescence.

### 2.7. Infectivity tests

*Nicotiana tabacum* L.NC89 (NC89), which was susceptible to TEV, was cultivated in a disease-free greenhouse and used as material to test the infectivity of TEV after concentration. In this test, 15 plants with 4 to 6 leaves were chosen and hydroponically cultivated. The composition of the liquid medium was $KNO_3$, $Ca(NO_3)_2$•4H2O, $MgSO_4$•$7H_2O$, $KH_2PO_4$, $Na_2EDTA$, $FeSO_4$•$7H_2O$, $H_3BO_3$, $MnCl_2$, $ZnSO_4$•$7H_2O$, $CuSO_4$•$5H_2O$, and $CoCl_2$•$6H_2O$, and the hydroponics were carried out according to the method of Yu et al. (2004). The original viral concentrate was diluted to $10^6$ viral copies/mL by the liquid medium. Five NC89 plants were planted in the TEV-mixed liquid medium, 5 plants were used as negative controls, and the 5 remaining TEV-infected plants were used as positive controls. The leaves and roots of all the plants were collected at 0, 7, 14, and 21 days after treatment (DAT). These samples were tested by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). DAS-ELISA was carried out using a commercially available kit (Neogen Corporation, USA) according to the manufacturer's instructions. Optical density at 405 nm ($OD_{450}$) was measured with a microplate reader (Thermo, USA) to estimate the virus load of the tested samples.

### 2.8. Clinical detection of TEV in field samples

In order to apply the concentration method to the analysis of environmental samples where TEV could not be directly detected, 180 water samples were collected from different tobacco production fields in Shaanxi Province, China. The concentration step and the qRT-PCR reactions were performed as previously described.

## 3. Results

### 3.1. TEV-specific quantitative RT-PCR

Through a series of orthogonal design tests, primer and annealing temperatures were optimized to achieve the best amplification. The results of the Ago-Gel and melting curve indicated that nonspecific amplifications were found in the reactions with F1/R1 and F3/R3, and that amplification efficiency was very low with F2/R2. With F4/R4, the amplification efficiency was increased, and no nonspecific amplifications were found in the reactions. The annealing temperature of 53 °C was optimum for primers F4/R4. The standard curves of qPCR were established from $1 \times 10^2$ to $1 \times 10^7$ viral copies/μL. The linear correlations ($R^2$) between the threshold cycle and the viral copy logarithm were 0.998 with a slope of −3.833, indicating a reproducible linear response in detection of the TEV (Figure 1). To evaluate the sensitivity of the qPCR assay, 10-fold serial dilutions of the standard plasmid from $1 \times 10^8$ to $1 \times 10^0$ viral copies/μL of each virus were tested. The detection limit of qPCR was determined to be 10 viral copies/μL. The threshold cycles for the standard plasmid of 10 viral copies/μL of TEV were 32.06 in the TEV samples (Figure 2).

### 3.2. Optimized method for concentrating TEV

In this study, the concentration factors (final concentration of PEG6000 and centrifugal force) were optimized. PEG6000 with final concentrations of 5%, 15%, 25%, and 35% and centrifugal forces of 3000 × *g*, 4000 × *g*, 5000 × *g*,

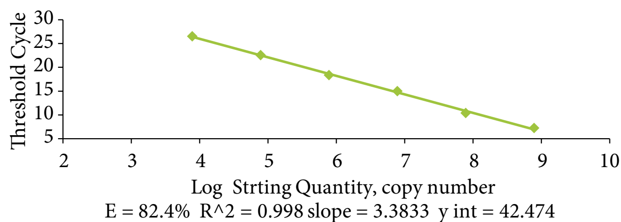


**Figure 1.** The standard curve of the qRT-PCR based on a 10-fold serial dilution of standard plasmids. The standard curves of qRT-PCR were established from $1 \times 10^2$ to $1 \times 10^7$ viral copies/µL. The linear correlations ($R^2$) between the threshold cycle and the viral copy logarithm were 0.998 with a slope of –3.833, indicating a reproducible linear response in detection of the TEV.

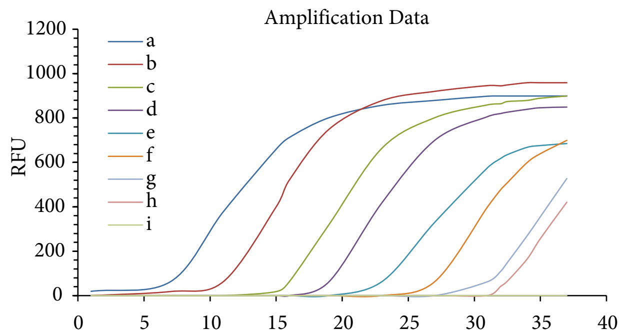


**Figure 2.** Sensitivity of the qRT-PCR for detection of TEV. Sensitivity was evaluated using nine 10-fold dilution series of TEV special standard plasmids, and the detection limit of the qRT-PCR was about 10 viral copies/µL.

6000 × *g*, and 7000 × *g* were tested by orthogonal test. The viral concentration from different treatments was assayed in triplicates in independent qRT-PCR runs. The recovery efficiency was used to assess the different treatments according to the equation E (%) = (viral copy of recovered TEV / viral copy of TEV in mother liquid) × 100. The results indicated that final concentrations of PEG6000 and centrifugal force had an effect on the recovery efficiency. Specifically, with the increase of the final concentration of PEG6000 from 5% to 25%, recovery efficiency increased; however, from 25% to 35%, the recovery efficiency decreased significantly. When the final concentration of PEG6000 was 25%, the recovery efficiency reached a maximum. Meanwhile, recovery efficiency increased significantly when centrifugal force increased from 3000 × *g* to 6000 × *g*; however, it decreased significantly when the centrifugal force was increased to 7000 × *g*. Recovery efficiency was the highest when the centrifugal force was 6000 × *g*. By comparing the recovery efficiency of different treatments, a treatment (25% PEG6000 final concentration; 6000 × *g* centrifugal force) with the best genome recovery of 92.05% was considered to be optimum (Table 2).

### 3.3. The infectivity of viral particles

The infectivity of viral particles after the concentrating procedure was tested. In the experiment in which NC89 was treated with TEV-mixed liquid medium, TEV was detected in roots at 7 DAT with an $OD_{450}$ value of 0.33 and in leaves at 14 DAT with an $OD_{450}$ value of 0.48 (Table 3). Furthermore, the average $OD_{450}$ value in the roots rapidly increased from 14 DAT with an $OD_{450}$ value of 1.07 to 21 DAT with an $OD_{450}$ value of 1.88, from 0.48 to 0.99 for leaves (Table 3). The results indicated that the concentrated virus could infect roots successfully and expand to leaves of NC89.

### 3.4. Detection in irrigation water samples

The detection assay was also used to test irrigation water samples from Long County, Xunyang County, Fu County, and Nanzheng County in Shaanxi Province. The distribution of the virus was greatly different. Nanzheng County was the most seriously infected region with a detectable rate of 44.19%, followed by the Xunyang County and Fu County (Table 4). Among these areas, Long County was the least-infected region with a detectable rate of 21.95% (Table 4). In summary, TEV can be detected in the irrigation water of Shaanxi Province. Fifty-six of 180 water samples were positive for TEV; the viral copies ranged from $1.43 \times 10^5$ to $6.33 \times 10^7$ with an average of 2.26 $\times 10^7$ (Table 4).

## 4. Discussion

In this study, a new and simple method was developed to monitor the sanitary conditions of irrigation water. In this method, PEG6000 as chemical flocculation was used to precipitate TEV, and TEV-specific qRT-PCR was developed to detect TEV in concentrates. The present study indicated that this method could be effective to concentrate and detect TEV in irrigation water.

The concentration of PEG in water samples is an important influencing factor on the absorption of viruses. Sobsey and Jones (1979) reported that efficient virus

**Table 2.** The orthogonal design tests for determining the efficiency of virus recovery.

| Centrifugal force (× *g*) | PEG6000 concentration (%) | | | |
|---|---|---|---|---|
| | 5% | 15% | 25% | 35% |
| 3000 | 0.00% | 9.03% | 33.75% | 11.56% |
| 4000 | 10.17% | 21.90% | 42.44% | 23.70% |
| 5000 | 22.64% | 51% | 56% | 23.77% |
| 6000 | 24.32% | 61.59% | 92.05% | 29.65% |
| 7000 | 18.74% | 41.78% | 46.04% | 9.03% |

**Table 3.** The ELISA results of the infection test for detecting the activity of TEV in concentrates.

| Treatment | The average OD value of 5 treated plants. | | | |
|---|---|---|---|---|
| | 0 DAT | 7 DAT | 14 DAT | 21 DAT |
| Roots | 0.16 (–)[a] | 0.33 (+) | 1.07 (+) | 1.88 (+) |
| Leaves | 0.15 (–) | 0.17 (–) | 0.48 (+) | 0.99 (+) |
| Positive control | 2.39 (+) | 2.38 (+) | 2.45 (+) | 2.41 (+) |
| ELISA buffer | 0.12 | 0.12 | 0.15 | 0.15 |

[a]: The positive threshold OD value was set to twice the OD value of the negative control; (–) or (+) indicates virus-negative or -positive samples, respectively.

recovery was generally different in different seasons. Filter or chemical flocculation is widely used in several techniques for concentrating aquatic viruses from water; those techniques could get better recovery efficiency in suitable pH conditions, since filter or chemical flocculation is sensitive to pH (Sobsey and Jones, 1979; John et al., 2011). Meanwhile, most of the results demonstrated that the recovery efficiency of several methods was significantly lower in concentrating large volumes of environmental samples than in small volumes of seeding samples (Atha and Ingham, 1981; Kramberger et al., 2004; Li et al., 2010; John et al., 2011; Suzuki et al., 2011). Although the temperature, pH, and volume of samples are common influencing factors on the absorption of viruses, only the role of PEG concentration in water samples was described in this study based on the following facts. First, the interaction between protein and PEG was insensitive to changes of solution conditions (pH, temperature, salts); second, the interaction between protein and PEG was based on excluded volume effects (Perez et al., 2011). In the interaction progress between protein and PEG, PEG can be viewed as an inert solvent sponge, which indiscriminately raises the effective concentration of the solute in water. Generally speaking, the interaction effectiveness of PEG increases along with the concentration of PEG in water samples. Because the interaction is not specific, all of the minor components, proteins, and viruses could be concentrated by PEG from water. Minor components, proteins, and viruses could be separated from water with an increase of PEG concentration according to the size of particle. Therefore, a suitable concentration of PEG does well in concentrating a special virus and improving recovery.

The qRT-PCR assay is one of the currently available methods for detecting TEV in concentrates. The largest obstacle in determining the infection route or behavior of a pathogenic virus is that the virus exists at an extremely low concentration in water. Although test methods utilizing conventional plaque assays or RT-PCR detection have been devised, it will remain very difficult to detect viruses in water (Boben, 2007). Consequently, more sensitive detecting methods combined with a procedure of concentration are necessary in studying WBPVs. In this study, a SYBR Green-based qRT-PCR assay was developed to detect TEV in concentrates, and its effect was evaluated. Through serial dilutions of standard plasmids, the detection limit of qRT-PCR was determined to be 10 viral copies/µL. The sensitivity of qRT-PCR makes it suitable for detecting TEV in environmental water samples compared to conventional methods such as PCR and ELISA.

The viability of TEV was confirmed. In the infectivity tests, TEV could be first detected in the roots of NC89 at 7 DAT; the concentration of TEV in the upper leaves began to accumulate after 7 DAT (Table 3). Those results showed that TEV maintained viability in the water environment and that it could reinfect plants though the roots. Although systemic symptoms were not observed, the concentration of TEV had reached a high level at 21 DAT with the $OD_{450}$ value of 0.99. Therefore, if the water used for irrigation is polluted by TEV, the risk of crops being infected by plant pathogenic viruses may increase.

Effective management measures can stop plant viruses from polluting irrigation water. Located in the western Kuan-chung Plain, the Long County tobacco region is one of the standard managed tobacco regions in Shaanxi Province. Due to standard management, water samples from this region were less polluted by the tobacco virus. However, for the Nanzheng tobacco region, most of the tobacco planting areas belong to mountain land, the

**Table 4.** Detection results of environmental samples.

| Location | Sample number | Positive sample | Average viral copies |
|---|---|---|---|
| Long County | 41 | 9 | $3.46 \times 10^6$ |
| Xunyang County | 47 | 15 | $1.92 \times 10^6$ |
| Fu County | 49 | 13 | $1.43 \times 10^5$ |
| Nanzheng County | 43 | 19 | $6.33 \times 10^7$ |
| Total | 180 | 56 | $2.26 \times 10^7$ |

management was relatively extensive, and detection rate was the highest. Effective field management techniques such as rational application of fertilizer, reasonable irrigation, or removing diseased plant debris timely play an important role in controlling WBPVs for the following reasons. First, it is important to eliminate or reduce the source of inoculums. Diseased plant debris is a significant source of WBPV (Zheng et al., 2000). Removing the diseased body in time and maintaining the cleanliness of the field can prevent WBPVs from polluting irrigation water. Second, it is also important to enhance plant disease resistance. Root release was one of the ways for the plant virus to pollute irrigation water (Yarwood, 1960); thus, increasing the plants' own disease resistance may be an efficient way to prevent WBPVs. Finally, it is important to stop WBPVs from spreading. They can be released into the irrigation water by a disabled body. These released viruses could infect the other plants through microwounds caused by soil microbes and nematodes and could cause diseases in very low concentrations (Zheng et al., 2000). Using drip irrigation, sprinkler irrigation, and other irrigation methods instead of the traditional flooding can not only save a large amount of water resources, but can also effectively reduce the possibility of WBPVs spreading. In summary, infected plants and disabled bodies are major sources of WBPVs, and the water itself is an important medium for the spread of WBPVs. The primary role of standardization management is to prevent WBPVs by clearing away sources of WBPVs and breaking down transmission routes. As a sensitivity assay, this method can be used as an elementary tool for the real-time monitoring of sanitary status.

Compared to WBAVs, WBPVs have not received enough attention. However, there are probably large amounts of WBPVs in the aquatic environment, although only a tiny portion can be detected. These WBPVs may lead to serious disease problems in agriculture. Currently, there are no effective measures to prevent them due to a lack of scientific knowledge regarding many aspects of the ecology and environmental properties of WBPVs. It is thus necessary to develop simple and accurate methods to concentrate and detect plant viruses in water and to further study the ecology of WBPVs and effective technologies for removing WBPVs, both of which are useful for controlling WBPVs.

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## References

Atha DH, Ingham KC (1981). Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. J Biol Chem 256: 12108–12117.

Balamurugan V, Sen A, Venkatesan G, Yadav V, Bhanot V, Bhanuprakash V, Singh RK (2012). A rapid and sensitive one step-SYBR Green based semi quantitative real time RT-PCR for the detection of *peste des petits ruminants virus* in the clinical samples. Virologica Sinica 27: 1–9.

Boben J, Kramberger P, Petrovic N, Cankar K, Peterka M, Strancar A, Ravnikar M (2007). Detection and quantification of *Tomato mosaic virus* in irrigation waters. Eur J Plant Pathol 118: 59–71.

Carrasco P, Daros JA, Agudelo-Romero P, Elena SF (2007). A real-time RT-PCR assay for quantifying the fitness of tobacco etch virus in competition experiments. J Virol Methods 139: 181–188.

Colombet J, Robin A, Lavie L, Bettarel Y, Cauchie HM, Sime-Ngando T (2007). Virioplankton 'pegylation': use of PEG (polyethylene glycol) to concentrate and purify viruses in pelagic ecosystems. J Microbiol Methods 71: 212–219.

Dai J, Penng H, Chen W, Cheng JL, Wu YF (2012). Development of multiplex real-time PCR for simultaneous detection of three Potyviruses in tobacco plants. J Appl Microbiol 114: 502–508.

Deboosere N, Horm SV, Pinon A, Gachet J, Coldefy C, Buchy P, Vialette M (2011). Development and validation of a concentration method for the detection of influenza A viruses from large volumes of surface water. Appl Environ Microbiol 77: 3802–3808.

Dougherty WG, Hiebert E (1980). Translation of potyvirus RNA in a rabbit reticulocyte lysate: Cell-free translation strategy and a genetic map of the potyviral genome. Virology 104: 183–194.

Fedick A, Su J, Treff NR (2012). Development of TaqMan allelic discrimination based genotyping of large DNA deletions. Genomics 99: 127–131.

Gao CX, Han LX, Qu LD, Luo YZ, Han JL (2012). Specific TaqMan probed real-time quantitative RT-PCR methods and their application to differentiate the transcripts of duplicated BF or BLB genes in chicken MHC. Vet Immunol Immunopathol 145: 590–596.

Guevremont E, Brassard J, Houde A, Simard C, Trottier YL (2006). Development of an extraction and concentration procedure and comparison of RT-PCR primer systems for the detection of hepatitis A virus and norovirus GII in green onions. J Virol Method 134: 130–135.

Huang PW, Laborde D, Land VR, Matson DO, Smith AW, Jiang X (2000). Concentration and detection of caliciviruses in water samples by reverse transcription-PCR. Appl Environ Microbiol 66: 4383–4388.

Huang T, Yuan ZL, Cheng JL, Wu YF, Wu ZM, Dai J (2012). Identification of resistance to TEV and CMV in tobacco varieties. J Northwest A&F University 40: 107–113.

Jaykus LA, De LR, Sobsey MD (1996). A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridization. Appl Environ Microbiol 62: 2074–2080.

John SG, Mendez CB, Deng L, Poulos B, Kauffman AK, Kern S, Brum J, Polz MF, Boyle EA, Sullivan MB (2011). A simple and efficient method for concentration of ocean viruses by chemical flocculation. Environ Microbiol Rep 3: 195–202.

Kramberger P, Petrovic N, Strancar A, Ravnikar M (2004). Concentration of plant viruses using monolithic chromatographic supports. J Virol Method 120: 51–57.

Li D, Shi HC, Jiang SC (2010). Concentration of viruses from environmental waters using nanoalumina fiber filters. J Microbiol Methods 81: 33–38.

Li JW, Wang XW, Rui QY, Song N, Zhang FG, Ou YC, Chao FH (1998). A new and simple method for concentration of enteric viruses from water. J Virol Method 74: 99–108.

Lian HX, Lu DX, Gao M (2009). Establishment of real-time TaqMan-fluorescence quantitative RT-PCR assay for detection and quantification of porcine lipoprotein lipase mRNA. Agric Sci China 8: 1256–1262.

Luigi M, Faggioli F (2011). Development of quantitative real-time RT-PCR for the detection and quantification of *Peach latent mosaic viroid*. Eur J Plant Pathol 130: 109–116.

Novo FG (2012). Moral drought: the ethics of water use. Water Policy 14: 65–72.

Perez LJ, Diaz de Arce H, Cilloni F, Salviato A, Marciano S, Perera CL, Salomoni A, Beato MS, Romero A, Capua I et al (2012). An SYBR Green-based real-time RT-PCR assay for the detection of H5 hemagglutinin subtype avian influenza virus. Mol Cell Probes 26: 137–145.

Perez LJ, Perera CL, Frias MT, Nunez JI, Ganges L, de Arce HD (2011). A multiple SYBR Green I-based real-time PCR system for the simultaneous detection of porcine circovirus type 2, porcine parvovirus, pseudorabies virus and Torque teno sus virus 1 and 2 in pigs. J Virol Method 179: 233–241.

Plese N, Juretic N, Mamula D, Polak Z, Krajacic M (1996). Plant viruses in soil and water of forest ecosystems in Croatia. Phyton-Ann REI Bot 36: 135–144.

Pocsai E, Horvath J (1997). The occurrence of plant viruses in waters of the Hungarian rivers and lakes. Novenyvedelem 33: 69–76.

Polischuk V, Budzanivska I, Shevchenko T, Oliynik S (2007). Evidence for plant viruses in the region of Argentina Islands, Antarctica. FEMS Microbiol Ecol 59: 409–417.

Radin D, D'Souza DH (2011). Simple and rapid detection of human norovirus from produce using SYBR Green I-based real-time RT-PCR. Food Environ Virol 3: 121–129.

Riechmann JL, Lain S, Garcia JA (1992). Highlights and prospects of potyvirus molecular-biology. J Gen Virol 73: 1–16.

Rosner A, Lachman O, Pearlsman M, Feigelson L, Maslenin L, Antignus Y (2006). Characterisation of cucumber leaf spot virus isolated from recycled irrigation water of soil-less cucumber cultures. Ann Appl Biol 149: 313–316.

Santhosh SR, Parida MM, Dash PK, Pateriya A, Pattnaik B, Pradhan HK, Tripathi NK, Ambuj S, Gupta N, Saxena P et al. (2007). Development and evaluation of SYBR Green I-based one-step real-time RT-PCR assay for detection and quantification of Chikungunya virus. J Clin Virol 39: 188–193.

Schwab KJ, De LR, Sobsey MD (1996). Immunoaffinity concentration and purification of waterborne enteric viruses for detection by reverse transcriptase PCR. Appl Environ Microbiol 62: 2086–2094.

Sevik MA (2011). Water pollution: water-borne plant viruses. Erciyes Üniversitesi Fen Bilimleri Enstitüsü Dergisi 27: 40–47.

Sharma S, Dasgupta I (2012). Development of SYBR Green I based real-time PCR assays for quantitative detection of *Rice tungro bacilliform virus* and *Rice tungro spherical virus*. J Virol Methods 181: 86–92.

Sobsey MD, Jones BL (1979). Concentration of poliovirus from tap water using positively charged microporous filters. App Environ Microbiol 37: 588–595.

Suzuki Y, Suzuki T, Kono T, Mekata T, Sakai M, Itami T (2011). The concentration of white spot disease virus for its detection in sea water using a combined ferric colloid adsorption- and foam separation-based method. J Virol Method 173: 227–232.

Toze S (2006). Reuse of effluent water—benefits and risks. Agric Water Manage 80: 147–159.

Varga A, James D (2005). Detection and differentiation of Plum pox virus using real-time multiplex PCR with SYBR green and melting curve analysis: a rapid method for strain typing. J Virol Methods 123: 213–220.

Wu QL, Liu WT (2009). Determination of virus abundance, diversity and distribution in a municipal wastewater treatment plant. Water Res 43: 1101–1109.

Yarwood CE (1960). Release and preservation of virus roots. Phytopathology 50: 111–114.

Yu XM, Fang P, Zhu RQ (2004). A hydroponic method for direct culture of *Arabidopsis thaliana*. Plant Physiol Comm 40: 81–82.

Zhang ML (1993). Studies on aphids transmitting TEV. J Northwest University 23: 193–196.

Zheng YT, Lin QY, Xie LH (2000). Plant viruses and their ecological effects in water. Virologica Sinica 15: 1–7 (article in Chinese with English abstract).