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Research Article

Multiplex RT-PCR Assay for Crinivirus Detection Using RNA Prepared from Three Extraction Methods on Tomato Plant

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ABSTRACT

Tomato infectious chlorosis virus (TICV) and Tomato chlorosis virus (ToCV) are members of the Crinivirus genus that induces yellowing symptoms in tomato plants. Detection of both viruses is generally carried out singly, thus it is necessary to develop a fast, accurate and efficient detection method to detect multiple viruses simultaneously in an effort to determine the suitable disease management strategies. This study was aimed to detect both viruses using the multiplex RT-PCR method and evaluate three methods of total RNA preparation used from tomato plants as RT-PCR templates. The methods evaluated were simple direct tube (SDT), simple dsRNA, and commercial kit (RNeasy Plant Mini Kit) as a comparison. The total source of RNA came from Crinivirus symptomatic tomato leaves from Kopeng, and Ketep (Central Java); Pakem (Yogyakarta); Malang (East Java); and Bogor (West Java). Single RT-PCR and multiplex RT-PCR using specific primers CPd I/CPd II and ToCV CF/ToCV CR with DNA band targets of 760 bp and 360 bp. The SDT and dsRNA methods have been successful in obtaining total RNA and viral RNA from tomato leaf samples. Total RNA RT–PCR with simple SDT and dsRNA methods followed by multiplex RT-PCR produces specific DNA band intensities that are comparable to Kit. RNA preparation with SDT and simple dsRNA methods is a simple, fast, easy and affordable method in providing templates for RT-PCR. Multiplex RT-PCR technique using two pairs of specific primers CPd I/CPd II and ToCV CF/ToCV CR is suitable for simultaneous testing of TICV and ToCV.

Keywords: dsRNA, *multiplex* RT-PCR., SDT, TICV, ToCV

INTRODUCTION

Tomato yellow–purple leaf diseases caused by Tomato Infectious Chlorosis Virus (TICV) and Tomato Chlorosis Virus (ToCV) have been reported in Indonesia (Hartono & Wijonarko, 2007). Both viruses are members of the genus *Crinivirus*, family *Closteroviridae* transmitted by whitefly (Wintermantel, 2004). TICV infection was first reported in the state of California, USA in 1993 (Duffus et al., 1996) whereas ToCV was first reported in Northern Florida, USA in 1996 (Wintermantel & Wisler, 2006). Since then, the distribution area of TICV and ToCV expanded to several countries in Europe and Asia (EPPO, 2005). TICV is exclusively transmitted by a vector insect, *Trialeurodes vaporariorum*, in a semi–persistent manner and untransmitted mechanically (Duffus *et al*., 1996; Wisler *et al*., 1996; *Li et al*., 1998). Whereas the ToCV is transmitted by *Bemisia tabaci* biotypes A and B, *T. vaporariorum*, and *T. abutilonea* with an incubation time of 1–2 days, in a semi-persistent manner. The incidence of tomato yellow disease is influenced by population density and activity of whitefly as the insect vector (Jacquemond *et al*., 2009). The symptoms of TICV and ToCV are difficult to distinguish, namely yellowing of leaves and stems, interveinal chlorosis and reddish necrotic spots, that can be observed first on the older tomato leaves and then develop into young leaves (Navas-Castillo *et al*., 2000; Vaira *et al*., 2002) (Figure 1). Hartono and Wijonarko (2007) reported that the intensity of tomato yellow diseases in Magelang reached 30% and seen a tendency to increase. This disease causes losses for farmers, especially if the attack occurred in the vegetative stage by causing stunted, small, and harden fruit, hence the fruits are not marketable.

Diagnosis based on symptoms or serological techniques has limitations due to the similarity between the infection and nutrient deficiency symptoms and low titers of antigens in plant tissue (Agrios, 2005). The real-time PCR multiplex (RT-PCR) is a detection

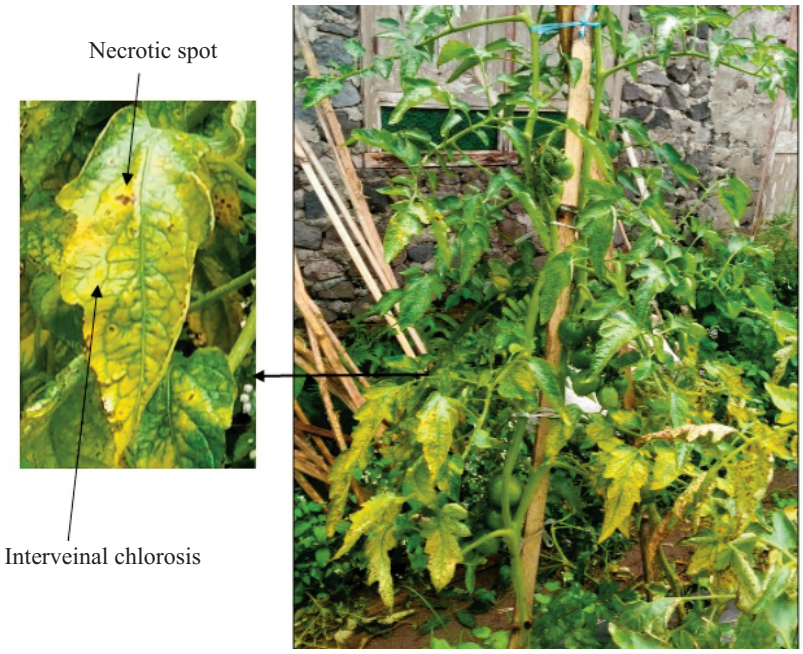

Figure 1. Yellowing symptoms in Crinivirus–infected tomatoes that started with old leaves, interveinal chlorosis leaf and necrotic spots on tomato plantations from Ketep, Magelang District, Central Java

more sensitive and able to detect the presence of viruses in low concentrations. In addition, RT-PCR can also be used to detect multiple virus infections in plants separately, quickly, easily, accurately, efficiently, and has been widely developed (Miftakhurohmah, 2013). The multiplex RT-PCR can detect the presence of ToCV and TICV viruses simultaneously in tomato plants in Greece and France (Dovas *et al*., 2002; Jacquemond *et al*., 2009). Multiplex PCR was able to distinguish between TYLCV (Tomato Yellow Leaf Curl Virus) and TYLCV–Mid clades (Lefeuvre *et al*., 2007). Papayiannis *et al*. (2010) reported that RT-PCR multiplex managed to distinguish the TYLCV and TYLCSV. In Indonesia there are not many studies using the multiplex RT-PCR for detecting multiple virus infections in tomato plants simultaneously.

Nucleic acid extraction is the first step to amplify DNA or RNA (Zou *et al*., 2017). Nucleic acid sample preparation involves various techniques for converting samples that cannot be directly analyzed into one in accordance with the requirements of the analytical techniques used, i.e. RT-PCR or PCR. In some developed-extraction methods, RNA extraction requires quite long and complicated stages and uses hazardous chemicals, i.e. phenol and chloroform (Diningsih *et al*., 2017). The long and complicated extraction stages can allow contamination present between samples hence the results are not as expected (Suehiro *et al*., 2005).

Various methods have been widely used in RNA extraction including simple direct tube (SDT) (Suehiro *et al*., 2005; Adiputra *et al*., 2012), simple dsRNA extraction (Endarsih *et al*., 2017), and commercial kit. Those methods are able to obtain RNA from plants and viruses. Simple RNA extraction using SDT method successfully detected several species of viruses, such as Turnip mosaic virus (TuMV), Green mottle mosaic virus (CGMMV), Cucumber mosaic virus (CMV), Sugarcane streak mosaic virus (SCSMV), TICV, ToCV, *Chrysanthemum* virus B (CVB), *Chrysanthemum* stunt viroid (CSVd), and Carnation mottle virus (CarMV) (Suehiro *et al*., 2005; Adiputra *et al*., 2012; Damayanti & Putra, 2012; Temaja *et al*., 2012; Diningsih *et al*., 2017). In addition, the simple dsRNA extraction method has succeeded in detecting several viruses, such as Rehmanian mosaic virus (ReMV), CMV, and ToCV (Fajarfika *et al*., 2015; Endarsih *et al*., 2017). Furthermore, tomato plants infected TICV is detected with RNA preparation using Kit.

However, evaluation of these methods is needed to produce RNA without contaminants such as DNA and protein and does not change the structure and function of RNA molecules. Besides that, the method must be effective and efficient and can be done for all species. Based on the description, this study was aimed to detect Crinivirus infection through multiplex RT–PCR using two specific primer pairs and RNA

preparations derived from three extraction methods. The results of this study are expected can be used as a rapid detection method of tomato plant viruses to determine suitable disease management strategies.

MATERIALS AND METHODS

Plant Material Preparation

The tomato leaves showed symptomatic Crinivirus in the middle and the bottom of the leaves was sampled. Tomato leaf samples were obtained from Ketep, Kopeng (Central Java); Pakem (Yogyakarta); Bogor (West Java); and Malang (East Java).

RNA Extraction

Simple Direct Tube (SDT). The Simple Direct Tube (SDT) method was carried out according to Suehiro *et al*. (2005). Tomato leaves were crushed in a phosphate buffer saline (PBS) solution containing 0.05% Tween-20 (PBST) in a ratio of 1: 1 (w/v), then the crude sap (50 µl) was transferred to the PCR tube (0.5 ml, polypropylene), and incubated at room temperature for 15 minutes. After that, the sap was removed by cutting the tip and the PCR tube was washed twice with 50 µl PBST to remove any residual tissue. 30 µl DEPC water and 1 µl RNAse inhibitor were added to the PCR tube, then incubated at 95°C for 1 minute and cooled in ice for 1 minute. Furthermore, the solution can be used for RT-PCR.

Simple dsRNA. dsRNA extraction using the method carried out by Endarsih *et al.* (2017). 0.2 grams of leaf tissue was crushed using liquid nitrogen, 500 µl extraction buffer was added (2X STE contains 0.1% (v/v) 2–mercaptoethanol and 1% (w/v) SDS), then the extract solution was transferred to 1.5 ml tube, and added ±10 mg polyvinyl pyrrolidone (PVPP) to remove phenol compounds that pollute the purity of nucleic acids. 500 µl Phenol-Chloroform-Isoamyl Alcohol/PCIAA (25: 24: 1) was added to the extract solution, homogenized for 1 minute, then centrifuged at 20,000× g for 5 minutes. This step was repeated if the supernatant is not clear. 400 µl of the supernatant was transferred to a 1.5 ml tube and then added 80 µl of ethanol (final concentration of 16.6%). The solution was centrifuged at 20,000× g for 3 minutes then the supernatant was transferred to the micro spin column. The column was centrifuged at 10,000× g for 5 minutes (some ssRNA and DNA are bounded to cellulose), after that the liquid remains in the bottom of the column was discarded. A washing buffer (STE–16% ethanol) of 400 µl was added to the column, centrifuged at 10,000× g for 5 minutes to eliminate ssRNA and DNA bound to cellulose, then the fluid collected under the column was discarded. This step was repeated 2 times. After the last washing, the column was centrifuged at 10,000× g for 5 minutes to remove ethanol from cellulose D. A tube of 0.6 ml was placed in a new 2 ml tube and 400 µl of elution buffer (STE 1X) was added to the column then centrifuged at 10,000x g for 5 seconds. In the elution process, a pure dsRNA would be obtained, then a 0.6 ml tube was discarded. 40 µl 3M sodium acetate (pH 5.2) and 1 ml of 99.5% ethanol were added, stored at -20°C for 30 minutes overnight. After that, the tubes were centrifuged at 20,000× g for 5 minutes for dsRNA precipitation. The tube was washed with 100 µl of 70% alcohol then centrifuged at 3000 rpm for 2 minutes, the alcohol was carefully discharged using a pipette and this step was repeated twice, and then air-dried, the dsRNA was dissolved in nuclease-free water.

Commercial KIT

0.1 g samples were crushed in a mortar with pestle using liquid nitrogen until a fine powder obtained. The extraction was carried out according to recommended protocol from the extraction kit manufacturer (RNeasy Plant Mini Kit from Qiagen, Germany).

Nucleic Acid Extraction Measurement

Nucleic acid was measured to determine the concentration and quantity of DNA/RNA after the extraction process. RNA/DNA was measured using a nanodrop spectrophotometer, by calculating the ratio and concentration of RNA/DNA in the absorption of light wavelengths (230 nm, 260 nm, 280 nm, and 320 nm). Absorbance measurements at 230, 260, 280 nm were reduced by the absorbance value of the blank (absorbance of 320 nm) before being used to calculate the concentration and purity of RNA.

Synthesis of Complementary DNA and Single RT-PCR

The extracted RNA was transcribed back to obtain cDNA using the RT–PCR technique. The RT-PCR reaction was made using a kit from Toyobo. 4 µl 5× RT Buffer (containing 25 mM Mg 2+), 1 µl Primary oligo (dT) 20 (10 pmol/µl), 2 µl dNTP mixture (10 mM), 1 µl Reverra Acc, 1 µl RNase Inhibitor (10 U/µl), and

8 µl RNase-free H_2O were added into the labeled microtubes. Then 3 µl extracted RNA was added into the PCR tube and homogenized using a vortex machine. The RT-PCR reaction mixture was incubated in a PCR machine with a 30°C program for 10 minutes, 42°C for 20 minutes, 99°C for 5 minutes and the incubation process was completed at 4°C. This cDNA prepared RT was used as a DNA template in the PCR reaction.

The DNA amplification reaction of each virus was conducted by the PCR method using MyTaqTM HS Red Mix and several specific primer pairs that can amplify the virus separately. Specific primer pairs used to detect the presence of TICV and ToCV viruses were shown in (Table 1). Single RT-PCR reaction with a total volume of 10 µl, consisting of 0.5 µl of each primer (10 µM), 5 µl MyTaqTM HS Red Mix, 3 µl dH2O, and 1 µl DNA template. Single RT-PCR for TICV and ToCV amplification was carried out on a Thermal Cycler PCR machine with a 95°C predenaturation for 1 minute, 32 cycles at 95°C for 15 seconds, primer attachment 58°C for 15 seconds, elongation 72°C for 10 seconds, and a final elongation of 72°C for 5 minutes.

Multiplex RT-PCR

The multiplex RT-PCR assay was carried out using two specific primer pairs (Table 1) and Crinivirus cDNA samples in one reaction mixture with a total volume of 20 µl, consisting of 0.5 µl of each primer (10 µM), 10 µl MyTaqTM HS Red Mix, µl dH_2O, and 2 µl DNA template. Multiplex RT-PCR for TICV and ToCV amplification was carried out with a 95°C predenaturation for 2 minutes, 35 cycles at 95°C for 30 seconds, primer attachment 58°C for 30 seconds, 72°C elongation for 20 seconds, and elongation final 72°C for 5 minutes.

RT-PCR Amplification Visualization

Visualization of the amplification product was carried out using 1% agarose gel in a 1×TBE buffer (Tris-borate EDTA) with electrophoresis at 50 volts for 50 minutes.

Nucleotide Sequences Analysis

Analysis of nucleotide sequences was conducted using the sequencer (ABI-Prism 3100-Avant Genetic Analyzer) in the Research and Development Center laboratory of PT Genetics Science, Jakarta, Indonesia. The DNA sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) to compare the target virus sequences with the virus nucleotide sequences from other countries registered in Genbank. The homology level of nucleotides and amino acids was obtained by the ClustalW multiple alignment programs and Sequences Identity Matrix using Bioedit 7.05 software.

RESULTS AND DISCUSSION

Comparison of Extracted RNA Quantity Protein Purity

The simple KIT and dsRNA extraction method can produce high RNA purity in several test samples (Table 2). In sample extracts from Ketep, Kopeng, and Pakem (commercial KIT); and from Kopeng and Bogor (simple dsRNA extraction) have the RNA purity of 1.8–2.1. However, Bogor and Malang sample extracts (commercial KIT); Ketep, Pakem, and Malang (simple dsRNA); and the overall sample extract by the SDT method obtained RNA purity below 1.8 or more than 2.1. The low purity of protein RNA in the commercial KIT method, simple dsRNA, and SDT (A260/A280 <1.8) indicated that there is contamination remain in the RNA solution from DNA or plant proteins absorbed in 280 nm. However, between the dsRNA and SDT methods, the dsRNA is still able to produce RNA purity higher than SDT.

Impurity Purity

The purity of impurities (A260/A230) of RNA produced by SDT, simple dsRNA simply, and the

Table 1. Primer sequences for TICV and ToCV amplification

Target	Primer	Primer Base (5'-3')	Product Size	Reference
TICV	CPd I	ATGAGGTCTTTCACAGTGG	760 bp	Li *et al.*, 1998
	CPd II	GTCCGAAACTGATTGAACC		
ToCV	ToCV-CF	GTGTCAGGCCATTGTAAACCAAG	360 bp	Wintermantel *et al.*, 2005
	ToCV-CR	CACAAAGCGTTTCTTTTCATAAGCAGG		

Table 2. Purity values and RNA concentrations measured by nano drop spectrophotometer

Sample	Absorbance Value			RNA Purity[a]		Concentration (µg/ml)
	A230	A260	A280	A260/A280	A260/A230	
Commercial KIT extraction Method						
Ketep	9.11	11.86	7.86	2.02	1.53	316.40
Kopeng	5.70	8.51	4.28	2.03	1.51	332.40
Pakem	9.86	12.20	8.25	1.96	1.41	322.40
Bogor	4.16	1.24	0.95	1.55	0.22	34.40
Malang	4.67	2.79	2.61	1.45	0.24	23.20
dsRNA simply Extraction Method						
Ketep	1.88	2.05	1.78	2.20	1.50	19.60
Kopeng	0.92	0.56	0.28	2.15	1.25	17.60
Pakem	1.41	1.45	1.29	2.26	1.19	11.30
Bogor	1.68	1.83	1.53	2.10	1.35	23.20
Malang	1.25	1.60	1.15	2.22	1.75	32.80
SDT Extraction Method						
Ketep	18.81	17.69	17.32	1.52	0.49	43.20
Kopeng	0.87	0.52	0.50	1.12	0.36	8.00
Pakem	20.17	19.62	19.64	0.98	0.36	12.40
Bogor	15.99	14.82	14.40	1.50	0.52	50.40
Malang	2.50	2.17	1.90	1.62	0.68	28.00

Remarks: [a]RNA purity is measured by calculating the absorbance ratio at 230, 260 and 280 nm (A260/A280 ratio:absorbance of RNA with protein, expressed as pure RNA in the range of 1.8–2.1; A260/A230 ratio:RNA absorbance compared to organic compounds, expressed pure RNA in the range of 1.8–2.2).
Concentration is measured at 260 nm × 40 µg/ml waves

KIT in all sample extracts was low (less than 2.0) (Table 2). This showed that the three extraction methods have not been able to remove contaminants optimally from organic compounds. Total RNA with lots of contaminants from organic compounds will be absorbed in 230 nm and will produce low RNA purity.

RNA Concentration

The RNA concentrations produced from the three extraction methods showed different results (Table 2). The simple dsRNA extraction method resulted in lower RNA concentrations because of the virus RNA was obtained while the SDT and Kit methods obtained total RNA (virus RNA and plant RNA). The highest concentration of RNA extracted from simple dsRNA was obtained in extracts from Malang (32.80 µg/ml) and the lowest was from Pakem (11.30 µg/ml). In the SDT extraction method, the highest RNA concentration was obtained from Bogor (50.40 µg/ml) and the lowest was from Kopeng (8.00 µg/ml). Whereas the highest RNA concentration extracted by Kit was from Kopeng (332.40 µg/ml) and the lowest was from Malang (23.20 µg/ml). To obtain total RNA or virus RNA with the high quality and quantity, certain chemicals in extraction buffers are needed to inhibit the RNase enzymes (Claros & Canovas, 1999; Diningsih *et al*., 2017). SDT method uses DEPC containing RNase inhibitors as RNA solvents to prevent RNA degradation by RNase (Amanda & Cartealy, 2015). The simple dsRNA uses 2-mercaptoethanol in extraction buffers to eliminate ribonuclease (RNase) which is released during cell lysis. Likewise, RNase degrading agents in the buffer are present in commercial kits (Diningsih *et al.*, 2017). In addition, sample selection also influences the concentration level resulted from the extraction as reported by Endarsih *et al.* (2017) that a higher concentration of virus would be obtained in leaf samples with symptoms of early viral infections than in old tissue with advanced infection symptoms.

Amplification of Plant Virus RNA Using a Single RT-PCR

The quality of RNA from three extraction methods was analyzed using the two-step RT-PCR technique. TICV and ToCV do not have poly-A compound, thus in the reverse transcription process a random primary was used to amplify some parts of the mRNA. Then the cDNA produced was used as a template in the PCR process. Complementary DNA templates from

the three extraction methods were well amplified and produce a single DNA band pattern with different intensities. In TICV detection, DNA bands with uniform thickness were obtained from all extraction methods, this showed that the three methods could produce a similar quality of DNA. While the ToCV detection showed different intensities between the three methods, the extraction using KIT showed very thick DNA bands from Ketep, Bogor, and Pakem. Whereas extraction using SDT and simple dsRNA methods produced the quite thick DNA bands from Pakem and Malang. The difference in the thickness of DNA bands shows that there is a difference in the quality of RNA formed, this can be caused by a factor of sample homogenisation (Endarsih *et al*., 2017).

A pair of CPd I and CPdII specific primers are designed to detect divergen coat protein (CPd) gene in terminal 3' RNA 2 TICV (Li *et al*., 1998) and successfully amplified cDNA samples from Kopeng, Ketep, and Bogor (Figure 2A). This CPd gene is a specific characteristic of the Closteroviridae virus (Martelli *et al*., 2002). The single DNA band produced is 760 bp, similar to DNA band size from America, Italy, Japan, Yogyakarta (Li *et al*., 1998; Hartono *et al*., 2003; Kusumaningrum *et al*., 2015), hence the virus is identified as TICV. Samples of tomato plants infected by ToCV were not amplified by TICV specific primer pairs so that the DNA bands would not appear. Furthermore, the double infected samples by TICV and ToCV, the DNA bands that appeared were only the TICV.

The specific primers of ToCV-CF and ToCV-CR are designed to amplify the ToCV protein envelope gene (Hirota *et al*., 2010), and successfully amplified cDNA samples from Kopeng, Ketep, Bogor, Pakem, and Malang (Figure 2B). The single DNA band produced is 360 bp, similar to DNA band size from Japan and Cipanas (Indonesia) (Hirota *et al.*, 2010; Nurulita *et al.*, 2013) hence the virus was identified as ToCV. Samples of tomato plants that were positively infected by TICV were not amplified by specific primer pairs of ToCV so that the DNA band would not appear as well as for samples that were double infected by TICV and ToCV, the DNA band that appeared was only the ToCV. Samples from Pakem and Malang were only amplified with ToCV primers whereas with TICV primers could not be amplified, this showed that the samples were positively infected by a single virus, ToCV. However, the Kopeng, Ketep, and Bogor samples could be amplified with TICV and ToCV single primer showed that in the sample there was a double infection.

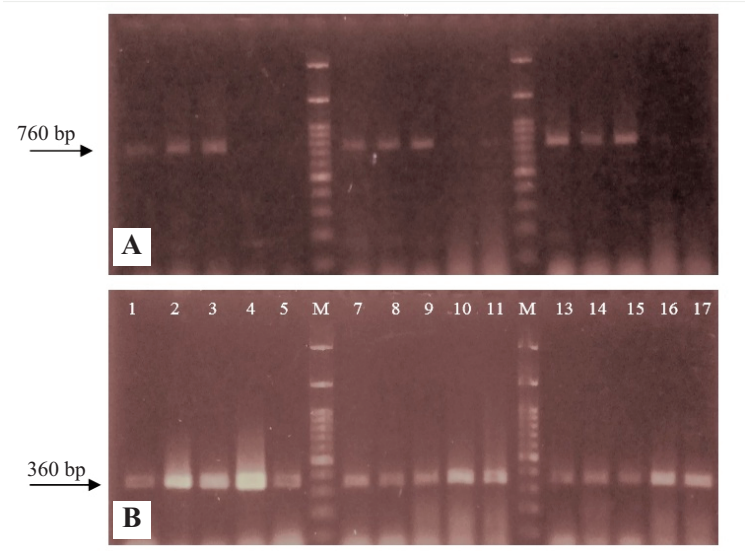

Figure 2. Visualization of a single RT-PCR using TICV CPdI/CPdII primers and ToCV-CF/ToCV-C on 1% agarose gel in 1X TBE. (A) DNA amplification of TICV by three RNA extraction methods [Commercial KIT: (1) Kopeng, (2) Ketep, (3) Bogor, (4) Pakem, (5) Malang; SDT: (7) Kopeng, (8) Ketep, (9) Bogor, (10) Pakem, (11) Malang; and the simple dsRNA: (13) Kopeng, (14) Ketep, (15) Bogor, (16) Pakem, (17) Malang]. (B) DNA amplification ToCV by three RNA extraction methods [Commercial KIT: (1) Kopeng, (2) Ketep, (3) Bogor, (4) Pakem, (5) Malang; SDT: (7) Kopeng, (8) Ketep, (9) Bogor, (10) Pakem, (11) Malang and the simple dsRNA: (13) Kopeng, (14) Ketep, (15) Bogor, (16) Pakem, (17) Malang)]; M: DNA marker 100 bp

Multiplex RT-PCR

The the single RT-PCR succeeded in amplifying TICV and ToCV with DNA sizes of 760 bp and 360 bp, respectively. This result showed that the two primers used have specificity in amplifying the target DNA. The combination of CPd I/CPd II and ToCV-CF/ToCV-CR primers with a concentration of 10 µM for each primer, the annealing temperature of 58^{o}C, and the number of cycles of 35 showed that both target DNA viruses were amplified hence they could be used to quickly detect both viruses. The simultaneous amplification resulted in a thinner TICV DNA band compared to ToCV DNA band however both showed a similar intensity in all extraction methods. The three extraction methods could be used to detect the two viruses from Kopeng, Ketep, and Bogor samples, whereas for Pakem and Malang samples only positive to amplify the ToCV (Figure 3). This showed that samples from Kopeng, Ketep, and Bogor were multiply infected by TICV and ToCV whereas in Pakem and Malang samples were only a single infection, ToCV.

In contrast to a single RT-PCR, where the two primer pairs are used separately, in multiplex RT-PCR, the TICV and ToCV specific primer pairs are used simultaneously, mixed with other PCR components. When both pairs of primers are used, they will attach to each DNA pairs. The TICV primer pair will attach to the TICV DNA sequence to form the TICV DNA band and the ToCV primer pair will attach to the ToCV DNA sequence to form the ToCV DNA band. To determine the success in the multiplex RT-PCR technique are the selection of primers with the same temperature melting (TM) for all sites, variations in the primer length to separate the product easily by TM and annealing temperature (Viljoen *et al*., 2005; Yang *et al*., 2013). The two specific primer pairs are used in multiplex RT-PCR are because they have various product sizes (thus it easier to read the results when the samples are visualized in agarose gel) and also has the same annealing temperature (where the annealing temperature used in multiplex RT-PCR must be able to optimize the two primer pairs used). The multiplex RT-PCR technique to detect ToCV and TICV has been developed by Tian *et al.* (1996) with degenerate primers corresponding to the motifs of the phosphate-1 and -2 homologous proteins of Closterovirus and Crinivirus HSP70.

The three extraction methods used could facilitate the simultaneous detection of more than one virus. Simple multiplex SDT/RT-PCR and simple dsRNA/ RT-PCR are suitable to be used for simultaneous detection of TICV and ToCV with results comparable to multiplex RT-PCR with commercial KIT extraction methods. James (1999) reported that the Tube capture/ RT-PCR method could detect Apple Stem Grooving Virus (ASGV) and Cherry Mottle Leaf Virus (CMLV) simultaneously with multiplex RT-PCR. The RT-PCR multiplex technique using two specific primer pairs in this study is suitable to be used to detect TICV and ToCV simultaneously.

DNA Sequencing

The alignment of the DNA sequencing of tomato from Ketep showed that the isolate was infected with TICV and ToCV (double infection). Based on the results of BLAST through NCBI, it was shown that TICV and ToCV isolates from Ketep has high homology with several isolates from other countries registered in the GenBank database (Tables 3 and 4).

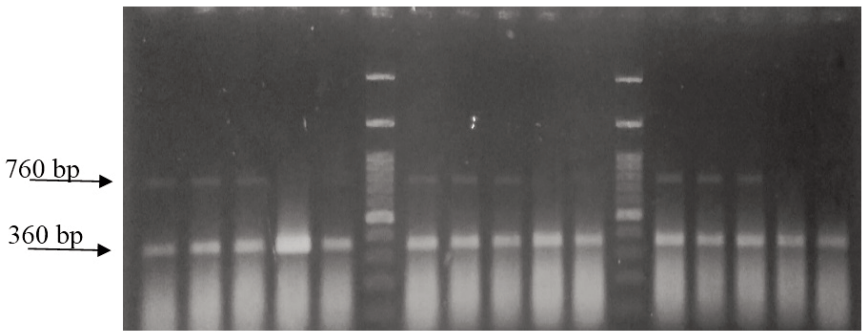

Figure 3. Visualization of the TICV and ToCV multiplex RT-PCR using primers of TICV CPdI/CPdII and ToCV-CF/ToCV-CR on 1% agarose gel in 1X TBE. Commercial KIT: (1) Kopeng, (2) Ketep, (3) Bogor, (4) Pakem, (5) Malang; SDT: (7) Kopeng, (8) Ketep, (9) Bogor, (10) Pakem, (11) Malang; and simple dsRNA: (13) Kopeng, (14) Ketep, (15) Bogor, (16) Pakem , (17) Malang; M: DNA marker 100 bp

Table 3. The homology level of CPd TICV nucleotide sequences in the test sample and several other TICV isolates obtained from the NCBI GenBank

No.	Isolate Identity	Homology (%)					
		1	2	3	4	5	6
1	TICV Ketep	ID					
2	AB085603.1 Tomato infectious chlorosis virus Japan	99.43	ID				
3	DQ355217.1 Tomato infectious chlorosis virus France	98.84	99.42	ID			
4	HG380082.1 Tomato infectious chlorosis virus Greece	98.99	99.57	99.86	ID		
5	FJ815441.1 Tomato infectious chlorosis virus USA	98.99	99.57	99.57	99.71	ID	
6	EU881362.1 Tomato infectious chlorosis virus Italy	98.85	99.43	99.71	99.86	99.86	ID

Remark: ID = Identical

Table 4. The homology level of the nucleotide sequence of the CP ToCV gene in the test sample and several other ToCV isolates obtained from the NCBI GenBank

No.	Isolate Identity	Homology (%)					
		1	2	3	4	5	6
1	ToCV Ketep	ID					
2	AY903448.1 Tomato chlorosis virus Florida USA	94.98	ID				
3	JN867337.1 Tomato chlorosis virus Tunisia	94.63	99.68	ID			
4	MF346383.1 Tomato chlorosis virus China	94.28	99.35	99.68	ID		
5	HG380090.1 Tomato chlorosis virus Greece	94.63	99.68	100.0	99.68	ID	
6	MG001347.1 Tomato chlorosis virus South Korea	94.28	99.35	99.68	99.35	99.68	ID

Remark: ID = Identical

Homology analysis using BioEdit showed that the TICV nucleotide sequences from Ketep with TICV from other countries have a similarity percentage of nucleotide bases of 98.84–99.43% (Table 3). Fauquet *et al*. (2005) stated that if a virus species has a nucleotide sequences similarity in the envelope protein gene more than 90% with other isolates in GenBank, then they are the same species. This finding showed that the TICV isolate from Ketep is the same species as those from Japan, France, Greece, USA, and Italy. Analysis of nucleotide sequences of ToCV envelope protein genes from Ketep with other countries has a high homology percentage of 94.28–94.98% (Table 4). Therefore, it can be concluded that the ToCV isolate infected tomato plantations in Ketep is the same species as the ToCV from the USA, Tunisia, China, Greece, and South Korea.

The Comparison of SDT, Simple dsRNA, and KIT Methods

The differences between the extraction methods of SDT, simple dsRNA simply and commercial kit are buffer composition, extraction time, complexity level, and cost (Table 5). Based on the chemical composition of the buffer used, the SDT uses the least buffer and does not use hazardous chemicals, such as phenol or chloroform as in the simple dsRNA. The dsRNA method requires several buffers prepared in advance and adjusted the pH. The grinding of samples for cell lysis is employed by adding liquid nitrogen and extract buffer according to Endarsih *et al.* (2017) by separating DNA from lipids using PCIAA and then adding 70% alcohol, and centrifugation. On the other hand, the extraction method using a commercial extraction buffer kit is available and ready for use in accordance with the default protocol of the kit manufacturer. The extraction method using a kit for the cell lysis stage is the samples are ground by adding lysis buffers. There are two types of lysis buffers, each containing guanidine thiocyanate, and guanidine hydrochloride to process cell lysis and to protect against RNase enzyme activity by denaturing the enzyme. A buffer containing guanidine thiocyanate is the preferred lysis buffer, yet this buffer can cause freezing of the sample depending on the number and type of secondary metabolites hence RNA extraction is difficult to be conducted, whereas for the extraction of plants containing many secondary metabolites is better to use a lysis buffer containing guanidine hydrochloride (Qiagen, 2012).

Table 5. Comparison of extraction methods of SDT, simple dsRNA and Commercial Kit based on buffer composition, extraction time, complexity level, and estimated cost of material for each sample

Criteria	Extraction Method		
	SDT	Simple dsRNA	Commercial Kit
Buffer Composition	Buffer PBST: NaCl KH_2PO_4 Na_2HPO_4 Tween 20 DEPC water RNase inhibitor	Buffer STE Polyvinylpyrrolidone PCIAA Ethanol absolute Ethanol 70% Cellulose Sodium acetate 3 M Nuclease free water	Lysis buffer Washing buffer 1 Washing buffer 2 Elution buffer
Extraction Time	30 minutes	1 hour	35 minutes
Complexity Level	Easy	Rather complicated	Rather complicated
Cost	Cheap	Cheaper	Expensive

Based on the complexity level to obtain RNA, the simple dsRNA requires the longest time (1 hour) to obtain pure viral RNA compared to SDT (30 minutes) and commercial kit (35 minutes). Those extraction time for the SDT and commercial kit are similar as reported by Diningsih *et al.* (2017). Therefore, based on the complexity level to extract the samples, the SDT is the easiest method compared to the other. The SDT requires several stages of extraction to get the total RNA, i.e. sample extraction, incubation, and tube washing repeatedly. The SDT using crude sap in small amounts, without centrifugation, and shorter incubation time to obtain RNA of virus target (Suehiro *et al*., 2005; Temaja *et al.,* 2012). Furthermore, the three methods are only carried out in one tube (Damayanti & Putra, 2012). Whereas in the simple dsRNA and commercial kit, the complexity level is rather complicated as reported by Diningsih *et al*. (2017) because it requires several steps before the RNA target is obtained (several steps of centrifugation and the transfer of sample extracts from one tube to others). This long extraction step may allow contamination present in the samples. Based on the extraction cost of the three methods used, the SDT and simple dsRNA are cheaper compared to the commercial kit (Diningsih *et al.* 2017; Endarsih *et al.* 2017).

CONCLUSION

The multiplex RT-PCR using two specific pairs of CPd I/CPd II and ToCV-CF/ToCV-CR is able to detect *Crinivirus* in tomato plants from Kopeng, Ketep, and Bogor with DNA sizes of 760 bp and 360 bp, respectively. Pakem and Malang samples are only infected by ToCV with a DNA size of 360 bp. Based on the results of DNA sequencing from Ketep showed positive infected with TICV and ToCV. The evaluation results of three RNA extraction methods showed that the preparation of RNA by SDT and simple dsRNA are better methods to detect TICV and ToCV than commercial KIT because they are simpler, faster, easier, and cheaper in providing templates for RT-PCR.

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