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Research Article

Double Infection of *Onion yellow dwarf virus* and *Shallot latent virus* in Garlic from Several Regions in Indonesia

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ABSTRACT

Viruses have been a problem on garlic cultivations in various countries. There are several viruses reported infecting garlic. Genera *Potyvirus* and *Carlavirus* are the most common viruses found infecting garlic. Mixed infection on garlic is often designated as a "garlic viral complex". These viruses can be transmitted through imported garlic seeds. Therefore, it is necessary to conduct early detection of garlic seeds to prevent the epidemic of these viruses. This study aimed to detect *Onion yellow dwarf virus* (OYDV) and *Shallot latent virus* (SLV) on garlic. Garlic samples were obtained from Enrekang, Magelang, Temanggung, Tawangmangu, and Yogyakarta. Total RNA was extracted from the samples and subsequently used for RT-PCR using two pairs of specific primers SLV-F/SLV-R and OYDV-F/OYDV-R. Primary pair SLV-F/SLV-R in amplicons sized 276 bp, while OYDV-F/OYDV-R in amplicons sized 112 bp. RT-PCR results showed that OYDV was found in all samples tested in this study. Meanwhile, double infections (OYDV and SLV) were found in eight out of ten samples tested. These results indicated that double infections on garlic were common in Indonesia.

Keywords: garlic; infection; OYDV; RT-PCR; SLV

INTRODUCTION

Garlic is an agamic crop, vegetatively propagated by bulbs over many centuries. Vegetative propagation has become the main mechanism of transmission of garlic viruses. This condition is believed to be linked to the vegetative propagation has resulted in it being infected by many virus species. Infection by many virus species is often designated as 'garlic viral complex' (Celli *et al*., 2016). Therefore, multiple infections are common and leading to result in yield losses and varietal degeneration (Nam *et al*., 2015; Pramesh & Baranwal, 2015). There are several viruses reported infecting garlic belong to genera *Potyvirus* and *Carlavirus*. Species on *Potyvirus* genus reported infecting garlic such as *Onion yellow dwarf virus* (OYDV), *Leek yellow stripe virus* (LYSV), and *Shallot yellow stripe virus* (SYSV) (Hu *et al*., 2015; Swari *et al*., 2015). Meanwhile, species belonging to the *Carlavirus* genus comprise *Garlic common latent virus* (GarCLV), *Garlic latent virus* (GLV), and *Shallot latent virus* (SLV) (Da Silva *et al*., 2019).

The viruses can induce both symptomatic and asymptomatic, indicating that latent infection is common. However, there is a constraint on virus detection based on visual symptoms only (Abraham *et al*., 2019). Therefore, detection methods with high accuracy are deployed, including serological-based and nucleotide-based detection. Serological detection such as DAS- ELISA has limitations in the production of specific antibodies against virus species from mixed infection (Pramesh *et al*., 2012). Nucleotide-based detection or molecular techniques such as Reverse Transcription - Polymerase Chain Reaction (RT-PCR) proved more highly specific, reliable and sensitive than ELISA for viral diagnostics (Mohammed *et al*., 2013; Jones *et al*., 2017).

In Indonesia, RT-PCR successfully detected the presence of OYDV, SYSV, GarCLV, and SLV

in shallot (Swari *et al.*, 2015; Harti *et al.*, 2020; Putri & Hidayat, 2020). Currently, there are not many studies using RT-PCR for detecting virus infection in garlic. Therefore, this study aims to detect OYDV and SLV infecting garlic through RT-PCR using two specific primer pairs. The RT-PCR followed by sequencing was applied to identify viruses infecting garlic in Indonesia accurately. The results of this study provide a method for rapid detection of viruses on garlic and help the government providing virus-free garlic seed bulbs.

MATERIALS AND METHODS

Plant Material Preparation

The leaves and bulbs of garlic were collected from several regions such as South Sulawesi (Enrekang), Central Java (Temanggung, Tawangmangu, Magelang), and Yogyakarta.

RNA Extraction

Total RNA was extracted from 0.1 gram leaves or bulbs of garlic. The extraction was carried out according to the protocol from the extraction kit manufacturer (RNesay Plant Mini Kit from Qiagen, Germany). The extraction RNA was used as a template for the synthesis of complementary DNA.

Synthesis of Complementary DNA

Total RNA extracted from the samples subsequently was used for cDNA synthesis. The reactions were performed using ReverTra Ace -α-, cDNA synthesis kit (Toyobo, Japan) with total volume of 10 µl, consisting of 3.5 RNase-free H_2O; 2 µl 5×RT buffer (contains 25 mM Mg^{2+}); 1 µl dNTPs mixture (10 mM); 0.5 µl primary oligo (dT) 20 (10 pmol/µl); 0.5 µl RNase inhibitor (10 U/µl); 0.5 µl Reverse transcriptase. This mixture was added with 2 µl RNA and homogenized with vortex. The RT-PCR used program of denaturation at 42°C for 20 minutes and heating 99°C for 5 minutes to activate the enzyme. The cDNA then used for the template.

PCR Amplification

The DNA amplification reaction of each virus was conducted by PCR method using MyTaqTM HS Red Mix (Bioline, USA) and several primer pairs (Table 1) that could amplify the virus. The PCR reaction with the total volume of 25 µl, consisted of 12.5 MyTaqTM HS Red Mix, 2 µl of each primer; 6.5 µl ddH_2O; and 2 µl cDNA templates. Meanwhile, duplex PCR reaction with a total volume of 25 µl, consisted of 12.5 MyTaqTM HS Red Mix, 1 µl of each primer (OYDV and SLV); 6.5 µl ddH_2O; and 2 µl cDNA templates. The PCR for *Potyvirus*, *Carlavirus*, and OYDV, SLV were amplified for 35 cycles: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 1 minute, annealing at temperature (50, 51, 52, 53, 54, 55, 56, 57, and 58°C) for 30 seconds, elongation at 72°C for 3 minutes, and the final elongation at 72°C for 10 minutes. The most optimum annealing temperature from all temperatures was 54°C. Consequently, PCR and duplex PCR used annealing temperature 54°C for 30 seconds in its PCR amplification. Subsequently, PCR products were electrophoresed in 1% agarose gel, then visualized by UV light. The PCR products were used in nucleotide sequencing.

Table 1. Primer sequences for *Potyvirus*, *Carlavirus*, OYDV, and SLV amplification

Primer	Sequence (5'-3')	Target	Product size	Reference
MJ1	ATGGTHTGGTGTGYATHGARAAYGG	*Potyvirus*	320 bp	Marie-Jeanne *et al.*, 2000
MJ2	TGCTGCKGCYTTCATYTG			
AlcarF	TGCTGCYTTTGATACYTTCGAT	*Carlavirus*	715 bp	Gambley, 2012 as cited in Swari *et al.*, 2015
Poty1	GGATCCCGGGTTTTTTTTTTTTTTTTV			Gibbs & Mackenzie, 1997
OYDV-F	CACTGATGCAGCTGAAGCATA	OYDV	112bp	This study
OYDV-R	CGAATGCGTAACGCGCCAAACTA			
SLV-F	CACAAGAAGTTAGCCCTTGCGAAG	SLV	276bp	This study
SLV-R	ATACTCTTAGCTCTACGAGCTCG			

Nucleotide Sequencing Analysis

Analysis of nucleotide sequences was conducted using the sequencer in the Research and Development Center laboratory of PT Genetics Science, Jakarta, Indonesia. Subsequently, the DNA sequence was analyzed by MEGA X and using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the target virus sequences with the virus nucleotide sequences from other countries registered in Genbank.

RESULTS AND DISCUSSION

Symptoms in Garlic Plants

Field observation was conducted to discover various symptoms of garlic leaves. The symptoms visual were varied from symptomless, mild, to severe yellow mosaic (Figure 1). The RT-PCR was carried out to assess whether the symptoms were caused by viral agents either OYDV or SLV. Interestingly, those viruses were also detected on symptomless garlic leaves. This might indicate the ability of the plant to tolerate the effects of virus infection or early infection (Jemal *et al*., 2015). Moreover, yellow mosaic symptoms in garlic plants were also reported elsewhere. Hu *et al*. (2015) reported the mixed infection of OYDV, SLV, and *Allexivirus* causing yellow mosaic symptoms in garlic plants was found in China. Meanwhile, in Brazil and Ethiopia, mixed infections of OYDV and *Allexivirus* have been reported causing yellow mosaic and yellow stripe symptoms on garlic leaves (Jemal *et al*., 2015; Araújo *et al*., 2018). Moreover, in West Nusa Tenggara (Indonesia), SLV and GCLV have reported causing yellow mosaic and yellow stripe symptoms in garlic plants (Pauzi *et al*., 2018). Although all garlic plants presented yellow mosaic symptoms, it was hard to conclude whether all viruses were associated with this symptom or because of the synergistic effect among the viruses. Therefore, in future research, a study for individual viruses in garlic plants was needed to fully understand the effects of each virus on disease symptoms (Takaichi *et al*., 2001; Da Silva *et al*., 2019).

Design and Test of Primers Suitable for RT-PCR Detection of Viruses in Garlic

In this study, all targeted fragments were successfully amplified for samples from Enrekang and Tawangmangu. Each primer pair was amplified to produced amplicon at size: 320 bp for *Potyvirus* using primer MJ1, MJ2, and 715 bp for *Carlavirus* using primer AlcarF and Poty1 (Figure 2). These universal primer pairs (MJ1, MJ2 and AlcarF, and Poty1) had used by Swari *et al*. (2015) and Laili & Damayanti (2019) for detecting *Potyvirus* and *Carlavirus*. In this study, two virus genera were found simultaneously infecting garlic plants from Enrekang and Tawangmangu. BLAST analysis of the nucleotide sequence showed that isolates from Enrekang and Tawangmangu had sequence similarity of 95.19%, 95.8% with OYDV from China, respectively, and 95.52%, 94.7% with SLV from Australia, respectively. Based on percent of homology these viruses were identified as OYDV

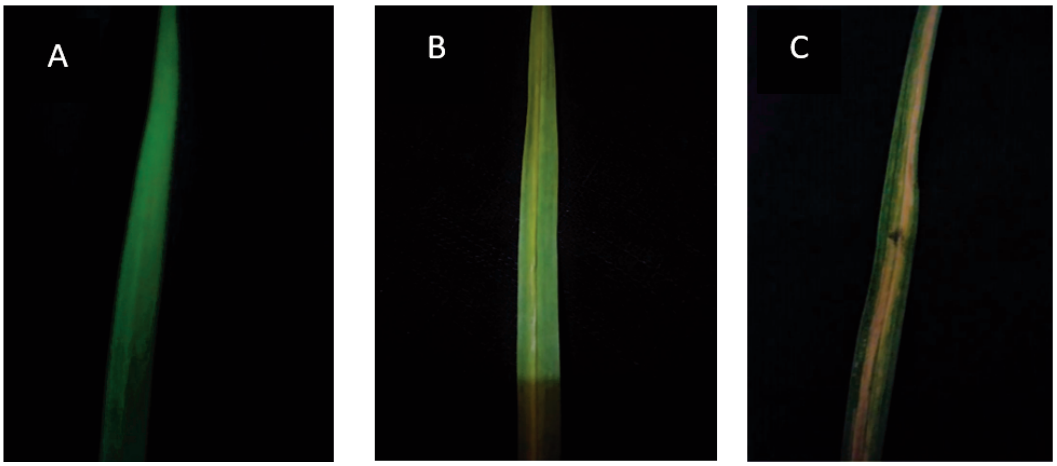

Figure 1. (A) Garlic leaves symptomless (asymptomatic), 34 days after sowing; (B) Garlic leaves with mild yellow mosaic symptoms, 34 days after sowing; (C) Garlic leaves with severe yellow mosaic symptoms, 60 days after sowing

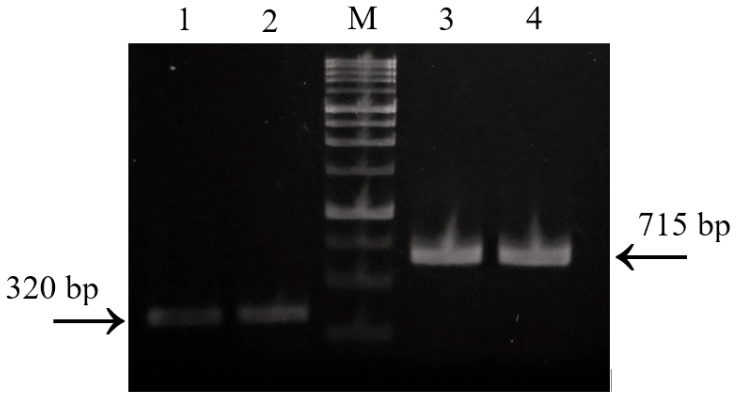

Figure 2. Analysis of PCR products obtained from *Potyvirus* (320 bp) and *Carlavirus* (715 bp) infected garlic leaves from two samples. Lane 1, 3: Enrekang, lane 2, 4: Tawangmangu, lane M: 1 kb DNA ladder

Table 2. RT-PCR detection of *Onion yellow dwarf virus* (OYDV) and *Shallot latent virus* (SLV) in bulbs and leaves of garlic from several regions in Indonesia

	Location	Sample	OYDV	SLV
1	Enrekang (farmer's field)	Leaf	+	+
2	Tawangmangu (farmer's field)	Leaf	+	+
3	Tawangmangu (farmer's field)	Bulb	+	-
4	Temanggung (farmer's field)	Leaf	+	+
5	Temanggung (farmer's field)	Bulb	+	+
6	Magelang (farmer's field)	Leaf	+	-
7	Yogyakarta (traditional market, local garlic)	Bulb	+	+
8	Yogyakarta (traditional market, imported garlic)	Bulb	+	+
9	Yogyakarta (planted bulbs from Temanggung)	Leaf	+	+
10	Yogyakarta (planted bulbs from Tawangmangu)	Leaf	+	+

of *Potyvirus*, and SLV of *Carlavirus*. Based on two pairs of specific primers targeting OYDV and SLV were designed. The specific primer sequences and amplicon sizes were shown in Table 1. Samples from several regions (Table 2) were used to test the performance of the specific primers designed in this study. Previously all samples were tested using universal primers (data not shown) to ensure that specific primers designed in this study were reliable. All targeted fragments were successfully amplified using the specific primer. Each primer pair amplified an amplicon at size: 112 bp for OYDV and 276 bp for SLV (Figure 3).

Specific Primers for Duplex RT-PCR Detection of Viruses in Garlic

In many countries, duplex and multiplex RT-PCR using specific primers have been successfully used for simultaneous detection of garlic viruses. In India, duplex RT-PCR using specific primers had successfully detected OYDV and SLV (Majumder *et al.*, 2008). Moreover, multiplex RT-PCR using specific primers was successfully detected OYDV, LYSV, and SLV in China (Hu *et al.*, 2015). In this study, the RT-PCR duplex test of used samples were confirmed with OYDV and SLV infection. Only one sample was successfully amplified from the six samples used. Meanwhile, the other samples only amplified OYDV target, while the SLV target was not amplified (Figure 4). According to Majumder & Baranwal (2014) and Hu *et al.* (2015), excellent virus-specificity, compatible annealing temperature, and easy to identify amplicon fragment length in gel-electrophoresis are needed to achieve success in duplex or multiplex RT-PCR. This duplex RT-PCR result gave many possibilities include are because different virus concentrations in each sample or

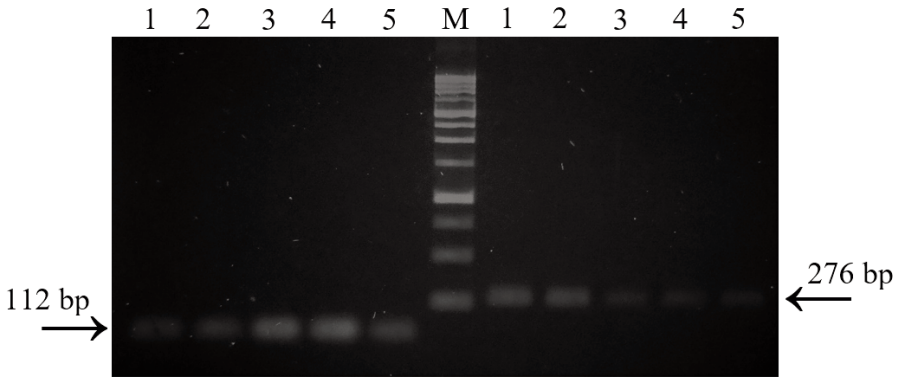

Figure 3. Analysis of PCR products obtained from OYDV (112 bp) and SLV (276 bp) infected garlic from different samples. Number (1) Enrekang, (2) Tawangmangu, (3) Temanggung, (4) traditional market (local garlic), (5) planted garlic bulb, (M) 1 kb DNA ladder

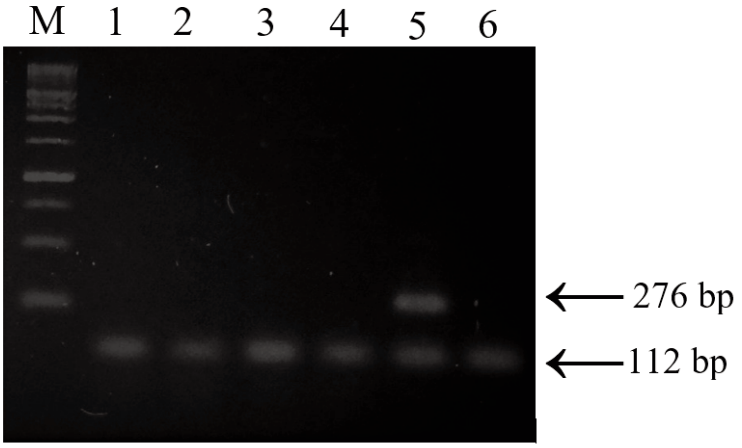

Figure 4. Analysis of PCR products obtained from OYDV (112 bp) and SLV (276 bp) infected garlic from different samples. Number (1) Enrekang, (2) Tawangmangu, (3) Temanggung, (4) traditional market (import garlic), (5) traditional market (local garlic), (6) planted garlic bulb, (M) 1 kb DNA ladder

annealing temperature have not been appropriated, even though when used for single PCR it is appropriate. The result of duplex RT-PCR was not stable, so further optimization was needed.

Identification OYDV and SLV from Indonesia

All garlic samples found were infected by OYDV. Meanwhile, double infection (OYDV and SLV) was found in eight out of ten samples (Table 2). Among these two viruses, OYDV was commonly found compared to SLV. Similar conditions were reported in Czech Republic (Leisova-Svobodova & Karlova-Smekalova, 2011), in Indian (Majumder & Baranwal, 2014), and Ethiopia (Abraham *et al*., 2019), that OYDV was commonly found if compared to other viruses. This indicates that OYDV was abundant in garlic fields in many countries. Mostly garlic farmers in many countries such as China, Ethiopia, and Indonesia, utilized garlic bulbs from seeds that have been previously infected with the viruses from previous season as seeds. Consequently, the virus infection rate in the garlic plants increased (Hu *et al*., 2015; Jemal *et al*., 2015). The presence of OYDV and SLV in most garlic plants in this study indicates that those viruses were spread mainly by vegetative propagation.

It was found that garlic viruses distributed from different geographical regions in Indonesia. Such as garlic plants in Bandung and West Nusa Tenggara have been infecting by GCLV and SLV (Kadwati & Hidayat, 2015; Pauzi *et al*., 2018). Garlic plants in

Magelang have been infecting by OYDV. Meanwhile, garlic plants in Enrekang, Temanggung, and Tawangmanggu have been infecting by OYDV and SLV. Due to some garlic production regions have been infecting by various viruses, the prevention of the spreading of those viruses into a new location becomes very important. Hence, the knowledge of the distribution of viruses should be taken into account during the movement trading garlic seed bulbs (Wylie *et al.*, 2014; Bereda *et al.*, 2017). China was the biggest garlic producer in the world and has been exported garlic to various countries including Indonesia (Amanda *et al.*, 2016). If it is traced furthermore, Indonesia has imported garlic for many years from China and isolates OYDV Indonesia has a 95% similar identity with isolate OYDV from China. Therefore, China is probably the source of the OYDV in Indonesia.

CONCLUSION

RT-PCR using specific primers was developed and it successfully detected OYDV and SLV from some regions in Indonesia. Double infection (OYDV and SLV) was commonly found in several regions in Indonesia. This procedure will be useful for the rapid detection of garlic viruses for quarantine applications.

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