

COMPARISON OF REAL-TIME PCR, CONVENTIONAL PCR AND RT-LAMP FOR THE DETECTION OF COCONUT CADANG-CADANG VIROID VARIANT IN OIL PALM

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ABSTRACT

Real-time quantitative polymerase chain reaction (real-time PCR) was designed for the detection of oil palm Coconut Cadang-cadang Viroid (CCCVd) 246 nt variant. The primers and probe specifically designed for the real-time PCR were optimised with 300 nM of probe against a primer concentration of 400 nM for the detection of oil palm CCCVd variants. Oil palm CCCVd 246 nt variant was successfully detected using real-time PCR in leaf samples collected from 14 symptomatic oil palm from various regions. The sensitivity of real-time PCR was compared with reverse transcription loop-mediated isothermal amplification (RT-LAMP) and conventional PCR. The latter two techniques were reported earlier to be able to detect CCCVd variants in oil palm. Conventional PCR analysis using CCCVd full-length primers detected the presence of CCCVd 246 nt variant in 10 while RT-LAMP detected only seven positive samples from the total 14 field samples. The real-time PCR was highly sensitive and reliable compared to the two detection techniques. The primers designed for the real-time PCR were specific as only the oil palm CCCVd variant plasmid was detected with high fluorescence and lowest quantification cycle (C_q value) compared to the other tested viroids (ASSVd, CTiVd, ELVd, HLVd and PLMVd). The present study has proven the reliability of the technique, thus, highly recommending the next step of developing the high-throughput diagnostic procedure for the eventual use in epidemiological monitoring programmes.

Keywords: CCCVd detection, conventional PCR, oil palm, real-time PCR, RT-LAMP.

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INTRODUCTION

Coconut Cadang-cadang Viroid (CCCVd) is a single-stranded, circular, and non-coding ribonucleic acid

(RNA) molecule with a small genome (246-297 nt), causing cadang-cadang disease in coconut. More than 40 million coconut palms have been affected and destroyed due to the viroid in the Philippines (Randles and Rodriguez, 2003). Although initial studies on the viroid were reported in coconut palms, the occurrence of its variants has also been reported in African oil palm (*Elaeis guineensis* Jacq.) as it is associated with orange spotting (OS) disorder in commercial oil palm plantations (Vadamalai *et al.*, 2006; Wu *et al.*, 2013).

Due to the devastation experienced by the coconut industry in the Philippines, there is an immediate need to monitor the presence of the variant in oil palm as part of the biosecurity

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mitigation approach. Hence, it is absolutely crucial to develop a reliable detection tool to detect the variant in oil palm to avoid and mitigate its impact on the oil palm industry. The development of a quick, reliable and sensitive method to detect CCCVd variants would be the ideal solution. However, the viroid lacks of protein coding (Lee and Koonin, 2022), consequently rendering any detection tools using serological techniques unfeasible. Nevertheless, detection via molecular technique would be the most practical approach in developing the viroid detection tool in oil palm. Additionally, the technique requires high sensitivity to detect the viroid in lower titre within the tested samples. A wide range of diagnostic techniques have been employed for the identification of orange spotting CCCVd (OS-CCCVd) in oil palm: Polyacrylamide gel electrophoresis (PAGE) (Randles *et al.*, 1980); northern blot hybridisation, two dimensional polyacrylamide gel electrophoresis (2D-PAGE), conventional polymerase chain reaction (PCR) and ribonucleic protection assay (RPA) (Vadamalai *et al.*, 2006) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Madiah *et al.*, 2020; Thanarajoo, 2014). The techniques mentioned above for detecting OS-CCCVd variants are mainly to detect the presence of the viroid in the palm through the application of radioactive label and colourimetric changes or turbidity. Apart from the safety hazards due to the use of radioactive labelling, these methods increase the risk of contamination because the dyes are added during the post RT-LAMP reaction, and the changes in hydroxy naphthol blue colour can be perceived variably by different observers (Scott *et al.*, 2020; Wastling *et al.*, 2010). Meanwhile, a weak hybridisation signal through 2D-PAGE and northern blot has been reported by Vadamalai (2005), showing that the CCCVd-like RNA in the oil palm samples was present at low concentration. Although the viroid concentration in oil palm is low, this weakness can potentially be solved by using a real-time PCR as reported by previous studies on other viroids (Botermans *et al.*, 2020; Leichtfried *et al.*, 2021; Seigner *et al.*, 2020; Tsushima and Fuji, 2022).

In this study, the hydrolysis probe PCR or often known as TaqMan probe has been adopted whereby two fluorescent dyes are added at the 5' end as reporter and 3' end as quencher of the hydrolysis probe. Together with the new specific primer target, the probe specifically binds to the target and the Taq polymerase extends the primer until the probe cleaves at the 3' reporter to eventually emit the fluorescence. Recently, real-time PCR has been used successfully to detect some viroids, such as *Potato spindle tuber viroid* (PSTVd), *Citrus exocortis viroid* (CeVd) and *Grapevine latent viroid* (GLVd) (Ghobakhloo *et al.*, 2019; Osman *et al.*, 2017; Tsushima and Fuji, 2022; Zhang *et al.*, 2018). For this study, dual-labelled probe

hydrolysis was selected due to the ease of designing this probe and their typically higher sensitivity in detecting and quantifying low concentration of viroid in oil palm. Although previous studies have demonstrated better amplification efficiency of RT-LAMP as compared to PCR, there is yet a comprehensive report to evaluate the sensitivity of CCCVd detection among RT-LAMP, conventional PCR, and real-time PCR. Therefore, the aim of this study was to compare the currently developed real-time PCR with two other available detection methods and concurrently determine the performance of detection. The two earlier reported techniques namely RT-LAMP (Madiah *et al.*, 2020; Thanarajoo, 2014) and conventional PCR (Vadamalai *et al.*, 2006) were compared with real-time PCR technique. Therefore, we reported the development of a detection tool using real-time PCR by incorporating hydrolysis Taqman probe to gauge the sensitivity of CCCVd detection in oil palm leaves. To the best of our knowledge, this is the first report of using TaqMan probe based on real-time PCR to detect the OS-CCCVd in commercial oil palm plantations.

MATERIALS AND METHODS

Field Samples

Leaf samples with OS from 14 oil palms of *Dura x Pisifera* (DxP) based on the OS symptoms were collected from local oil palm plantations in different states of Malaysia, including Sabah and Sarawak (Table 1). The samples were collected from symptomatic palms with isolated cases (Figure 1a) in the field that expressed typical OS with irregular shapes (Figure 1b) as described by Sundram *et al.* (2017). The leaves were harvested from frond 20 (middle portion of the crown), wrapped in paper and transported to the lab within 24 hr. Approximately 500 g of leaflets from the sampled frond 20 were cut to 30 cm in length and rinsed with tap water before being soaked in 5% bleach (sodium hypochlorite) in distilled water for 2 min. The leaflets were wiped with tissue paper and sealed in plastic bags before being kept at -80°C. Viroid-related samples from other countries could not be used in this study because of the quarantine regulations in Malaysia that strictly prohibit the entry of any invasive alien species.

Total Nucleic Acid Extraction, Purification by Polyacrylamide Gel Electrophorom (PAGE) and Complimentary Deoxyribonucleic Acid (cDNA) Synthesis

Natrium-EDTA-Tris-Mercaptoethanol extraction (NETME) was adopted from Thanarajoo (2014).



Figure 1. (a) Oil palm deemed as an isolated case in the field with typical symptoms of orange spotting (OS). (b) A closer view of the affected leaflets with OS symptoms.

TABLE 1. OIL PALM SAMPLES USED FOR CCCVd DETECTION WITH THE LOCATIONS AND THE YEAR OF PLANTING

Sample No.	Sample ID	Location	Year
1.	KLJ 3	Johor	2000
2.	KLJ 1	Johor	2000
3.	KLJ 5	Johor	2000
4.	KLJ 7	Johor	2000
5.	KLJ 6	Johor	2000
6.	KLJ 2	Johor	2000
7.	KLJ 4	Johor	2000
8.	S 1	Sarawak	2003
9.	S 2	Sarawak	2003
10.	DSR 1	Selangor	2003
11.	DSR 2	Selangor	2003
12.	AP 1	Perak	2008
13.	AP 2	Perak	2008
14.	AP 3	Perak	2008

Approximately 2.5 g of oil palm leaves were ground using liquid nitrogen and mixed with 8 mL of NETME buffer (2 M NaCl; 0.1 M NaOAc; 0.05 M Tris-HCl, pH 7.5; 0.05 M EDTA, pH 8 and 20% ethanol) in which 4 mL SDS and 20 μ L β -mercaptoethanol were added. The mixture was shaken at 151 rpm (Daihan, Japan) using an incubator shaker for 30 min at room temperature and then centrifuged at 11 000 rpm (Eppendorf, Germany) for 15 min. The supernatant was reextracted with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and Chloroform: Isoamyl alcohol (24:1). Isopropanol (1 volume) was added to the supernatant and incubated overnight at -20°C . The

mixture was centrifuged at 11 000 rpm (Eppendorf, Germany) for 15 min. The pellet was washed with 1 mL 70% ethanol and centrifuged at 11 000 rpm (Eppendorf, Germany) for 10 min. The resulting pellet was air dried, eluted in 30 μ L of distilled water and stored at -80°C .

Due to the low level of CCCVd in oil palm and the specific aim to separate and enrich the viroid circular RNA of 246 nt in size from all other linear RNAs of various host or viral origins, the nucleic acid was fractionated using 5% non-denaturing PAGE buffered with $1\times$ Tris-Borate EDTA (TBE) at 20 mA for 150 min (Vadamalai, 2005). Briefly, 20 μ L of nucleic acid sample was mixed with loading dye (1:1 ratio) (Ambion, USA) and the region of the gel where CCCVd RNA was expected to migrate was cut for elution. The excised gel was crushed until homogenous and soaked in 3 volumes of elution buffer (0.5 M ammonium acetate, 0.1% SDS and 1 mM EDTA pH 8.0) for 16 hr at 37°C with shaking at 121 rpm (Daihan, Japan) using incubator shaker. The eluted samples were centrifuged at 12 000 rpm (Eppendorf, Germany) for 2 min at room temperature. The supernatant was collected, mixed with 3 volumes of absolute ethanol and incubated at -20°C for 6 hr followed by centrifugation at 12 000 rpm (Eppendorf, Germany) for 20 min. The resulting pellet was air-dried for 30 min, dissolved in 20 μ L of distilled water and stored at -80°C until further use.

Complementary deoxyribonucleic acid (cDNA) was synthesised using GoScriptTM Reverse Transcription Systems kit (Promega, USA) according to the manufacturer's protocol. Briefly, 5 μ L RNA eluted from PAGE was added with 1 μ L of reverse primer of CCCVd Vir R, mixed, heated at 80°C for 12 min and then chilled in ice. Subsequently, 15 U of AMV reaction mix, 1 mM dNTP, $1\times$ RT-Buffer, 5 mM MgCl_2 and 20 U RNasin were mixed and incubated at 25°C for 5 min and 55°C for 30 min. The reaction was inactivated by incubation at 70°C for 15 min.

Primers and Probe Designing

In this study, the primer for conventional PCR was retrieved from Vadamalai *et al.* (2006) and RT-LAMP from Madihah *et al.* (2020). The sequences are listed in Table 2. The primers and probes in this study were designed from the sequences of CCCVd oil palm variants 270, 297, 293 and 246 nt (GenBank Accession: HQ608513, DQ097183, DQ097184 and DQ097183) retrieved from NCBI. A multiple sequence alignment was generated using MEGA X software (Kumar *et al.*, 2018). A single probe was designed using the sequences of the central conserved region (CCR) with partial coverage of the variable domain. The forward primer was designed from the pathogenic

TABLE 2. SEQUENCE AND POSITION OF THE PRIMERS AND PROBE

Name	Sequence	Position	References
Vir probe	CCTCAAGCGAATCTGGGAAGGGAGCGT	69-95	This study
Vir F	GCAGGAGAGGCCGCTTGA	35-52	This study
Vir R	GATCGTGCGCGTTGGAG	105-121	This study
GV4 F	ACTCACGCGGCTCTTACC	191-172	Vadamalai <i>et al.</i> (2006)
GV4 R	TGTATCCACCGGGTAGTCTC	192-209	Vadamalai <i>et al.</i> (2006)
CCCvdF3	PSQIRL-N-N-N-N-N-N-KPLQER	PI 2017702332	Madihah <i>et al.</i> (2020)
CCCvdB3	VRVGGDSP-N-N-N-N-N-N-SPK	PI 2017702332	Madihah <i>et al.</i> (2020)
CCCvdFIP	GKS-N-N-N- N-GT	PI 2017702332	Madihah <i>et al.</i> (2020)
CCCvdBIP	AA- N-N-N-N-N-N-VS	PI 2017702332	Madihah <i>et al.</i> (2020)

region (P) and CCR while the reverse primer was designed from the terminal right region. The probe and both primers were designed (Table 2) using Beacon Designer Software Bio-Rad by considering the melting temperatures, secondary structure, base composition and amplicon lengths. The specificity of the probe and primers for CCCVd was confirmed through a homology search. Highly purified salt-free primers and hydrolysis probes were obtained commercially from Integrated DNA Technologies (IDT).

Preparation of *in vitro* Oil Palm CCCVd 246 nt Variant Plasmid

Plasmid with CCCVd 246 nt cloned between *EcoRI* and *Sall* in pBlueScript II KS (+) vector with T7 promoter was used for the *in vitro* transcription. The plasmid was linearised by digestion using *KpnI* restriction enzyme during the incubation at 37°C for overnight and the *in vitro* transcription was performed using HiScribe™ T7 Quick High Yield RNA Synthesis kit (New England Biolabs, USA) according to the manufacturer's protocol. The DNA was removed with DNaseI (Promega, USA) and the RNA was recovered by extraction using a Phenol: Chloroform (1:1), followed by ethanol precipitation.

Testing Primer and Probe Combination

The primer and probes were tested using a fixed template (10^{-4}) cDNA prepared from *in vitro* oil palm CCCVd 246 nt variant plasmid. The cDNA was prepared using GoScript™ Reverse Transcription Systems kit (Promega, USA) according to the manufacturer's protocol. A combination of five concentrations (300 nM, 350 nM, 400 nM, 450 nM and 500 nM) of both forward and reverse primers with a constant probe concentration was determined. The combination showing the highest fluorescence was subsequently tested

at four different probe concentrations (50-300 nM). Real-time PCR was performed using CFX96 (Bio-Rad, USA), following thermal cycling conditions: 95°C for 1 min, initial denaturation at 9°C for 15 s followed by 40 cycles of 65°C for 60 s for annealing. The combination of primer and probe concentration that yielded the optimal assay performance was selected for further experiments.

Real-time PCR

The final optimised real-time PCR reaction mixture used contained 5 µL of 5× Luna® Universal Probe PCR Master Mix (New England Biolabs USA), 0.45 µL (0.45 mM) of Vir F primer and Vir R primer, 0.3 µL Vir Probe, and 1 µL template in final reaction volume of 10 µL. The following thermal cycling conditions were used: 95°C for 1 min, initial denaturation at 95°C for 15 s followed by 40 cycles of 65°C for 60 s for annealing. All tests were run on a CFX96 real-time thermocycler (Bio-Rad, USA) by two steps of PCR reaction in three replicates for each sample. Data were analysed with Bio-Rad CFX manager 2.0 software (Bio-Rad, USA), applying a default threshold (relative fluorescent units) for BHQ and FAM.

Standard Curve

Data collected were analysed using Bio-Rad CFX manager 2.0 software (Bio-Rad, USA) by applying a default threshold (relative fluorescent units) for BHQ and FAM. In this study, the cDNA prepared from *in vitro* oil palm CCCVd 246 nt variant transcript was serially diluted from 15.4 ng/µL; 1.54 ng/µL; 0.154 ng/µL; 0.0154 ng/µL and 0.00154 ng/µL. The result was analysed by plotting the log of the template concentration against the number of cycle/cycle threshold (Cq value) using the formula $E = [10^{(-1/\text{slope})} - 1] \times 100$ where E is the amplification efficiency. The reaction mixture for

the real-time PCR and the amplification conditions were as above.

Determination of Specificity

Specificity of primers and probe was verified using artificially synthesised plasmid of *Apple scar skin viroid* (ASSVd), *Coconut tinangaja viroid* (CTiVd), *Egg latent viroid* (ELVd), *Hop latent viroid* (HLVd) and *Potato Mosaic Latent Viroid* (PLMVd).

Sensitivity Comparison of Real-time PCR with Conventional PCR

Comparison between the sensitivity of real-time PCR and the conventional PCR was carried out using 10-fold dilution (10^{-1} to 10^{-6}) of *in vitro* oil palm CCCVd 246 nt variant transcripts as templates. Samples with cycle (C_q value) greater than 35 were ignored as these were unreliable on weak positive samples (Khan *et al.*, 2015).

Comparison of Detection Methods

Conventional PCR. The real-time PCR was compared with conventional PCR using full-length primer CCCVd 246 bp, GV4 primer. The synthesised cDNA was amplified by PCR using GoTaq® Green Master Mix (Promega, USA) containing 12.5 µL of 2× GoTaq® Green Buffer and 0.4 µM of GV4 forward and reverse primers (Table 2), nuclease-free water and approximately 5 µL of cDNA was added to a final volume of 25 µL reaction volume. The conventional PCR reaction described by Vadamalai (2005) was followed. The initial denaturation was set at 96°C for 3 min and amplification was done under the following conditions: 93°C for 45 s; 55°C for 45 s and 72°C for 3 min for 40 cycles; and 72°C for 15 min. All the PCR products were analysed on a 1% agarose gel in 1× TBE buffer and stained with ethidium bromide for UV visualisation. The PCR products were purified and sent for direct sequencing to confirm.

Loop-mediated Isothermal Amplification (RT-LAMP). RT-LAMP reaction was performed in an 8-strip tube in a 20 µL total reaction, each containing 12.5 µL Isothermal Master Mix (Optigene Ltd., United Kingdom) and 5 µL of cDNA. RT-LAMP primers for CCCVd specific amplification, as optimised by Madihah *et al.* (2020), were added to the reaction mixture at the following final concentrations: External F3 and B3 primers at 0.2 µM, inner FIP and BIP primers at 1 µM, respectively. The RT-LAMP reactions in the 8-strip tube were conducted at 65°C for 70 min and deactivation at 100°C for 5 min. The reactions were carried out using Genie® III (Optigene Ltd., United Kingdom).

RESULTS AND DISCUSSION

Optimisation of Real-time PCR using Hydrolysis Probe Dually Labelled Fluorescent

In order to develop a reliable real-time PCR, various technical parameters should be considered. The reaction and sample volume, primer and probe concentration and melting temperature are among the parameters that should be well designed and optimised (Bharucha *et al.*, 2018; Faggioli *et al.*, 2017). The best combination of concentration primers and probe was optimised based on the number of cycles (C_q value) of real-time PCR assays as described in Table 3. It was observed that for Vir F and Vir R

TABLE 3. QUANTIFICATION CYCLE (C_q) AMPLIFICATION PLOT WITH VARIOUS PRIMER AND PROBE CONCENTRATIONS

Optimisation of primer concentrations		
Primer/Probe	Concentration (nM)	Quantification cycle (C _q)
Vir F	300	
Vir R	300	18.60
Vir probe	300	
Vir F	350	
Vir R	350	18.42
Vir probe	300	
Vir F	400	
Vir R	400	19.13
Vir probe	300	
Vir F	450	
Vir R	450	18.08
Vir probe	300	
Vir F	500	
Vir R	500	37.21
Vir probe	300	
Optimisation of probe concentrations		
Vir F	450	
Vir R	450	21.81
Vir probe	50	
Vir F	450	
Vir R	450	19.52
Vir probe	100	
Vir F	450	
Vir R	450	18.53
Vir probe	200	
Vir F	450	
Vir R	450	17.87
Vir probe	300	

Note: The optimisation was done with constant primer concentration followed by constant probe concentration.

primers, the concentration of 300 nM showed the Cq value of 18.60, 350 nM showed the Cq value of 18.42, 400 nM showed the Cq value of 19.13, 450 nM showed the Cq value of 18.08 and 500 nM showed the Cq value of 37.21. Meanwhile, for the optimisation of Vir probe with constant 450 nM of Vir F and Vir R primers, the probe concentration of 50 nM, 100 nM, 200 nM and 300 nM showed the Cq value of 21.81, 19.52, 18.53 and 17.87, respectively.

Conventional PCR can be used to detect CCCVd 246 nt but it possesses several inherent drawbacks, such as primer dimer formation, low viroid titre in samples and the unequal distribution of CCCVd throughout the plant. These drawbacks have later paved the way for the emergence of several innovated PCR, such as real-time PCR. The usage of primers that yield long fragments of PCR product leads to possible risk of saturated levels of fluorescence or depletion of PCR reagents rapidly (Jansson and Hedman, 2019). The short viroid genome sequence limits the choice of probe and primers selection. Therefore, the probe and primers designed in this study yielded specific and efficient amplification of an 80 nt fragment. The primers and probe were suitable for real-time PCR, required shorter polymerisation time for replication and reduced the amplification of contaminating genomic DNA. A range of primer and probe concentration is essential for an optimal reaction as if it is too low, no fluorescent signal is observed. If it is too high, the result can lead to a high fluorescent background, which can mislead the detection and quantification (Kralik and Ricchi, 2017). Prior to performing a real-time PCR reaction, optimisation of primers and probe was done. In this study, primer concentration of 450 nM and probe concentration of 300 nM were observed with the lowest Cq value and high fluorescent signal, and therefore, was selected as the suitable conditions for the technique.

Preparation of Standard Curve

A standard curve was constructed using cDNA constructed from *in vitro* transcript with 450 nM of primer and 300 nM of probe to determine the PCR efficiency and linearity of template amplification. The standard curve showed slope close to -3.3 with strong linear relationship along with a correlation coefficient of 0.98 and amplification efficiencies at 100.2%, indicating maximum PCR efficiency (Figure 2a). The ability to detect low concentrations indicated the effectiveness of optimised probes and primers to develop precise and reliable markers in detecting CCCVd in oil palm.

Specificity and Sensitivity Assessment of Real-time PCR

The primers designed for this assay detected the oil palm CCCVd variant with high fluorescence

and lowest Cq value as compared to other tested viroids, such as ASSVd, CTiVd, ELVd, HLVd and PLMVd with Cq value of 36.61, 36.99, 37.05 and 36.64, respectively (Figure 2b). The specificity of other viroids of different genus and closely related viroids in the same genus revealed that the primer and probe design did not specifically match the sequences. It was only the CCCVd variants 246 nt plasmids that was detected by the real-time PCR while other viroids had low Cq values, suggesting the specificity of the real-time PCR assay developed to detect CCCVd variants in oil palm. In addition, compared to RT-LAMP's specificity, the real-time PCR was more specific since the reported RT-LAMP primers also detected the HLVd and ELVd apart from the CCCVd (Madiah *et al.*, 2020). This is another major drawback of the RT-LAMP technique which may contribute to false positive presence of CCCVd.

The real-time PCR sensitivity was assessed with a series of dilutions of cDNA from the *in vitro* transcript. The real-time assay was able to detect the lowest concentration of 10^{-5} of the *in vitro* oil palm CCCVd 246 nt variant transcript with Cq value of 34.02 (Figure 2c). Meanwhile, the evaluation of sensitivity using conventional PCR showed that it only detected with no multiple bands up to 10^{-4} of *in vitro* oil palm CCCVd 246 nt variant transcript (Figure 2d). However, the developed real-time PCR in this study had around 10-fold higher sensitivity as compared to the conventional PCR.

Application of Real-time PCR and Comparison with Conventional PCR and RT-LAMP

A total of 14 oil palm leaves with typical symptoms of OS were subjected to the testing using real-time PCR in comparison to conventional PCR and RT-LAMP for the detection of CCCVd. The real-time PCR successfully detected the presence of CCCVd in all 14 samples with Cq values ranging from 25.12 to 30.64 cycles, suggesting higher sensitivity (Table 4). On the contrary, the conventional PCR analysis showed detection in only 10 samples. All the positive samples produced an amplicon of 246 bp in size, but primer dimer formation was shown in Figure 3. Meanwhile, the RT-LAMP only managed to detect seven samples. Figure 4 illustrates the annealing curve which shows that the RT-LAMP primer is CCCVd specific.

To enhance the reliability of the assay, a complete control system was introduced including cDNA from *in vitro* transcript as positive control followed by healthy oil palm leaves, spiked in with cDNA from the *in vitro* transcript as reaction control and non-template control (NTC). Our observations suggested that the real-time PCR performed more efficiently in detection when compared to the conventional PCR and RT-

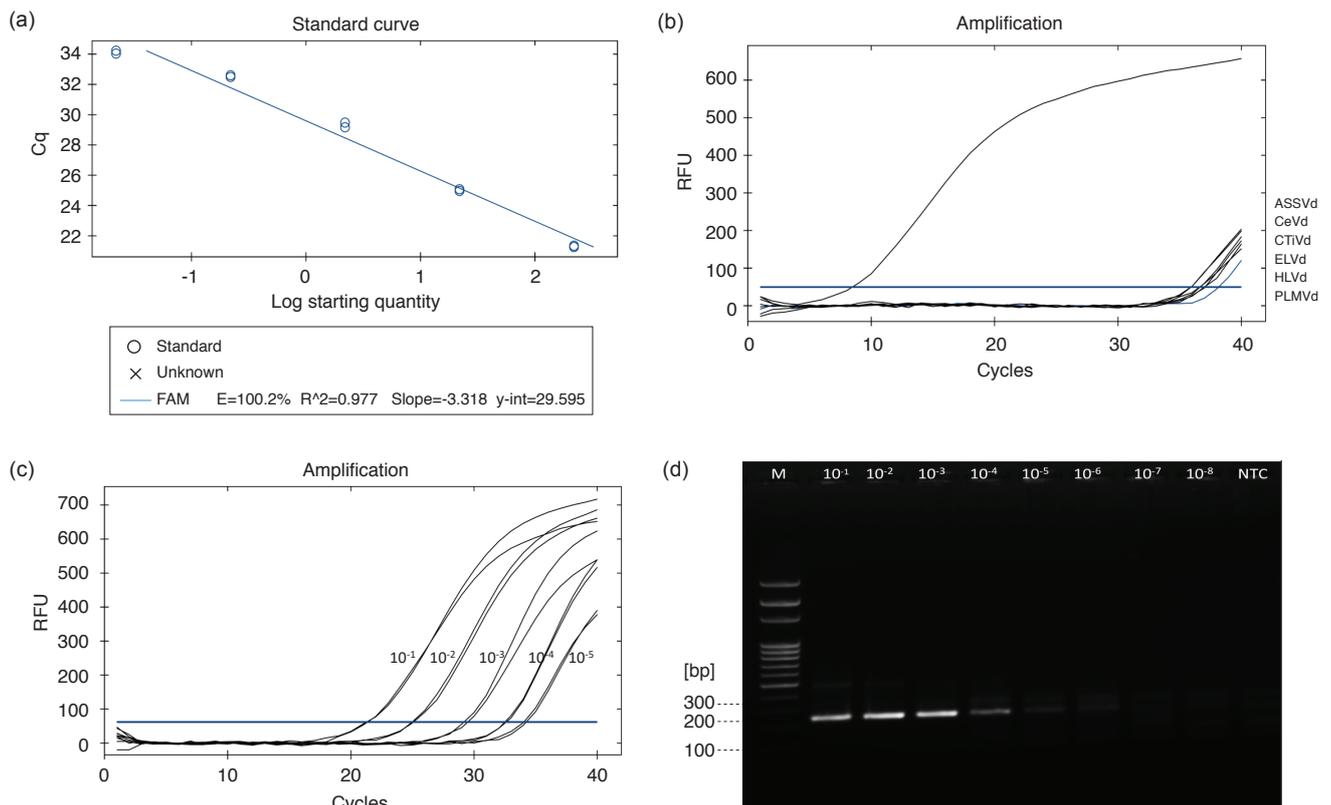


Figure 2. (a) Standard curve obtained from oil palm CCCVd 246 nt variant quantification for the efficiency of the real-time PCR. The horizontal axis represents a series of 10-fold dilutions of in vitro oil palm CCCVd 246 nt variant plasmid while the vertical axis represents the Cq value obtained from the reaction with three replications. The E-value represents the efficiency of the assay and the R² is used to evaluate the accuracy between replicates; (b) Evaluation of specificity of real-time PCR on ASSVd, CTiVd, ELVd, HLVd and PLMVd; (c) Evaluation of sensitivity of 10-fold dilutions of in vitro transcript of CCCVd using real-time PCR; (d) Evaluation of sensitivity of 10-fold dilutions of in vitro transcript of CCCVd using conventional PCR.

LAMP. In addition, the conventional PCR may be unsuitable in detecting the lower concentration of viroid in oil palm because the result showed that four samples of the 14 palms were observed with negative detection when subjected to conventional PCR, affecting the detection assessment. In some instances, multiple non-specific products in the conventional PCR were obtained, further reducing the reliability of the technique (Khan *et al.*, 2015). This highlights the practicality of real-time PCR in detecting CCCVd 246 nt. The optimised CCCVd real-time PCR was able to detect the viroid at a lower concentration. Its high sensitivity proves its potential as an ideal diagnostic tool for the use of epidemiological monitoring programmes.

The presence of CCCVd was detected with the amplification of conventional PCR using adjacent specific CCCVd primer GV4 and templates concentration optimised by Vadamalai (2005) while with the RT-LAMP using optimised RT-LAMP primers and templates concentration by Madihah *et al.* (2020) using Genie® III (Optigene Ltd). Based on the result, the conventional PCR amplified the CCCVd but it showed multiple bands which may affect the detection assessment. RT-LAMP only managed to detect seven out of 14 palms as positive with CCCVd presence (Table 4).

The inconsistency of detecting CCCVd circular form and high detection limits in the reaction suggests the possibility of the less sensitive RT-LAMP results in this study. Although RT-LAMP has been demonstrated to be practical for pathogen detection in the field, it may not be sensitive enough to detect low concentrations of the pathogen. This is demonstrated by a study comparing the detection of *Alternaria solani* and *Xylella fastidiosa* using RT-LAMP, conventional PCR, and real-time qPCR, showing that real-time qPCR was more sensitive than RT-LAMP (Harper *et al.*, 2010; Khan *et al.*, 2018), implying the necessity for comparative detection.

To the best of our knowledge, this study is the first validation report on comparison and evaluation of different PCR-based and RT-LAMP techniques using similar nucleic acid sources for the detection of CCCVd circular form, targeting variant 246 nt. Although RT-LAMP was reported as a convenient method for rapid pathogen detection, there is a high potential risk of false negative and false positive. As an example, RT-LAMP is prone to contamination due to the carryover of the LAMP product from one assay to another assay as the product increases three-fold every half cycle (Panno *et al.*, 2020). As a consequence, it is usually

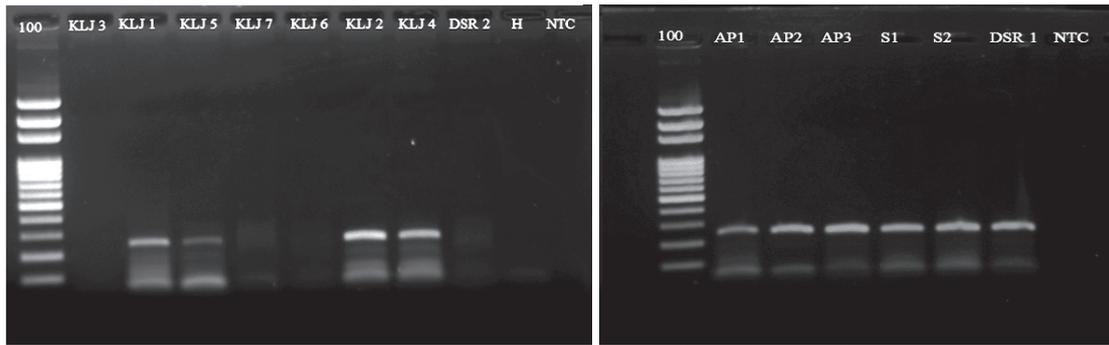


Figure 3. Detection of 14 oil palm samples from field by conventional PCR. The 100 bp ladder was labelled as 100 (Solis Biodyne, Estonia). The 246 bp amplicon corresponds to oil palm CCCVd 246 nt RNA.

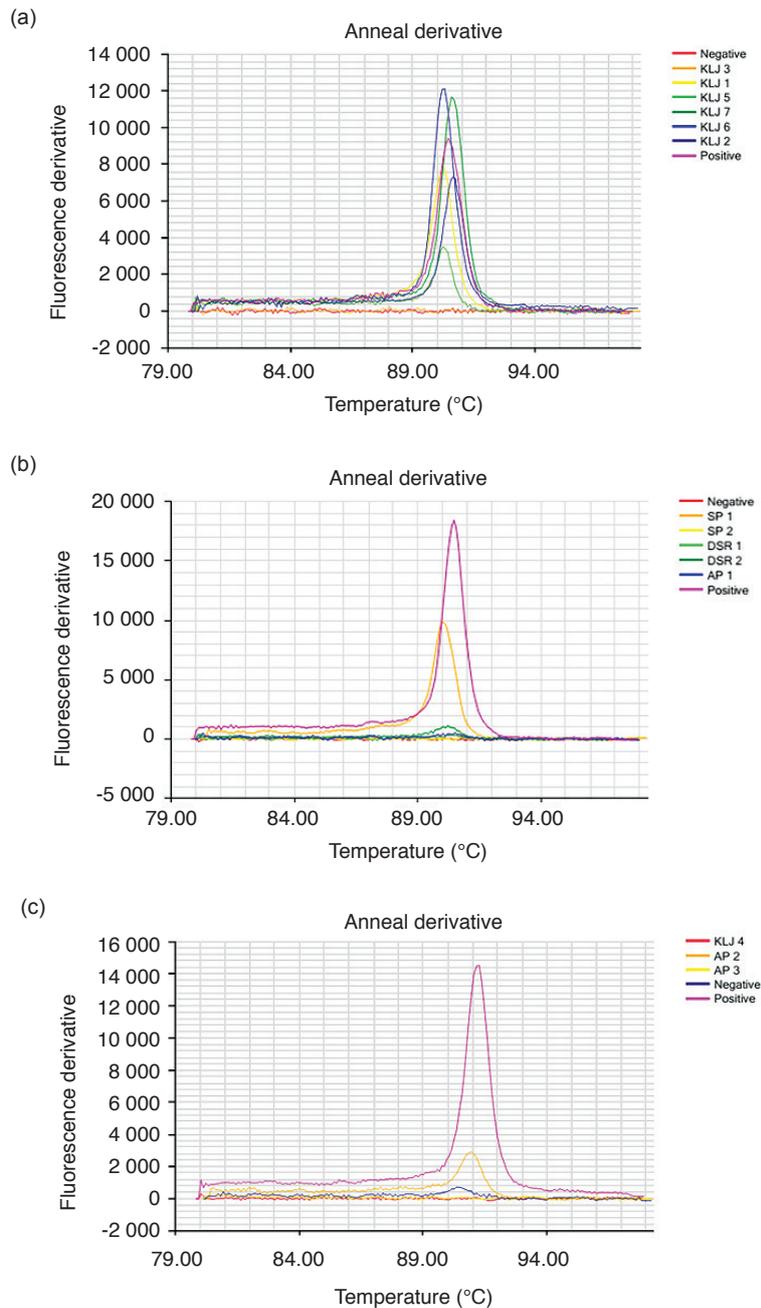


Figure 4. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) annealing derivative curve detected for oil palm from field with threshold value of 2000 after 65 min incubation time in 63°C. Any peak below the threshold was considered as negative, (a) KLJ 1, 2, 5, 6, 7 and 2 = positive, whereas KLJ 3 and KLJ 4 = negative; (b) only SP 2 = positive; (c) KLJ 4, and AP 3 = negative whereas AP 2 = positive.

TABLE 4. COMPARATIVE ANALYSIS OF REAL-TIME PCR ASSAY IN OS-CCCVd SAMPLES WITH RT-PCR AND RT-LAMP

Samples	Real-time PCR Mean Cq value		
Std-1	7.2 ± 0.65		
Std-2	10.8 ± 0.46		
Std-3	13.8 ± 0.03		
Std-4	17.3 ± 0.34		
Std-5	21.6 ± 0.79		
Healthy oil palm leaves	37.0 ± 0.19		
Healthy oil palm leaves + spiked in cDNA from <i>in vitro</i> transcript	15.1 ± 0.49		
cDNA from <i>in vitro</i> transcript	14.9 ± 0.48		
NTC	Underdetermined		
OS-CCCVd samples	Real-time PCR Mean Cq value	Conventional PCR	RT-LAMP
KLJ 3	+ (30.06 ± 0.31)	-	-
KLJ 1	+ (26.80 ± 0.34)	+	+
KLJ 5	+ (28.74 ± 0.48)	+	+
KLJ 7	+ (29.45 ± 0.80)	-	+
KLJ 6	+ (28.06 ± 2.89)	-	+
KLJ 2	+ (28.88 ± 1.28)	+	+
KLJ 4	+ (28.98 ± 0.73)	+	-
S 1	+ (28.98 ± 0.73)	+	+
S 2	+ (23.24 ± 2.02)	+	-
DSR 1	+ (25.83 ± 0.33)	+	-
DSR 2	+ (30.64 ± 0.49)	-	-
AP 1	+ (25.55 ± 1.45)	+	-
AP 2	+ (25.77 ± 1.39)	+	+
AP3	+ (23.12 ± 2.01)	+	-

Note: The assays included five points of dilution for standard curve, healthy oil palm leaves, healthy oil palm leaves spiked in with cDNA from *in vitro* oil palm CCCVd 246 nt variant and cDNA from *in vitro* oil palm CCCVd 246 nt variant for control reactions. NTC - non-template control; - - Negative; + - Positive; Cq - quantitative cycle.

recommended that at least two diagnostic methods are compared for viroid detection purposes (Murcia *et al.*, 2009).

A previous study employed RT-PCR with NETME extracted nucleic acids to detect CCCVd and recognised partial CCCVd sequence. This resulted in fewer positive samples with CCCVd presence (Roslan *et al.*, 2016). In addition, according to the International Committee on Taxonomy of Viruses (Owens R A *et al.*, 2012), the identification of viroids should be based on the analysis of their complete genome. However, complete sequences are still difficult or impossible to be obtained from samples with low viroid levels because traditional RT-PCR techniques lack the required sensitivity to generate full-length amplicons. Furthermore, conventional RT-PCR is generally more prone

to inhibition by matrix components (Botermans *et al.*, 2020). Therefore, to address the weakness in the detection methods reported earlier, the viroid was enriched by PAGE following the NETME extraction, allowing the complete length sequence/copies of CCCVd to be effectively amplified, and more samples to be evaluated. Using a full-length primer in RT-PCR detection allowed further sequence characterisation to identify sequence variants that may occur in field samples and accumulation analysis. This finding also addressed a gap in a previous study (Thanarajoo, 2014), which claimed that RT-LAMP could only identify the CCCVd variants in six oil palm samples out of 15 oil palm samples collected randomly from the field. However, the technique has yet to be verified for CCCVd detection sensitivity.

The ever-evolving real-time PCR may be the answer and future strategy in mitigating continuous spread of plant diseases by screening and culling contaminated materials. The early detection of CCCVd variants in oil palm allows planters to carefully select and breed only CCCVd-free materials. The detection using real-time PCR typically takes an hour as compared to using conventional PCR with GV4 primers which take approximately 3 hr. Meanwhile, in this study, the amplification of CCCVd 246 nt in the RT-LAMP was observed within 60-65 min, similar to studies by Thanarajoo *et al.* (2014) and Madihah *et al.* (2020). The runtime required to perform a real-time PCR is almost equal to RT-LAMP for maximum production of amplified product for CCCVd detection. The detection of other viroids using RT-LAMP was reported to be distinctly rapid where the turbidity was observed within 20-40 min for *Pepper chat fruit viroid* (PCFVd) (Boubourakas and Kyriakopoulou, 2022; Tangkanchanapas *et al.*, 2018). However, some observations could detect in 50 min for PSTVd (Verma *et al.*, 2020) and 60 min for *Peach latent mosaic viroid* (PLMVd) (Lee and Jeong, 2020). Hence, the duration depends on the viroid species and their genome sequence (Bhuvitarkorn *et al.*, 2019). Higher consideration in developing modern detection methodology is encouraged for reliable detection as it is critical for the quarantine and certification of oil palm planting materials. In Japan, real-time PCR detection was used for inspecting dahlia's seedlings to prevent the invasion of PSTVd at the border, leading to the quick eradication of the viroid (Tsushima and Fuji, 2022). Similar approach will be applicable in Malaysia especially in allowing exotic materials entering into the country as part of the biosecurity plan of the commodity crop.

CONCLUSION

In conclusion, an optimised and validated real-time PCR for CCCVd with higher sensitivity was successfully developed with the reaction specific to CCCVd. The real-time PCR was the only technique that managed to detect CCCVd presence in all 14 oil palm samples with a range of Cq value from 25.12 to 30.64 compared to RT-LAMP, which only detected seven samples while the RT-PCR detected 10 samples. The real-time PCR enables rapid detection with higher sensitivity. A complementary method such as the real-time PCR increases the confidence of detection level assessment by comparing the Cq value. In the past, epidemiology research on CCCVd variants in affected oil palms, particularly the OS, has been hampered by the lack of reliable detection of the viroid. Therefore, this improved real-time PCR technique provides a valuable tool to enhance the knowledge of the viroid in oil palms.

This is essential for future strategies involving the control and mitigation of any outbreaks in oil palm to ensure a sustainable crop production.

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