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Performance of Ready-To-Use Isothermal Detection Kits in Plum pox virus (PPV) Detection

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ABSTRACT

Plum pox virus (*Potyvirus plumpoxi*-PPV), one of the most significant diseases affecting stone fruit trees, has been extensively studied worldwide since its initial detection in 1917, with various diagnostic approaches explored. This study investigates the performance of colorimetric and real-time diagnostic kits developed by DOSA Information and Communication Technologies Ltd. for detecting PPV under isothermal conditions. These isothermal diagnostic kits, developed and optimized with domestic resources, stand out as highly practical diagnostic tools, leveraging an advantage of containing proprietary primer sets that enable direct testing of RNA from infected plants, independent of existing isothermal methods. In this study, the isothermal diagnostic kits enabled successful amplification of PPV at 65°C within 60 minutes, without cross-reaction with potential host genomes or other viruses that could co-infect. The colorimetric and real-time diagnostic kits demonstrated detection sensitivities of 10^{-4} and 10^{-5} ng/µL of PPV RNA, respectively, meeting the desired standards for PPV detection. These kits also facilitated effective amplification of PPV-M, D, and T strains, commonly found in Türkiye. Colorimetric and real-time isothermal diagnostic kits can be utilized as rapid and cost-effective tools in PPV screening programs. The application of these kits, especially in field settings and areas with limited laboratory infrastructure, should be encouraged. This successfully developed and optimized isothermal diagnostic platform could also be adapted for the detection of other significant plant viruses, offering a strategic advantage in managing viral diseases in agricultural regions such as Türkiye.

Key words: PPV, Sharka Disease, Isotermal, LAMP, Kit

Kullanıma Hazır Izotermal Tanı Kitlerinin Plum pox virus (PPV) Tanısındaki Performansları

ÖZET

Sert çekirdekli meyve ağaçlarını etkileyen en önemli hastalıklardan birine neden olan Plum pox virus (*Potyvirus plumpoxi*-PPV), ilk tespit edildiği 1917 yılından bu yana dünya çapında kapsamlı bir şekilde incelenmiş ve çeşitli tanı yaklaşımları araştırılmıştır. Bu çalışmada, DOSA Bilgi ve İletişim Teknolojileri Ltd. tarafından izotermal koşullar altında PPV'yi tespit etmek için geliştirilen kolorimetrik ve gerçek zamanlı tanı kitlerinin performansı araştırılmıştır. Yerli kaynaklarla geliştirilen ve optimize edilen bu izotermal tanı kitleri, mevcut izotermal yöntemlerden bağımsız olarak enfekte bitkilerden RNA'nın doğrudan test edilmesine olanak tanıyan tescilli primer setleri içerme avantajından yararlanarak son derece pratik tanı araçları olarak öne çıkmaktadır. Bu çalışmada, izotermal tanı kitleri, potansiyel konukçu genomları veya birlikte enfekte edebilecek diğer virüslerle çapraz reaksiyona girmeden 65°C'de 60 dakika içinde PPV'nin başarılı bir şekilde çoğaltılmasını sağlamıştır. Kolorimetrik ve gerçek zamanlı tanı kitleri, PPV tespiti için istenen standartları karşılayan, sırasıyla 10^{-4} ve 10^{-5} ng/µL PPV RNA tespit hassasiyetini göstermiştir. Bu kitler ayrıca Türkiye'de yaygın olarak bulunan PPV-M, D ve T ırklarının etkili bir şekilde amplifikasyonunu kolaylaştırmıştır. Kolorimetrik ve gerçek zamanlı izotermal tanı kitleri, PPV tarama programlarında hızlı ve uygun maliyetli araçlar olarak kullanılabilir. Bu kitlerin, özellikle saha koşullarında ve sınırlı laboratuvar altyapısına sahip alanlarda uygulanması teşvik edilmelidir. Başarıyla geliştirilen

ve optimize edilen bu izotermal tanı platformu, Türkiye gibi tarım bölgelerinde viral hastalıkların yönetiminde stratejik bir avantaj sunarak diğer önemli bitki virüslerinin tespiti için de uyarlanabilir.

Anahtar kelimeler: PPV, Şarka Hastalığı, İzotermal, LAMP, Kit

INTRODUCTION

PPV belongs to the genus *Potyvirus* within the family *Potyviridae*. The virus has a single-stranded, positive-sense RNA genome approximately 9.8–10 kb in size. The genome has a long poly(A) tail at its 3' end and a protein (VPg) attached to its 5' end. This polyprotein structure is processed into 10 protein products encoded by the virus genome: P3, CP, HCPro, NIa-VPg, P1, 6K1, NIaPro, 6K2, NIb, and CI proteins (Salvador et al. 2006, Koç 2010, Çelik 2020).

The symptoms of PPV, also known as Sharka, vary depending on the virus strain, environmental conditions, host species, and cultivar. The virus typically causes chlorotic streaks and spots on flowers, leaves, and fruits, especially in spring, and also affects seeds, as well as fruit peels (Cambra et al. 2006). PPV is the most significant viral pathogen affecting stone fruit trees, including peach, plum, sour cherry, cherry, and apricot. The virus causes substantial yield and quality losses, rendering the fruit unmarketable (Garcia et al. 2014). Infected plants have shown reductions of 21–34% in fruit density and 0.14–2.11% in sugar content, with increased acidity. A recent study estimated the economic cost of the disease over the 28-year period from 1995 to 2023 at €2.4 × 10^9 (Cambra et al. 2024). PPV causes visible symptoms, especially on leaves, flowers, and petals of peach, apricot, and plum varieties. Similarly, in almonds, PPV causes reduced fruit yield and quality, with deformed fruits and chlorotic leaves, although the symptoms may be rapidly masked (Koç 2010).

The primary reason for the widespread dissemination of the disease is the use of infected propagation materials to establish new orchards. The virus is transmitted by different aphid species, particularly *Myzus persicae*, through a non-persistent mechanism (Cambra et al. 2006).

PPV was first identified in Türkiye approximately fifty years ago on plum trees in Edirne (Sahtiyanci 1969, Kurçman 1973). Subsequent studies have detected the virus in various provinces across the country (Candresse et al. 2007, Akbaş et al. 2011, Serçe et al. 2011, Gürcan and Ceylan 2016, Morca et al. 2020; 2021; 2022, Coşkan et al. 2022). Among the 10 identified PPV strains, only 4 (PPV-D, -M, -Rec, and T) have been detected in Türkiye. Additionally, a unique strain named PPV-Mist has been discovered in Türkiye, specifically in Istanbul. Recent studies have shown that the PPV-D, -M, -Rec, and -T strains have been prevalent in Türkiye for a long time, exhibiting significant genetic diversity and thus being considered native to the region. Various studies indicate that Türkiye is the origin of these four strains, with PPV-T isolates being dominant within the country (Elibüyük 2003, Serçe et al. 2009, Gürcan and Ceylan 2016, Gürcan et al. 2019, Teber et al. 2019, Gürcan et al. 2020, Gürcan et al. 2020, Teber et al. 2023).

For PPV diagnosis, in addition to biological methods, serological methods, particularly the ELISA (Enzyme-Linked Immunosorbent Assay) test, were widely used in the early years. Using monoclonal antibodies has made differentiating and diagnosing variations among virus strains easier. Specifically, D and M strains can be identified using monoclonal antibodies against structural and non-structural PPV proteins.

The ease of application and cost-effectiveness of the ELISA test have made it one of the most preferred techniques for virus diagnosis. However, low virus concentration in plants and high levels of plant inhibitors can sometimes make detecting viral agents via ELISA challenging (Lopez Moya et al. 2000). Therefore, more sensitive molecular methods, such as molecular hybridization and PCR (Polymerase Chain Reaction), have been introduced as alternatives. These techniques enable the accurate diagnosis of agents even at very low concentrations (Koç 2010). In addition to serological tests, serological and molecular techniques, including ELISA and RT-PCR (Reverse Transcription-Polymerase Chain Reaction), have been employed together to detect PPV in various stone fruit species (cherry, peach, apricot, almond, plum, nectarine) exhibiting different symptoms across different regions of Türkiye. These diagnostic methods have been applied to validate one another (Serçe et al. 2009, Koç 2010, Gürcan and Ceylan 2016). Furthermore, in addition to the RT-PCR method, PPV's detection in quantitative analyses, particularly in aphids, has been successfully implemented. Using RT-PCR, SYBR Green, melting curve analysis has been identified as a simple and robust method for identifying and testing various pathogens (Varga and James 2006).

In addition to the methods mentioned above, which are still effectively used in PPV diagnosis today, the Loop-mediated isothermal amplification (LAMP) technique, which is the subject of this study, has been developed for the sensitive, rapid, and high amplification of low amounts of DNA. This technique utilizes a primer set specifically designed to recognize six different regions on the template genome, enabling the reaction (Notomi et al. 2000). A LAMP reaction can be performed using a simple water bath or heat block to maintain 60–65 °C. Amplification results can be determined by agarose gel electrophoresis, and the amplified DNA or

cDNA (Complementary Deoxyribonucleic Acid) can be validated through visual observation in the reaction tube, either with the naked eye or under UV light. Although originally developed for DNA amplification, the LAMP technique has been adapted for the amplification of viral RNA (Ribonucleic Acid) targets under the name RT-LAMP by incorporating a reverse transcriptase enzyme (Varveri et al. 1987, Laín et al. 1989, Candresse et al. 1994, Martin et al. 2000, Notomi et al. 2000, Varga and James 2006; Fernandez-Soto et al. 2014, Panno et al. 2020). It has also been applied to detect plant viruses. Using this method, Varga and James (2006) developed an RT-LAMP procedure for PPV detection, identifying five PPV isolates, including D, M, EA, C, and W strains. Additionally, Hadersdorfer et al. (2011) developed a modified Blue LAMP based on Varga and James' method, which was applied to PPV suspensions obtained from infected plant tissues. In Türkiye, a study by Çelik and Ertunç (2021) developed a classical RT-LAMP method for PPV-T, and findings were evaluated by adding components after the reaction.

In recent years, the adaptability of the LAMP method at non-variable temperatures and its non-requirement for advanced laboratory instrumentation have made it the subject of various studies aiming for potential commercial application. In this study, as supported by the relevant literature mentioned above, the diagnostic performance of locally developed diagnostic kits designed for the rapid colorimetric and real-time detection of PPV-D, PPV-M, and PPV-T strains, which are commonly found in Türkiye, in a single-tube format was investigated.

MATERIALS AND METHODS

Virus Source

The virus isolates used in this study consisted of peach plant parts infected with PPV-M, PPV-T, and PPV-D, stored at -80°C in the Virology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Bolu Abant İzzet Baysal University. The isolates and strain diversity were confirmed by Çelik (2020).

RNA Isolation and DNase treatment

A total of six infected peach leaves (two from each strain) were used in this study. Plant tissues from plums, peaches, apricots, and almonds, which are common hosts of PPV, were subjected to RNA isolation for cross-reaction and specificity tests.

To isolate RNA from plant tissues infected with all three PPV strains and from common hosts, the Nucleozol RNA Isolation Solution (Macherey-Nagel, Germany) was used. The DNase I treatment was performed using Thermo Fisher Scientific DNase I (Catalog Number: EN0525) in accordance with the manufacturer's instructions, with volumetric modifications. A final concentration of 2000 ng/µL total RNA was used in the process.

Kits Used for Isothermal Detection of PPV

This study used detection kits developed by DOSA Information and Science Technologies Biological Development & Research were used to diagnose PPV. These kits enable the colorimetric and real-time isothermal amplification of the three PPV strains prevalent in Türkiye, all within a single reaction tube. Unlike conventional counterparts, the diagnostic kit employed in this study contains specially designed primer sets targeting the coat protein region of PPV for accurate detection. The RT-LAMP test can be conducted directly using viral RNA, allowing results to be evaluated visually or in real time.

Evaluation of Isothermal Test Results

Three different methods were used to assess the results of the reaction. The first method involved observing the color change from pink to yellow in the reaction tubes, which indicates a positive result. This color change was evaluated with the naked eye to confirm the presence of the sample's target nucleic acids (PPV RNA). The second evaluation method utilized agarose gel electrophoresis, following the protocol by Gallitelli and Minafra (1994). This technique allows for the separation and visualization of amplified products based on their size, confirming the presence and specific amplification of PPV-related sequences in the sample. According to the working principle of the real-time kit, amplification curves' formation and development time were monitored at 65°C for 60 minutes, with readings taken every minute on a real-time PCR system (LightCycler® 480; Roche, Basel, Switzerland). This method provides quantitative data on the amplification process, allowing for precise real-time monitoring of the reaction dynamics.

Initial Reaction

According to the manufacturer's instructions, RNA from the PPV-D isolate, along with negative and positive controls provided by the kit, was subjected to colorimetric and real-time reactions at an incubation

temperature of 65°C for 60 minutes. The reaction outcome was evaluated through three methods: direct visual observation of color change, agarose gel electrophoresis, and real-time monitoring of the amplification saturation curve.

Specificity, Sensitivity, and Detection Performance of Isothermal Diagnostic Kits in Common Strains

To determine the specificity of LAMP primers, in addition to PPV-D RNA, the DNA of PPV's natural hosts, such as peach, plum, apricot, and almond, were tested for cross-reactivity. The PPV-D RNA, isolated from plant samples, was employed in sensitivity studies to determine the minimum detectable nucleic acid concentration.

Six different dilutions were made, starting from a 10 ng/µL concentration. These dilution series were used in both colorimetric and real-time reactions to investigate the sensitivity of the kits. The minimum amount of nucleic acid that both kits could amplify was approximately determined. The reaction outcomes were evaluated through both visual observation and real-time PCR monitoring.

Additionally, samples known to be infected with PPV-D, M, and T strains, selected from the isolated pool of the Department of Plant Protection, Faculty of Agriculture, Bolu Abant İzzet Baysal University, were subjected to detection performance reactions in duplicate.

RESULTS AND DISCUSSION

Results

For the initial reaction, 10 ng/µL of PPV-D RNA was used, and both the positive and negative controls provided by the kit were included in the colorimetric and real-time RT-LAMP reactions. At the end of the initial reaction, after approximately 55 minutes, a color change from pink to yellow was observed in the positive control provided by the kit. After 60 minutes, a similar color change was observed in the PPV-D RNA tube (Figure 1a). Additionally, the presence of a ladder-like band pattern, which confirmed positive amplification, was verified by agarose gel electrophoresis (Figure 1b). Furthermore, based on the initial reaction results, real-time RT-LAMP showed that saturation curves indicating positive amplification appeared at approximately the 20th minute in the positive control provided by the kit, and at the 22nd minute in the PPV-D infected sample (Figure 1c). These findings concluded that the colorimetric test kit enabled a color change within 60 minutes, while the real-time test kit allowed amplification to occur in the PPV-D infected sample after approximately 22 minutes.

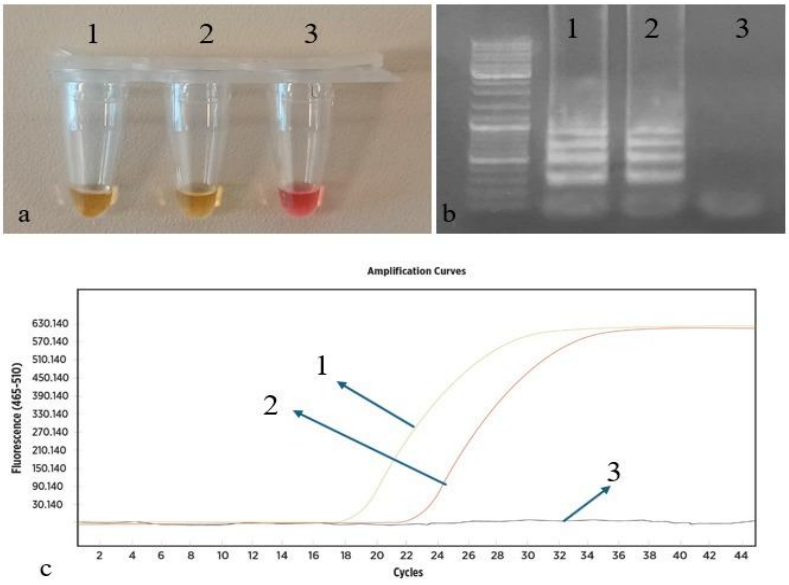

Figure 1. Initial LAMP reaction results. a. Visual evaluation for the colorimetric test after the initial reaction with 60 minutes of incubation at 65°C. b. Agarose gel electrophoresis image showing ladder-like band formation in positive samples. c. Real-time RT-LAMP results indicating amplification with saturation curves at 20 and 22 minutes (1; kit positive control, 2; PPV-D RNA, 3; kit negative control).

RT-LAMP Diagnostic Kit Specificity and Sensitivity Analysis Results

To test the specificity of the isothermal diagnostic kits used, in addition to the PPV-D infected sample, DNA from peach, plum, apricot, and almond, as well as PDV and PNRSV RNAs, were subjected to a 60-minute reaction at 65°C. At the end of the incubation period, no color change was observed in any tube except for the

one containing PPV-D RNA (Figure 2a). Additionally, the results were consistent with the real-time RT-LAMP analysis, where no saturation curves were observed for any RNA other than PPV-D (Figure 2b). Furthermore, no unwanted amplification or banding was detected (Figure 2c). Based on these findings, the developed test kits are highly specific for PPV amplification and do not exhibit cross-reactivity.

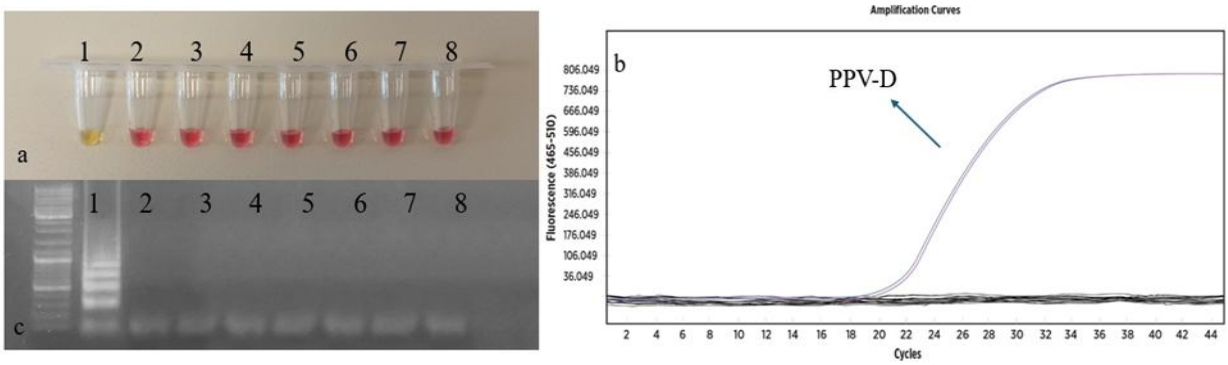

Figure 2. Specificity analysis of developed kits a. Color change observed in tube 1 containing PPV-D RNA after a 60-minute reaction, b. Saturation curve indicating amplification in PPV-D infected sample (tube 1) observed via real-time LAMP, c. Agarose gel electrophoresis images confirming amplifications (2; peach DNA, 3; plum DNA, 4; apricot DNA, 5; almond DNA, 6; PNRSV RNA, 7; PDV RNA, 8; kit negative control, 9; water control. Real-time RT-LAMP test was conducted in duplicate).

To determine the detection levels of the developed diagnostic kits, dilution series adjusted within the range of 10^{-6} to 10^{-4} ng/µL were reacted. The colorimetric reaction results showed that PPV RNA at a concentration of 10^{-4} ng/µL could be successfully amplified (Figure 3a), while in the real-time RT-LAMP test, amplification was detected in the sample with a concentration of 10^{-5} ng/µL (Figure 3b). However, as the RNA amount decreased, it was observed that the saturation curves in the real-time kit formed later. No unwanted amplification bands were observed (Figure 3c). Additionally, it was noted that as the RNA concentration decreased, the color change in the colorimetric reaction became harder to distinguish. Based on these findings, it can be concluded that the developed diagnostic kits can successfully amplify PPV RNA up to 10^{-4} and 10^{-5} ng/µL.

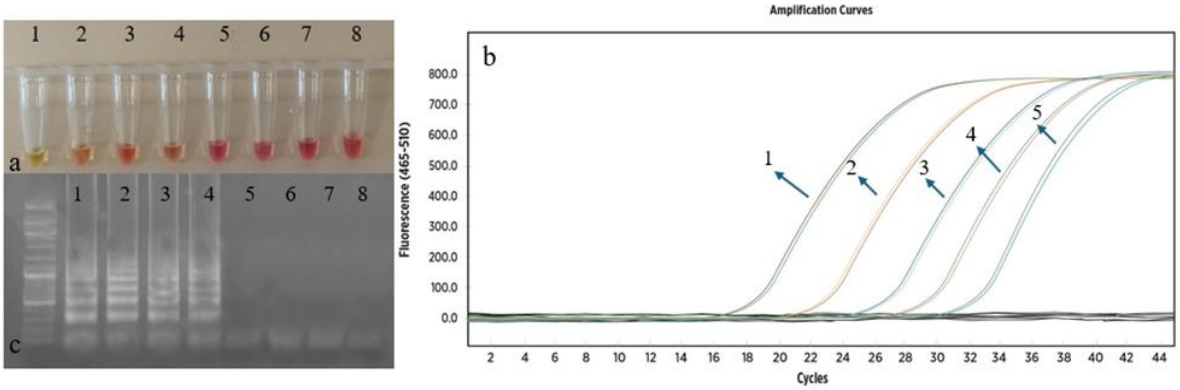

Figure 3. Sensitivity Levels of the Diagnostic Kits Used. a. Visual representation of amplifications in the colorimetric reaction. b. Saturation curves obtained with the real-time kit. c. Agarose gel images confirming amplifications (Dilution series from 10^{-6} to 10^{-4} were used in the experiments, with real-time RT-LAMP tests performed in duplicate for the samples).

Testing of Isothermal Diagnostic Kits on Samples Infected with Three PPV Strains

Three RNA samples, selected from the isolated pool in the Virology Laboratory of the Plant Protection Department at Bolu Abant İzzet Baysal University, which is known to be infected with PPV-D, M, and T strains, were tested in duplicate. According to the colorimetric RT-LAMP reaction results carried out at 65°C for 60 minutes, all three PPV strains were successfully amplified (Figure 4a). Additionally, real-time RT-LAMP analysis revealed that saturation curves for real-time amplification appeared after 22 minutes for each strain (Figure 4b). Gel images confirming the amplification were also obtained (Figure 4c). These findings conclude that the developed isothermal diagnostic kits successfully detect the widely found PPV-D, M, and T strains in Türkiye.

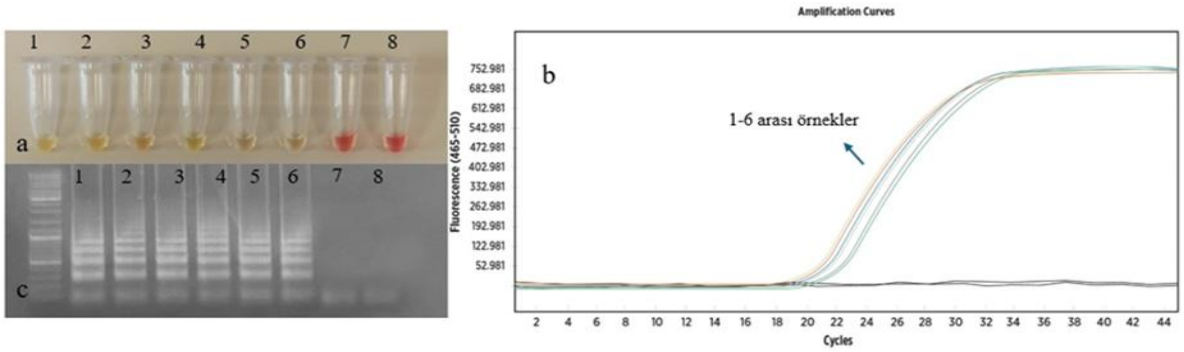

Figure 4. Detection of PPV-D (1 and 2), M (3 and 4), and T (5 and 6) strains using the developed isothermal diagnostic kit, a. Visual color change observed for the three strains in the colorimetric reaction, b. Saturation curves observed in the real-time LAMP test, c. Agarose gel electrophoresis images, 7. kit negative control, 8. water control.

Discussion

This paper addresses the detection of PPV, a significant viral issue in *Prunus* species, using isothermal diagnostic methods. The virus can infect stone fruit species such as apricot, peach, almond, plum, and cherry and is widely disseminated via infected propagation materials. Since its first detection in 1917, many studies have reported the virus spreading to different countries (Atanassov 1932, Levy et al. 2000, Thompson et al. 2001, Spiegel et al. 2004, Navratil et al. 2005, Zotto et al. 2006, Gürcan and Ceylan 2016). Despite over a century since its first detection, the spread of PPV continues to increase, and quarantine and eradication measures are still being applied globally and nationally (Akbaş et al. 2023). This situation has led PPV to maintain its position as a major stone fruit viral disease and remains the subject of numerous studies. The global and specific situation of PPV in Türkiye has prompted this research.

Various methods have been applied for PPV detection to date. Among these methods, biological indexing (Akbaş et al. 2011), ELISA, PCR, and sequencing techniques have been used (Gürcan and Ceylan 2016, Akbaş et al. 2023). Varga and James (2006) developed the RT-LAMP procedure for PPV detection and reported detecting five PPV isolates, including D, M, EA, C, and W, using the RT-LAMP method. Additionally, Hadersdorfer et al. (2011) modified the method developed by Varga and James with Blue LAMP and applied it to PPV suspensions from infected plant tissues. Furthermore, research on classical LAMP methods for PPV-T, a strain found in significant concentration in Türkiye has been conducted (Çelik and Ertunç 2021). The isothermal detection studies of PPV mentioned above emphasize the preparation of external primer sets, optimization, and the need for agarose gel electrophoresis, making these studies stand out. Some studies show that minimizing the components of a LAMP reaction and using single-tube assessments reduce the risk of false-positive results (Sarkes et al. 2020, Çelik and Morca 2021, Golabi et al. 2021, Lalli et al. 2021, Peltzer et al. 2021). This consideration makes simplified, ready-to-use, and practical LAMP reactions preferable.

In this study, contrary to the mentioned studies, two ready-to-use isothermal diagnostic kits were investigated for colorimetric and real-time detection of PPV performance. These kits offer a practical detection method by directly amplifying PPV RNA, thanks to special primer sets that differentiate them from other isothermal methods.

Various studies have explored the development and validation processes of ready-to-use diagnostic kits with different organisms (Cook et al. 2015, Kaarj et al. 2018, Moehling et al. 2021, Jawla et al. 2023). However, ready-to-use isothermal diagnostic kits in plant pathology are limited. We are aware of the existence of the AmplifyRP® Acceler8® isothermal real-time diagnostic kit (ACS 31505/0008) for PPV. However, as this product could not be obtained in the present study, a performance comparison could not be made.

Specificity studies are crucial for proving the reliability of all methods. The isothermal diagnostic kits used in this study successfully facilitated PPV amplification and showed no cross-reaction with the potential host genomes or other viruses that may share common infections. This indicates that the developed diagnostic kits are specific to PPV. Since its development by Notomi et al. (2000), the LAMP method has been used as a highly target-specific diagnostic tool. Various LAMP studies in plant virology have demonstrated that the reactions were aimed at the target organism and did not cause cross-reactive positive amplification (Çelik and Ertunç 2021, Çelik and Morca 2021, Çelik et al. 2024). The specific findings obtained in this study align with those of previous studies, supporting the intended development goals of the LAMP technique.

A diagnostic protocol is expected to have a high sensitivity level. In this study, the colorimetric and real-time diagnostic kits successfully detected PPV RNA at 10-4 and 10-5 ng/µL sensitivity levels, reaching the desired

thresholds. Compatible with our findings, several studies show that isothermal diagnostic methods offer higher sensitivity compared to alternative methods (Zhao et al. 2015, Suzuki et al. 2016, Iftikhar et al. 2020, Sarkes et al. 2020, Stehlíková et al. 2020). The findings in this study indicate that the isothermal diagnostic kits used here have very high detection sensitivity.

Besides possessing strong sensitivity and target specificity, a diagnostic protocol should also be comprehensive for the target organism. The isothermal diagnostic kits used in this study enabled successful amplification of PPV strains D, M, and T found in Türkiye. The findings are consistent with previous studies on PPV (Varga and James 2006, Hadersdorfer et al. 2011, Çelik and Ertunç 2021). This inclusivity is likely due to the selection of the virus's coat protein region as the target region for detection.

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Author Contributions

A.Ç. designed the study and supervised the experiments, S.Y.A and B.Ö contributed in methodology of experiments and supply of detection kits, A.F.M made contributions in validation, K.R. was responsible for conducting all experiments and writing original draft.

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