T.C.
NİĞDE ÖMER HALİSDEMİRUNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF PLANT PRODUCTION AND TECHNOLOGIES

MASTER THESIS

INVESTIGATION OF CHERRY VIRUS DISEASES IN NİĞDE

QURAT-UL-AIN SAJID

June 2018

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Supervisor

Assistant Professor Dr. EMİNUR ELÇİ

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Qurat-ul-Ain SAJID tarafından **Dr. Öğr. Üyesi Eminur ELÇİ** danışmanlığında hazırlanan **"Niğde İlinde Kiraz Virüs Hastalıklarının Araştırılması"** adlı bu çalışma jürimiz tarafından Niğde Ömer Halisdemir Üniversitesi Fen Bilimleri Enstitüsü Bitkisel Üretim ve Teknolojileri Ana Bilim Dalı'nda Yüksek Lisans tezi olarak kabul edilmiştir.

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THESIS CERTIFICATION

I certify that the thesis has been written by me and that, to the best of my knowledge and belief. All information presented as part of this thesis is scientific and in accordance with the academic rules. Any help I have received in preparing the thesis, and all sources used, have been acknowledged in the thesis.

Qurat-ul-Ain SAJID

ÖZET

NİĞDE İLİNDE KİRAZ VİRÜS HASTALIKLARININ ARAŞTIRILMASI

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Türkiye'nin en önemli sert çekirdekli meyve ağaçlarından biri olan kiraz (*Prunus avium* L.) *Rosaceae* ailesine aittir. Niğde ili 23.660 metrik tonluk kiraz üretimi ile önemli bir konuma sahiptir. Bu çalışmanın amacı, Niğde ili kiraz ağaçlarındaki olası virüslerin moleküler yöntemlerle araştırılmasıdır. Niğde'nin farklı bölgelerinden toplanan 90 örnek, *Little cherry virus 1* (LChV1), *Cherry necrotic rusty mottle virus* (CNRMV), *Apple mosaic virus* (ApMV), *Prune dwarf virus* (PDV), *Prune necrotic ring spot virus* (PNRSV), *Cherry green ring mottle virus* (CGRMV), *Cherry leaf roll virus* (CLRV), *Cherry mottle leaf virus* (CMLV), *Plum bark necrotic stem pitting associated virus* (PBNSPaV), *Cherry twisted leaf virus* (CTLV), *Apple stem grooving virus* (ASGV), *Little cherry virus 2* (LChV2), *Cherry rusty leaf virus* (CRLV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV)gibi farklı virüse spesifik primerler kullanılarak PCR aracılığıyla taranmıştır. PCR analizleri sonucunda LChV1 virüsü dışında herhangi bir enfeksiyona rastlanılmamıştır. LChV1' in genetik çeşitlilik analizi çalışmaları amacı için 4 farklı gen bölgesine özgü primerler kullanılmış ve tarama yapılmıştır. Fakat herhangi bir çoğalma gözlenememiştir. dsRNA analizleri sonucunda sadece bir örnekte şüpheli bir profil tespit edilmiş olup cDNA sentezi ve PCR analizleri sonucunda pozitif sonuç elde edilememiştir. Sonuçlarınteyidi amacı ile hassasiyeti daha yüksek olan TaqMan Real-Time PCR sistemi kurulmuş ve sadece bir örnekte LChV1'e karşı pozitif bir sonuç elde edilmiştir. Yapılan bu analizler sonucunda, taranan ağaçlarda düşük konsantrasyonda LChV1 tespit edilmiş olup genel itibari ile taranan diğer virüsler bakımından sağlıklı oldukları sonucuna varılmıştır.

*Anahtar Sözcükler:*Kiraz, virüs, RNA, cDNA, dsRNA, TaqMan RealTime PCR, Niğde.

SUMMARY

INVESTIGATION OF CHERRY VIRUS DISEASES IN NIĞDE

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Niğde Ömer Halisdemir University

Graduate School of Natural and Applied Sciences

Department of Plant Productions and Technologies

Supervisor : Assistant Professor Dr. Eminur ELÇİ

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Cherry (*Prunus avium L.*) is one of the important stone fruit crops in Turkey belongs to family*Rosaceae.*Niğde is one of the important provinces in Turkeywith the production of 23.660 metric tons' cherries. The objective of this study is to determine the sanitary status of cherry plants grown in Niğde province.For this purpose, 90cherry plant samples collected from different parts of Niğde were screened against different viruses using their specific primers as*Little cherry virus 1*(LChV1)*, Cherry necrotic rusty mottle virus*(CNRMV)*, Apple mosaic virus*(ApMV)*, Prune dwarf virus*(PDV*), Prune necrotic ring spot virus*(PNRSV*), Cherry green ring mottle virus*(CGRMV*), Cherry leaf roll virus*(CLRV*), Cherry mottle leaf virus*(CMLV*), Plum bark necrotic stem pitting associated virus*(PBNSPaV),*Cherry twisted leaf virus* (CTLV*), Apple stem grooving virus*(ASGV*), Little cherry virus 2* (LChV2), *Cherry rusty leaf virus* (CRLV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV). Based on the PCR analysis, no any amplification was observed, beside LChV1. To determine genetic diversity of LChV1 isolates, four different gene regions of LChV1 were screened and no any amplification was detected. dsRNA analysis revealed one suspicious profile and cDNA-PCR analysis using dsRNA as a template was also did not give any amplification. For the confirmation of those results, more sensitive TaqMan Real-Time PCR system was used and only one sample found to be positive to LChV1. It can be concluded that only low quantity of LChV1 infections were observed on some of the screened trees and none of them are infected by remained viruses which are used in this study.

*Keywords:*Cherry, virus, RNA, cDNA, dsRNA, TaqMan Real-Time PCR, Niğde.

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SYMBOLS AND ABBREVIATIONS

Symbols/Abbreviations	Descriptions
P.	Prunus
PDV	*Prune Dwarf virus*
ApMV	*Apple mosaic virus*
PNRSV	*Prunus necrotic ringspot Virus*
ACLSV	*Apple virus chlorotic leaf spot virus*
CGRMV	*Cherry green ring mottle virus*
CNRMV	*Cherry necrotic rusty mottle virus*
CLRV	*Cherry leafroll virus*
ASPV	*Apple stem pitting virus*
ASGV	*Apple stem grooving virus*
CMLV	*Cherry mottle leaf virus*
CTLV	*Cherry twisted leaf virus*
CRLV	*Cherry rasp leaf virus*
PBNSPaV	*Plum bark necrosis stem pitting associated virus*
LChV1	*Little cherry virus 1*
PCR	Polymerase chain reaction
ml	Micro liter
LChV2	*Little cherry virus 2*

CHAPTER I

INTRODUCTION

Stone fruits are important in several parts of the world. Cherry is one of the most important stone fruit majorly grown for its sour and juicy fruit. Cherry belongs to family*Rosaceae*one of the largest family with 3400 species also includes apple, peach, apricot, strawberry, and pears. Cherry is mainly divided into two types sweet cherry (*Prunus avium* L.) and sour cherry (*P. cerasus* L.*).*

The origin of both types are very old, originally it derived from Armenia or Black Sea region and Caspian Sea. Its name cherry derived from Cerasus, a Turkish city (Herbst, 2001). In Turkey more than about 100 varieties of sweet cherries can be found. Meanwhile growers have started to produce new cherry varieties like Sweet Heart, Celeste, Kordia, Regina and Sunburst. Cherry is basically tetraploid (2n = 4× = 32), Cross fertilized and by nature self-incompatible.The domestic variety named0900 Ziraat (famous as Turkish Napoleon) is the most known cherry type in Turkey because of its unique features according to need of growers.

Cherries for human consumption are imitative from two different varieties, the wild cherry (*P. avium* L.*)*, generally called sweet cherry most important and famous type of cherries and sour cherry *(P. cerasus* L.*)* which is main source of food industry (jam and juices), third one is black cherry (*P. serotina* L.*),* is source of wood for construction, and the Japanese cherry (*P. serrulata* L.*)* mainly grown as ornamental purposes because of its beautiful fragrances.

Sour and sweet cherry recorded as 13 million trees which produces about 17,000 tons fruits collectively. The share of Turkey in Cherry production is almost 12.83%, and in the sense of export Turkey is on second number with about 12.78% after USA (FAOSTAT, 2002a, FAOSTAT, 2002b). Turkey is the important cherry growing country and among the main three cherry exporters in the world. According to data calculated cherry production amounted about 2.29 million metric tons (Statistica, 2017).

In 2015/16 Turkey cherry production is 565.000 metric tons (FAOSTAT, 2016). The production rate was high in Konya and Manisa. Because of the frost in upcoming year it is forecasted that cherry yield may be decrease. Meanwhile Isparta province of Turkey plays a very important role in cherry production with about 6.17% of total Turkey's cherry production. District Uluborlu Isparta is one of the famous places for cherries growth.

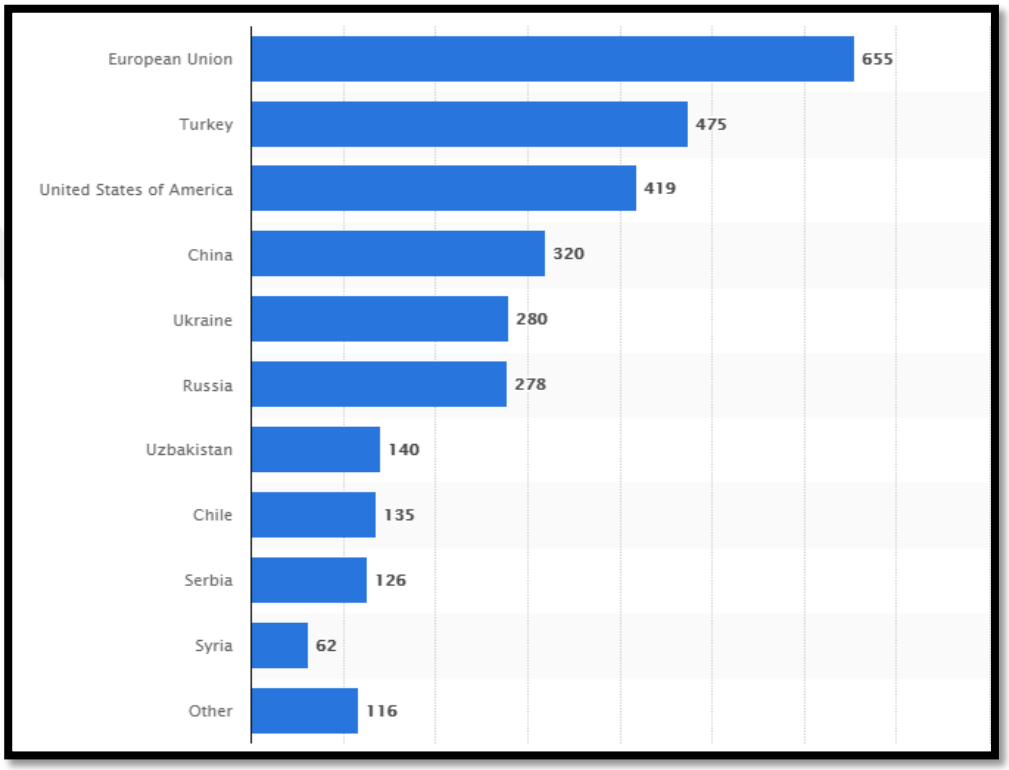


Figure 1.1. Global leading cherry producing countries in 2016/2017 (Statistica, 2017)

The major cherry producing areas in Turkey are Kemalpaşa (Izmir), Manisa, Akşehir, Ereğli, Hadim, Taşkent (Konya), Sultandağı (Afyon), Andırın (Kahramanmaraş), Ulukışla (Niğde).Niğdeis producing 23.660 tons of cherries while Ulukışla is producing about 19.913 tonnes of total Niğdeproductionaccording to 2017 (TUIK., 2017).

Cherries are known to be susceptible to a number of pests and viral diseases, and this enhances the risk of beneficial production. Cherries are infected by almost 30 viruses a approximately (Németh, 1986; Myrta and Savino, 2007). Although diverse disease epidemics are very general in cherry trees (Isogai et al.,2004; Bajet et al., 2008; Cevik et al.,2011).Well known viruses to infect cherries include: *Prune dwarf virus* (PDV), *Prunus necrotic ring spot virus* (PNRSV),*Apple chlorotic leafspot virus* (ACLSV),

Apple mosaic virus (ApMV), *Plum bark necrotic stem pitting associated virus* (PBNSPaV), *Cherry necrotic rusty mottle virus* (CNRMV), *Cherry green ring mottle virus* (CGRMV), *Cherry leaf roll virus* (CLRV), *Cherry raspleaf virus* (CRLV), *Little cherry virus 1* (LChV1*), Little cherry virus 2* (LChV2*), Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), *Cherry mottle leaf virus* (CMLV) and *Cherry twisted leaf virus* (CTLV)(Ghanem-Sabanadzovic et al., 2001; Myrta et al., 2003; Bouani et al., 2004).

Table 1.1. Viruses names, their families and genome sizes

Virus Names	Families	Genome Size	Genus
Apple mosaic virus	*Bromoviridae*	RNA1:3476 bp RNA2:2979 bp	*Ilarvirus*
Apple chlorotic leaf spot virus	*Flexiviridae*	7474-7561 bp	*Trichovirus*
Apple stem pitting virus	*Betaflexiviridae*	9265 bp	*Foveavirus*
Apple stem grooving virus	*Betaflexiviridae*	6495 bp	*Capillovirus*
Prune dwarf virus	*Bromoviridae*	RNA1:3324 bp RNA2:2593 bp RNA3:2129 bp	*Ilarvirus*
Prunus necrotic ring spot virus	*Bromoviridae*	RNA1:3332 bp RNA2:2591 bp	*Ilarvirus*
Cherry green ring mottle virus	*Betaflexiviridae*	8372 bp	*Foveavirus*
Cherry necrotic rusty mottle virus	*Betaflexiviridae*	8432 bp	*Foveavirus*
Cherry virus A	*Betaflexiviridae*	7434 bp	*Capillovirus*

Table 1.1. (Continue) Viruses names, their families and genome sizes

Virus Names	**Families**	**Genome Size**	**Genus**
Cherry mottle leaf virus	*Betaflexiviridae*	7987 bp	*Trichovirus*
Cherry twisted leaf virus	*Betaflexiviridae*	8431 bp	*Foveavirus*
Plum bark necrotic stem pitting associated virus	*Closteroviridae*	14213 bp	*Ampelovirus*
Little cherry virus 1	*Closteroviridae*	16933 bp	*Velarivirus*
Little cherry virus 2	*Closteroviridae*	15045 bp	*Velarivirus*

CNRMV and CGRMV belong to family *Betaflexiviridae*but these are unallocated member of this family. These are positive stranded single sense viruses(Adams et al., 2012). These two viruses are very much identical with each other in organization of genetic makeup (Gentit et al., 2001; Li et al., 2005; Wang et al., 2013).

Typically,symptoms ofCNRMV and CGRMV disease varies from development of brown, angular, necrotic spots, rusty chlorotic, and shot hole on the surface of leaves and cell darkening (Rott and Jelkmann, 2001). This is accounted as the serious disease of sweet and sour cherries early found in North America, Europe, and New Zealand (Wadley and Nyland, 1976). The vector for this virus is still unknown (Martelli, 1998).

Little cherry disease has two important members LChV1and LChV2,which belongs to family *Closteroviridae*.LChD is usually transmitted by apple mealybug which is an insect vector. This virus was first time identified in British Columbia, Canada in 1933 (Raine et al., 1986). This viral disease has also been reported in other different countries as New Zealand, Japan, United States, Poland and other European countries (Fry, 1970; Isogai et al., 2004; Uyemoto el al., 1992; Komorowska et al., 2004; Rott et al, 2001; Vitushkina, et al., 1997). LChV1 first time recorded in 2007 in Osmaniye Turkey (Serçe et al., 2011).

ACLSVwhich belongs to family *Betaflexiviridae*was firstly explained by Mink and Shay,(1999) in *Malus spp.* (Burnt et al., 1996; Carstens, 2010). First complete nucleotide sequences of ACLSV was isolated from Apple and followed by cherry (Jelkmann, 1996, German et al., 1997).This virus causes sharp green mottle, harsh leaf and fruit bend (Suticetal., 1999). ACLSV was reported in Turkey from fruit trees in Yalova. It was also detected in Malatya, Izmir, Eastern Mediterranean Regions (Elibüyük, 1998; Sipahioglu et al., 1999).

PNRSVbelongs to family *Bromoviridae* also a member of genus *Ilarvirus* having 3 single stranded RNA (Aparicio et al., 2010; Bujarski et al., 2012). Its transmission method is via pollen seed and vegetative propagation (Greber et al., 1991; Vaskova et al., 2000). This disease is great source of economic loss in different Prunus species as apple pear and cherries(Aparicio et al., 1999; Cui et al., 2012a; Myrta et al., 2001; Oliver et al., 2009; Uyemoto and Scott, 1992). This virus easily spread by pollen, seed and vegetative propagation materials (Greber et al., 1991; Vaskova et al., 2000). Its symptoms vary from none to severe mosaic on leaf upper surface (Crosslin and Mink, 1992; Howell and Mink, 1988). PNRSV is initially reported in China (Hou et al., 2002; Yu et al., 2013; Zhou et al., 1996). PNRSV firstly detected in Turkey by incidence of infection (Kurcman, 1977) in samples from Izmir. For Turkish isolate reverse transcription polymerase chain reaction (RT-PCR) was applied for detection (Ulubaşand Çağlayan, 2001; Myrta et al., 2001).

ApMV belongs to family *Bromoviridae*and genus *Ilarvirus*having 19 species (Anonymous, 2015). This virus infects a many woody and herby plants in about 65 species like apple, hazelnut, strawberry, apricot, cherry, almond, red currant, raspberry (Aramburu et al., 1998, Grimova et al., 2013, Lakshmi et al., 2011, Tzanetakis et al., 2007). Methods of transmission of this virus are mechanically and vegetative(Aramburu et al., 2000). Vector for this virus is still unknown (Postman et al., 1994). Its symptoms include Chlorosis, yellow signs, rings and mosaic.This virus is basically single stranded positive sense virus (Roosincket al., 2005). ApMV distributed almost wholeworld including Europe, Asia Wherever its host are available (Fulton, 1972). About 70% of ApMV infection was detected in Czech Republic. (Svoboda and Polák, 2010). In Romania and Albania, 6 %and 0.4 %viruses detected (Popescuet al., 2004;Myrtaet al.,

2004). In Turkey almost 8 %plants were found infected by this virus (Yardimci and Culal, 2009).

CTLVwas first reported in British Columbia, Canada, in 1943 by Lott (Lott, 1943). It causes adverse signs in some types of sweet cherries (Nemeth, 1986).It is transmitted by budding because it is linked with virus (Hansen et al., 1976; Lott, 1943; Nemeth, 1986).

PDVbelongs to the genus Ilarvirus, family*Bromoviridae*(King et al., 2012). PDV is distributed worldwide and infects many Prunus species, including almond, sour- and sweet cherry, peach, apricot, and plum trees (Németh, 1986).Studies showedthat PDV infection was found in different regions of Turkey (Çevik et al., 2011; Gümüş et al., 2007). It was also recorded in Aegean regions, (Dunez, 1986; Gümüş et al., 2007), Eastern Mediterranean (Çağlayan-Yıldızgördü and Hurugil, 1996; Sertkaya et al., 2004), WesternMediterranean (Çevik et al., 2011), Eastern Anatolia (Sipahioglu et al., 1999), Central Anatolia (Elibüyük, 2003).

Table 1.2. Symptoms of some important viruses

Viruses	Family	Symptoms	References
ApMV	*Bromoviridae*		Grimová et al., 2016
PDV	*Bromoviridae*		Pallas et al., 2012

Table 1.2. (**Continue**)Symptoms of some important viruses

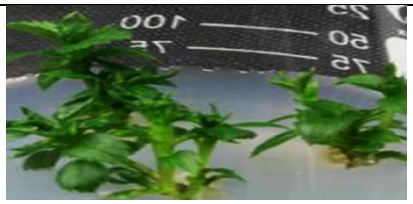
Viruses	Family	Symptoms	References
ASPV	*Betaflexiviridae*		Mathioudakis et al., 2007
CMLV	*Betaflexiviridae*		Hadidi et al., 2011
CTLV	*Betaflexiviridae*		Villamor et al., 2015
CRLV	*Comoviridae*		Villamor et al., 2014
LChV1 and 2	*Closteroviridae*		Eastwell et al., 2001
PBNSPaV	*Closteroviridae*		Uyemoto et al., 1995
ASGV	*Capilloviridae*		Chen et al., 2014
CGRMV	*Betaflexiviridae*		Eastwell et al., 2001

Table 1.2. (**Continue**)Symptoms of some important viruses

Viruses	Family	Symptoms	References
ACLSV	*Flexiviridae*		Smith et al., 1988
CNRMV	*Betaflexiviridae*		Villamor et al., 2014
CLRV	*Comoviridae*		Jalkanen et al., 2007

CVAbelongs to family *Betaflexiviridae* and genus *Capillovirus.* The structure of this virus is single stranded and positive sense RNA. This virus was initially reported in 1995 (Jelkmann W., 1995). Complete genome sequence of CVA was first recorded in 2016 (Koinuma et al, 2016)

PBNSPaVbelongs to family *Closteroviridae* and genus is *Ampelovirus*. It is first recorded in plum in USA (Marini et al., 2002). This virus is closely linked with bark necrosis. It shows severe symptoms as harsh pits on wood trunk of trees (Amenduni et al., 2005; Cui et al., 2011, Sipahioglu et al., 2011). PBNSPaV virus identifies in Turkey in stone fruits as cherry, prune and plum (Gumuşet al., 2007; Usta et al., 2007).

ASGVbelongs to family *Capillovirus.* This virus is important and cause severe disease of some cultivars and reduces the yield (Nemeth, 1986). It is also included in EPPO list of quarantine diseases (EPPO, 1999).

CRLV is the member of *Comoviridae*. It can cause severe yield loss in different plants like pome fruits (apple), stone fruits (cherry and peach) and potatoes (James et al., 2001; Thompson et al., 2004; James, 2011).

CMLV belongs to family *Betaflexiviridae.* It was initially recordedin Oregon in the 1920s and also spread in different parts of North America and Europe and South Africa (Nemeth, 1986).

Cherry plants are most likely to be infected by manyviruses and virus like organisms. These viral diseases cause a strong decrease of fruit sizewith mild fruit deformations. In very sensitive cultivars, however, infection of a tree often results in a total loss of its crop. Some of the viruses are very dangerous economically as they affect the yield of cherry fruits as Elche can reduce the size of fruit also fruit turned pale or some time colorless (Serçe et al., 2011).It can be easily transmitted by young trees, scions, woods. So, if there is need of cutting of tree all the remains should be discarded to avoid the spread of infection.Although occurrence of these infections were dramatically reduced by using of different certification programs and by maintaining some cultural practices

Virus specific primers are the tools used in polymerase chain reaction for specifically targeted a desired region. And provide accurate genetic information about that specific region of our desired virus genome. It can also be easily used in genetic analysis.Many researchers have been done for overcoming the viral disease infection of cherries to increase the yield in efficient way. However, in Turkey only PDV, ApMV, ACLSV, PNRSV, LChV1, PBNSPaV and CGRMV infections were observed in cherries. While in Niğde province is famous for cherry production,growers need to know about best methods to overcome the yield loss and for best production of cherries in Niğde. Till to now no any studies available for the incidence of virus in this province. Therefore, the current study was planned to screen cherry plants for virus incidence with molecular techniques in Niğde. The studies would be helpful for virus free cherry production.

CHAPTER II

LITERATURE REVIEW

Virus diseases of sweet and sour cherries are known to cause severe yield loss in production of cherries. It is widely disseminated by vectors, wind and insects. Most of these viruses are included in EPPO list of quarantine diseases as LChV1,CNRMV, CGRMV, ACLSV, ASGV, ASPV, ApMV, CLRV, PDV,LChV2and CRLV. All of the Virusesact as great threat in production of cherries in Turkey (EPPO, 2012). So, it has been causing disastrous effects yield and quality in several regions of the world and broad studies have been carried out on the management and development of overcoming theses serious problems. These studies have extensively been reviewed in this chapter. As the subject of this study is molecular detection of cherry viruses in Niğde, Turkey. Furthermore, the studies and literature are limited to molecular detection of viruses to possible extent. The emphasis is mostly reserved on the molecular screening of cherry viruses.

2.1 GlobalDistribution of Cherry Viruses

Katsianiet al.,(2018) worked on LChV1which are in one of the sweet cherry pathogens which have been reported in other *Prunus*species. TaqManprobe and Primers were prepared for conserved regions of the CP gene. Various divergent viral isolates were used for different detection range evaluation purpose. About 96.7% were estimated for the amplification efficiency of the method. While the detection limit was approximately 100 RNA copies. The protocol was used in the research of virus variation withinphloem tissue and leaves. Comparative analysis of mentioned method showedthat the higher analytical and diagnostic sensitivity of the new test making was reliable tool that couldbe used in monotonous testing and authorization programs.

Cieslinska et al., (2016) worked on ApMV. This causes damage in more 65 plants species of *Rosaceae* family along with hazelnut where viruses cause rings and mosaic symptoms on greeneries and also cause losses in nuts yield. About 14 plants of hazelnut were evaluated for the genetic and incidence diversity of ApMV in Poland. Using ELISA and reverse transcriptase-polymerase chain reaction 13 samples out of total 125

hazelnut samples tested positive results. Forthe amplification of the CP and movement protein MP genes along with the complete RNA-3 of the ApMV several primer pairs were used for RT-PCR. Genetic diversity was observed in a full length sequences of RNA-3 of the ApMV was isolated from hazelnut. The similarity between nucleotide sequence of MP and CP genes were isolated as 99.9 to 90.3 respectively. Phylogenetic analysis result showed that ApMV isolated from hazelnut grouped distinctly from the virus strains found in other plant species.

Cui et al., (2015) worked on PNRSV which is vital pathogen worldwide of stone fruit trees. About 21% (35 out of 166) of Prunus species were casually collected from various seven areas of China and were tested for PNRSV by reverse transcription-polymerase chain reaction for positive results. The movement protein (MP) gene of 15 isolates and coat protein (CP) gene of 28 isolates showed nucleotide sequence identity of 82.9 to 99.9% and 87.1 to 100% respectively. Phylogenetic study of CP and MP gene sequences exposed three distinct phylogroups characterized by isolates PE5, PV32 and PV96, with distribution frequencies of 6.3%, 56.3% and 37.5%, respectively. Sequence differences around a hex nucleotide addition at sites 124 to129 in the CP gene in six PV32-type isolates from (*P.avium L.)* resulted in an alteration of secondary structure.

James et al.,(2014) worked on a virus genome linked with the CTLV. This was sequenced and consists of 8431 nucleotides. The genome study revealed that CTLV signifies a new and separate species and with a genome organization that similar to those of unassigned viruses in family *Betaflexiviridae*. The CTLV genome has five open reading frames (ORFs), with supposed ORFs within ORF5 and ORF2, found as ORF5a and ORF2a respectively. The initial AUG start codons of ORF5a and ORF2a suggested being suited for effective translation.

Kalinowska et al., (2014) conducted an experiment on PDVinvestigation for genetic diversity. An amino acid sequences and a full length nucleotide of viral CP were collected from various stone fruit trees such as wild cherry tree, sour and sweet cherry trees, peach tree, plum tree and almond tree) from 23 extracts collected from different countries like Italy, Israel, Germany, Poland and USA were studied and compared to 57 others available in GenBank. Comparison of all sequenced virus isolates showed the

diversity of 79–100 % at amino acid and 86– 100 % at nucleotide level. It also showed that the polymorphic positions specified that purifying selection dominated in case of PDV. However, including the codon located inside the structure involved in RNA binding activity were found under strong positive selection.

Katsiani et al., (2014) worked on the genetic diversity and evolution of LChV1, member of genus*Velarivirus.* LChV1is a sweet cherry pathogen stated to infect other Prunus species and also associated with other plant disorders. A new nested RT-PCR method was developed and applied because of some problems encountered with LChV1 detection. Sequences correspondingto the partial RNA-dependent (RdRp), CP genes and heat-shock protein homologue (HSP70h) were studies from Greek LChV1. The phylogenetic results of these three genes shown the segregation of four evolutionary different groups showing no geography based clustering. The genetic distance among the various four were found high with the CP region showing the highest divergence, while intragroup inconsistency levels were found low. The mean ratio of non-synonymous substitutions per synonymous site to synonymous substitutions per synonymous site (dN/dS) for the partial HSP70h, RdRp, and CP showed that these genomic areas wereunder negative selection pressure.

Komorowska et al., (2013) worked on the detection of CNRMV and CGRMV using real-time PCR. For the detection of CGRMV and CNRMV infection in sweet cherry trees the real-time PCR method wasjoint with the HRM. Detection of CGRMV and CNRMV was performed in a RT PCR by using a set of primer that contains one specific primer set for each virus. 17 field samples show positive results for CGRMV and CNRMV. The HRM study made it also possible to differentiate clearly between CGRMV and CNRMV. The HRM results showed that three were infected with CGRMV and seven samples were positive for CNRMV.

Lin et al., (2013)worked on some pathogens infecting *Prunus* species as PBNSPaV and PLMVd. A single tube multiplex, TaqmanReal-time assay was developed for the simultaneous detection and identification of these pathogens. The competence and precision of the assay was evaluated by testing stone fruit trees from positive control collections and several orchard locations. According to this study this technique is most

stable that can be used by different programs for the identification of different stone fruits from infections.

Noorani et al., (2013) multiplex reverse transcription-polymerase chain reaction was designed and standardizedfor simultaneous detection purpose of four cherry viruses: CVA, LChV1, CNRMV, and PNRSVwith a nad5 as plantinternal control. In order to minimize the primer dimmer formation, a single antisense primer for CNRMV and CVA was used. All four viruses were detected successfully by RT-PCR in artificially created viral RNA mixture and field samples of sweet cherry. These virusesidentity was confirmed by sequencing. The designed RT-PCR will not only be valuable for the detection of viruses frommultiple or single infections of various sweet cherryplants but also for some otherpome and stone fruits. For quarantine and certification programme these methods proved very helpful according to this study.

Rana et al., (2010) conducted an experiment on complete sequencesof ACLSV from India. The extracts were obtained from various fruits such as stone (peach, plum, almond, wild Himalayan and cherry apricot) and pome (apple, quince and pear) trees. ACLSV isolates from India and from elsewhere in the world contributed 70–98% and 91–100% sequence identities at the amino acid and nucleotide levels. The highest unpredictable degree was detected in the medium share with 9 amino acid substitutions in contrast to N-terminal and C-terminal ends. ACLSV was phylogenetically closely related with CMLV and PMV.This was the first evidence of homologous recombination in ACLSV and also first report of complete CP sequencevariability study from India.

Oliver et al., (2009) showed the presence of PNRSV by reverse transcription polymerase chain reaction and amplification of the CP gene in total RNA from infected leaf tissue. Samples were carried out by 8-year-old rootstock trial of Sweet and Sour Cherries established on the Research NorthFarm at the New York State AgriculturalExperiment Station (NYSAES), CornellUniversity, and Geneva. Latent infection was widespread in the majority of trees infected (87%) while a little of them show shock symptoms or had severely reduced development (13%).

Bajet et al., (2008) conducted experiment on the one of main viral diseases of sweet cherry across the world, which mainly linked with two viruses as LChV1 and LChV2. They belong to family *Closteroviridae*. Primers of two sets consistent to a main part of the replicase gene of LChV1 and LChV2 were used in one tube reverse transcription polymerase chain reactions in order to detectthese viruses in the total RNA extracts of collected sweet cherry tissues. Both LChV1 and LChV2were identified alone and also in combination in five sweet cherry orchards in Washington State. The Sequence analysis of 240-nucleotide fragments of replicase open reading frame(ORF)1b and 232-nucleotide fragments from a part of ORF8 and the 3′ untranslated region (UTR) ofLChV1 showed that North American detaches shared about 90% to 99% nucleotide identity inboth genome segments studied. In contrast, comparisons of North American isolates to two Eurasian isolates of LChV1 specified shared 89% to 90% in the ORF8/3UTR fragment and nucleotide identities of 79% to 82% in replicase fragment. The sequence differences in the replicas region did not affect detection of LChV1 in 12 isolates using the replicas precise primers stated. This research article represents the first report of LChV1 and LChV2 in sweet cherry in Washington area.

Mandic et al., (2003) conducted an experiment of various cultivar on about hundred and twenty five trees of sweet and sour cherry from two large scale collections in Serbia which were visually examined for different virus symptoms and also tested for presence of cherry viruses by herbaceous host assays, ELISA, graft-indexing on*P. serrulatacv. Kwanzan,* and RT-PCR. Samples were examined by ELISA forPDV, PPV, ACLSV, ApMV, ACLSV, PNRSVandPPV. While ACLSV, PDV and PNRSV were 63% revealed overall. Additionally ELISA tests were conducted on 80 trees for CLRV, ToRSV, PetAMV, RpRSV, TBRV, ArMV, TMV, and SLRSV. In these tests, one tree showed positive results for PetAMV. RT-PCR testing of 44 trees detected another five viruses: CNRMV, CVA, PBNSPaV, CGRMV, and CMLV. In graft-indexing tests on Kwanzanalong with all 125 trees, samples from 38 trees induced symptoms of necrotic crook disease (causal agent unknown). Where the viruses described for the first time in Serbia were CNRMV, CGRMV, ERMaV, PBNSPaV, and CVA.

Hassan et al., (2006) used pentaplex reverse transcription polymerase chain reaction (Pentaplex RT-PCR) in a single tube was developed for the simultaneous detection of

Pome fruit viruses: ASPV, ASGV,ACLSV, ApMV. This is the first report of the simultaneous detection of all four viruses and host mRNA as an internal specific control.

Herranz et al., (2005) worked on the developing of the discovery of plant viruses by molecular hybridization. Randomly viralsequences were combined in tandem and transcribed to provide exclusive riboprobes and chosen as polyprobe. These polyprobe enclosed some virusesthat affect stone fruit trees including APLPV, ApMV, PNRSV, and PDV.While other needs to evaluate other viruses as PPV, ACLSV. These two viruses are important in nature that infects stone fruits. Finally they designed polyprobeto identify viruses under study. The confirmation of infection was established bythe examination by almost 46 samples with 7 various hostscollected from different locations.

Li et al.,(2005)develop a RT-PCR for the detection of CGRMV and CNRMV in naturally infected Prunus species and also in woody indicators. Viral RNA appropriate for RT-PCR was gotten by simple trapping method that did not require any extraction of double-stranded RNA or even total RNA. The RT-PCR results shows that the CGRMV detection rate in viral RNA and total RNA differs variable especially in case of diseased leaf tissues respectively. CGRMV was spotted in root tips,tender shoots, bark and leaves while thehighest bands were obtained in young leaves. In hot seasons as temperature increased incidence and observation percentage lowered down.

Isogai et al., (2004) conducted a research on molecular detection of various cherry viruses in Japan. Leaf samples were takenfrom sweet cherry trees in Aomori, Yamagata and Iwate regions. dsRNA analysis from sweet cherry leaves revealed that 73% of 49 samples contained dsRNAwith more than 6kbp in size. RT PCR study of cherry viruses specified that 49% CVA, 14% of the sampleshad LChV1, 65% in LChV2, 14%in CNRMV, and 92% had CGRMV. It was the first molecular detection of these five cherry viruses inJapan.

James and Chris (1999) conducted a research study on PMV and CMLV viruses. Real Time-PCR process using single oligonucleotide primer pair which allows instantaneous

detection and differentiation of two viruses were developed. A primer with 83% to the site of the CMLV and 100% complementarily to PMV genome was combined with either of two antisense primers one of CMLV origin and the other of PMV origin with 3% end comparing at variable sites. This allowed the differential amplification of PMV and CMLV specific fragments, 419 and 705 bp. Oligo was used to generate cDNA template, differential amplification was not observed, while only amplification of homologous virus linked with the antisense primer, which shows that polyadenylation of both viruses. Combination of these antisense primers into cDNA at reverse transcription step was shown to be essential for this approach. The PMV primer pair detected all isolates of PMV tested by Real Time -PCR analysis, both in bud wood tissue and peach leaf.

2.2 Distribution of Cherry viruses in Turkey

Ertunç (2016) conducted a research onApMV, a worldwide pathogen of pome and stone fruits along with hazelnut, which shows great unpredictable changes in its molecular, biological and serological properties. The CP sequences of fifteen ApMV variants from various hazelnut varieties and the 'Granny Smith' apple variety were taken from Turkey. The phylogenetic study of sequences of Turkish isolates along with the additional sequences of other ApMV variants from the NCBI database shows the presence of various ApMV groups worldwide. Sequences from hazelnut shown somewhat different amino acid and nucleic acid composition compared to the sequences taken from apples in Turkey and from various locations in the world.

Öztürk et al., (2015) conducted a study on cherry viruses. A series of viruses cause diseases and economical losses in sweet cherry. PDVis one of the most common viruses of stone fruits including sweet cherry around the world.PDVwas identified from 316 of 521 sweet cherry samples were collected from 142 orchards in 10 various districts of Isparta province of Turkey. The presence of PDVin ELISA positive samples was confirmed in 37 isolates by RT-PCR method. Amplified DNA fragments of these isolated samples were sequenced and purified for molecular characterization in order to determine genetic diversity of PDV.Sequence comparisons showed to 81-100% to84-99% sequence identity at amino acid andnucleotide level, respectively, of the CP genes

of PDV isolated from Isparta and other various parts around world. Some association between phylogenetic groups and geographical origins or hosts was observedwhile isolates were not grouped solely based on their geographical origins or hosts.

Serçeet al., (2011) worked on sour and sweet cherry disease especially little cherry disease. This was firstly reported in Osmaniye Turkey. LChV1 and LChV2 are linked with little cherry disease. The infections revealed by the plants were discolored small fruit with bitter taste. They collected 7 samples from cv. Napoleon and the *P. mahaleb*from Osmaniye province of Turkey. They did RNA extractions and used as a template for cDNA synthesis and PCR assays were done with virus specific primers.The results showed that two of them (onecherry cv. Napoleon and the *P. mahaleb*) found positives against LChV1but no result shown for LChV2.

Yardimci et al., (2011) conducted some experiments on stone fruits orchards mainly in Mediterranean region of Turkey. Suspicious samples were collected from different location of Isparta province. Samples which were collected included sweet cherries, sour cherries for checking the incidence of viruses. Samples were first verified by ELISA for different five RNA viruses including ACLSV, ApMV, PDV, PNRSV and PPV. While no PPV and ApMV infection was found, 46, 24 and 16 samples were tested positive for ACLSV, PDV and PNRSV, respectively, in ELISA screening about 45% of indicative trees in the region were infected with at least one of these viruses. Sweet cherry is the major stone fruit growing in the region, more than half of the symptomatic samples tested in this study were collected from sweet cherry.

Sipahioglu et al., (2011) conducted a research PBNSPaV. The virus has been for the first time spotted in Malatya region in plums and cherries. An Hsp70hfragment gene located on ORF3 of viral genome was cloned, analyzed and sequencedphylogenetically. The PBNSPaV-K1 isolation showed 93–96% nucleotide sequence character to sequences of Italian and American isolates in databases.

Serçeet al., (2009) conducted research on different parameters of PDV in stone fruits broadly. They explained 10 different sequenced information’s of PDV variants of Turkey. The length of each nucleotide is 657. The sequence comparison of Turkish and

International variants explain mainly existence of four lasses of PDV variables. Firstly, Turkish variants having specific amino acids, next one is apricot and cherries, and the third category is mixed between both. This study basically explained nomenclature based on their hosts.

Usta et al., (2007) describes effects of two different viruses as ApLV and PBNSPaV of Turkey which are mainly known for stone fruit as Eastern Anatolia. PBNSPaV and ApLV was firstly identified or reported in western Anatolia.Some molecular techniques were done for detection of both viruses. Severe symptoms on the stem observed during collection. Some cherries and plums detected infected with PBNSPaV while other virus showed negative results.

Gümüşet al.,(2006) worked on various symptoms of virus along with the viriods infections which have been observed during past ten years in stone fruit crops, while, no relative occurrences of virus and viroids diseases have been described on stone fruit crops in western Anatolia of Turkey. Surveys were directed from June 2004 to August 2006 in the main stone fruits growing orchards to govern the seven most important virus and two important viroids affecting *Prunus* species. The results from serological ELISA and molecular tests demonstration showed the incidence of PNRSV, PPV, ACLSV, ApMV, PDV, and PBNSPaV, tested 1732 specimens of stone fruits spp. Overall infection level with these graft transmissible agents was about 30%. The results showed that PDV wasmajor in stone fruit crops. Report shows that PBNSPaV was stated for the first time in western Anatolia in Turkey.

CHAPTER III

MATERIALS AND METHODS

3.1 Field Surveys

3.1.1ExperimentalArea Description

This thesis research related with Investigation of Virus Diseases of Cherries in Niğde was done in Laboratories of Department of Plant Production and Technologies, Faculty of Agriculture Science and Technologies, Niğde Ömer Halisdemir University Niğde, Turkey.

3.1.2 Plant Material

Sweet cherry and sour cherry leaf samples showing suspicious virus symptoms collected from different locations of Niğde province.

Totally ninety (90) samples were collected from three different locations, fifty two (52)samples from Darboğaz village (Niğde), 15 from Niğde Ömer Halisdemir University campus (research area) and 23 from Niğdecity center were collected at the end of June-July 2017. Details regarding samples collection with their symptoms summarized in table as follows (Table 3.1).

Table 3.1. Details of sample collection and their symptoms

Sample #	Sample	Site	Sample	Sample Notes	Site	Sample #	Sample	Site
1	Leaf yellowing	Orchard 1	31	Sour cherry	Orchard 2	61	Leaf curling	Orchard 4
2	Deformation		32	Sour cherry		62	Vein wrinkling*	
3	Yellow cherry		33	Yellow cherry		63	Yellow cherry	
4	Yellow cherry		34	Yellow cherry		64	Yellow cherry	
5	Yellow cherry		35	Yellow cherry		65	Yellow cherry	
6	wrinkling	Orchard 1	36	Deformation		66	Leaf curling	
7	Curling		37	Yellow cherry		67	Vein wrinkling*	
8	Leaf curling		38	Leaf curling		68	Leaf curling	
9	Leaf curling		39	Leaf curling		69	Leaf curling	
10	Vein wrinkling		40	Leaf curling		70	Vein wrinkling*	University
11	Yellow cherry	Orchard 2	41	Vein wrinkling*	Orchard 3	71	Orchard corner	
12	Yellow cherry		42	Yellow cherry		72	Orchard starts	
	Yellow cherry		43	Yellow cherry		73	Yellow cherry	
13	Malformation		43	Deformation		73	Yellow cherry	
14	Leaf curling		44	Yellow cherry		74	Yellow	

Table 3.1. (Continue) Details of sample collection and their symptoms

Sample	Sample note	site	Sample	Sample note	site	Sample	Sample note	site
15	Vein wrinkling*	Orchard 2	45	Leaf curling	Orchard 3	75	Leaf curling	University
16	Leaf curling		46	Vein wrinkling*		76	Vein wrinkling*	
17	Vein wrinkling*		47	Leaf curling		77	Leaves Wrinkling	
18	Yellow cherry		48	Leaf curling		78	Leaf curling	
19	Malformation		49	Vein wrinkling*		79	Leaf curling	
20	Yellow cherry		50	Orchard corner		80	Vein wrinkling	
21	Yellow cherry		51	Yellowing		81	Yellow cherry	
22	Yellow cherry		52	Yellow cherry		82	Dwarfing	
23	Sour cherry		53	Yellow cherry		83	Yellow cherry	
24	Malformation		54	Yellow cherry		84	Yellow cherry	
25	Leaf curling		55	Vein wrinkling*		85	Yellow cherry	
26	Leaf curling		56	Deformation		86	Dwarfing	
27	Vein wrinkling*		57	Leaf curling		87	Yellowing	
28	Deformation		58	Leaf curling		88	Leaf curling	
29	Genetic disorder		59	Vein wrinkling*		89	Leaf curling	
30	spots on stem		60	Genetic disorder		90	Vein wrinkling*	

3.2Molecular Screening of Cherry Samples

3.2.1 Total RNA Extraction

Total RNA was extracted from cherry leaves by using two different methods. Firstly RNA was extracted from RNeasy Plant minikit -Zymo Research and following the manufacturer's instructions and extractions of all samples were done by GuanidiumThiocyanate method. To perform Guanidium Thiocyanate method based RNA extraction, 1 ml solution D (250g Guanidium Thiocyanate, 293ml dH_2O, 17.6ml of 0.75M sodium citrate, 26.4ml 10% sarkosyl) per 100 mg fresh tissue were added and minced on ice.Tissue or cell lysate transferred to a 4 ml polypropylene tube. 0.1 mL of 2 M sodium acetate were added in 1 mL of lysate:, pH 4.0, mixed thoroughly by inversion;1 mL (1000 mL) water-saturated phenol, mixed thoroughly by inversion; 0,2 mL (200mL) of chloroform/ isoamyl alcohol (49:1), shaked vigorously by hand for 10 s. Samples were cooled on ice for 15 min.Centrifuged for 20 min at 10.000g at 4 °C. Upper aqueous phase were transferred carefully by using pipette, which contained mostly RNA to a clean tube. 1000ml isopropanol added to aqueous phase to precipitate the RNA.Samples were incubated for at least 1h at -20 °C.Centrifuged the samples for 20 min at 10.000g at 4 °C and discarded the supernatant. RNA pellet was dissolved in 300ml solution D. and transferred to a 1.5 ml micro centrifuge tube. Iso-propanol (0.3 ml) was added in the same tube. Samples were incubated again for at least 30 min at -20°C,and then centrifuged for 10 min at 10,000g at 4°C, Supernatant was discarded. RNA pellet re-suspended with 0.5-1ml of 75% ethanol and vortexed for a few seconds. Samples were incubated for 10-15 min at room temperature to dissolved possible residual traces of guanidinium. Samples were centrifuged for 5 min at 10,000g at 4 °C and supernatant was discarded again. RNA pellet air-dried for 5-10 min at room temperature. RNA pellet had been dissolved in 100µl of either DEPC-treated water or 0.5% SDS. RNA pallet was incubated for 10-15 min at 60 °C to ensure complete solubilization. Yield and quality of RNAs were measured by spectrophotometer (Thermo Sci., USA).Total RNAs were stored in -20 for further analysis.

3.2.2 cDNA synthesis

A two-step protocol was used for cDNA synthesis. The first strand cDNA synthesized from total RNAs using random hexamer primers and the Superscript Choice System for cDNA Synthesis Kit according to instructions (Abm's Easy Script™). In the first step for each,5µl of RNA, 1 µl of Random hexamer, 1 µl of dNTP and 7.5 µl of dH_2O were used. For second step, 4µl of 5XRT buffer, 0.5 µl RNase inhibitor, and 1µl of RNase Enzyme were added. The conditions were as follows., Step 1:(65°C 5 min., -20°C 1 min), Step 2:(42°C 50 min, 85°C 5 min).

3.2.3 PCR analysis

The targeted virus specific primers were used for PCR analysis. The details on the sequence of these primers were given in table 3.2. The PCRs were carried out with 16.8 µl sterilized water, 0.5 µl of 10 µM dNTP mix, 2 µl of 25 mM $MgCl_2$, 2.5 µl of 10X Taq buffer, and 0.5 µl of 10 µM of each primer with 0.20 µl of 5 Unit/µl Dream Taq DNA polymerase and 2 µl of cDNA. Total final reaction mixture of PCR was 25 µl. denaturation 94°C 5 min, 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min; and a final extension for 10 min at 72°C .

3.2.4Gel electrophoresis

The PCR products was visualized under UV light after electrophoresis on 1.5% agarose gel and stained with Ethidium bromide witha UV-trans illuminator BIO-RAD (Gel Doc).

Table 3.2. List of virus specific primers used in the molecular screening studies.

Virus Names	Primer Sequences (5'-3')	Coding region	Product length	References
ACLSV	F: TTCATGGAAAGACAGGGGCAA	CP	677 bp	Hassan et al., 2006
	R:AAGTCTACAGGCTATTTATTATAAGTCTAA			
ASPV	F: ATGTCTGGAACCTCATGCTGCAA	CP	370 bp	
	R:TTGGGATCAACTTTACTAAAAAGCATAA			
ApMV	F: ATCCGAGTGAACAGTCTATCCTCTAA	MP	262 bp	
	R: GTAACTCACTCGTTATCACGTACAA			
ASGV	F: GCCACTTCTAGGCAGAACTCTTTGAA	CP	273 bp	
	ASGVR:AACCCCTTTTTGTCCTTCAGTACGA			
PDV	F:GTGTAGAAAGAAGAGAAGTCCGACAAG	CP	874 bp	Serçe et al., 2009
	R:ATCTAGAAGCAGCATTTCCAACTACGA			
PNRSV	F: GAACCTCCTTCCGATTTAG	CP	346 bp	Sanchez et., 2005
	R: GTCTCCCTAACGGGGCATCCAC			
CGRMV	F: TAAACCCCTGCAATTCCACTC	CP	192 bp	Zong et al., 2015
	R: CTCTAAGGAAACTGAAGGAAAA			
CNRMV	F: TCCCACCTCAAGTCCTAGCAG	CP	584 bp	Osman et al., 2012
	R: TGAACTTGGCCAGTTCTGCC			
CLRV	F:GTTACTTTTACCTCCTCATTGTCCATGGTTG	CP	283 bp	Kumari., 2009
	R:GACTATCGTACGGTCTACAAGCGTGTGGCGTC			
CMLV	F: GACTCTTCAGGGTTGGTTCG	CP	425 bp	Zong et al.,2015
	R:CTCAATGTGATTTCGCAAGG			

Table 3.2. (Continue) List of primers used in the molecular screening studies.

Virus Names	**Primer Sequences (5'-3')**	**Coding region**	**Product length**	**References**
CTLV	F : TCAGCAAGATTAAGGAGGTTG	CP	562bp	Villamor et al., 2013
	R: ATNGGTTGAATTTGGCCAGT			
CRLV	F : GCTATGTGCGGGATAATGGAT	RNA 2	423 bp	F. Osman. et al., 2017
	R : CACAAGCAAACTGATGAGCTCC			
PBNSPaV	F : TACCGAAGAGGGTTTGGATG	CP	270 bp	Sanchez-Navarro et al. 2005
	R : TAGTCCGCTGGTACGCTACA			
LChV1	F: GGTTGTCCTCGGTTGATTAC	ORF	300 bp	Serçe et al., 2011
	R : GGCTTGGTTCCATACATC TC			
LChV2	F: CTCGGCGTATATGGTGGATGTTTA	CP	438bp	Rott. et al., 2001
	R : CCGAATGCAGTGGGGATAGG			
nad5 (internal control)	F : GATGCTTCTTGGGGCTTCTTGTT	CP	181bp	Hassan et al., 2006
	R : TAGTCCGCTGGTACGCTACA			

3.3 Genetic Diversity Analysis of LChV1

Different primers were used to study the genetic diversity of LChV1. It includes Coat protein(CP) 456bp, RNA dependent RNA polymerase(RdRp)300 bp, Open reading frame(p21)828bp, Open reading frame (p27) 951bp and heat-shock protein homologue (Hsp70h) 455bp region as well(Wang 2016)(Table 3.3). They cover partialgenome of LChV1.

Table 3.3. List of *Little cherry virus 1* primers for genetic diversity studies

Primer Sequences (5'-3')	Region	Product size	Reference
F: TCAAGAAAAGTTCTGGTGTGC R: CGAGCTAGACGTATCAGTATC	CP	456 bp	Nagyova et al., 2015
F:ATAAGATGATGAATACTTTTGAACG R: GCTGGTCGAAAAGAAAGTTGA	ORF 7	828 bp	Katsiani., 2015
F: CATGTATAACAAAGGACGTAGGAATG R: ATTCAGATTTTATAATAAACACTCAG	ORF 8 (p21)	951 bp	Katsiani., 2015
F: GGTATTAGTCCTGAACTAGA R: AACATCACCACCACCACTGACTC	p27	499-577 bp	Candresse et al., 2013

3.4 Double Stranded RNA (dsRNA)Analysis

Extraction buffer in a 15 ml Falcon (1 ml STE 2X, 70 µl SDS 20%, 20 µl Bentonite, 1.425 ml Phenol-TE) were prepared with 0.750g of samples.The samples in the presence of liquid nitrogen with the precooled mortar and pestle were pulverized. Frozen powder to a 15ml falcon tube containing extraction buffer with the spatula and funnel were transferred.After spinning aqueous phase to a new 1.5 ml tube were transferred. Then the sampleswere Spinnedat 10000 g for 20 min at 20°C. Absolute alcohol volume to add = 0.176 x phase aqueous volume wereadded. After washing the silica with 1 ml STE 1X + 15% alcoholsupernatantwere discarded and mixed well and pellet were taken off with a tip. The cellulose CF11 was dried carefully with the help of tip. The supernatant (about 400 µl) were retained.3M sodium acetate pH5.2 (40 µl) was added into the tubes. Isopropanol (320 µl) were added in to the same tubes. Samples were incubated overnight at -20°C or 1h at -80°C.Pellet was washed with 500 µl 70% alcohol. Supernatant was removed and RNA pallet was dissolved in 10 µl DEPC-treated water in final step.

3.5 TaqMan Real-Time PCR Analysis for the Universal Detection of LChV1

The universal primers and TaqManprobe designed for this experiment are explained in Table 3.4. LChV1 Real-Time PCR reaction was performed in PCR plates using each

reaction (20µl final volume) contained 2 µl of cDNA, 2x TaqMan buffer (10 µl), forward and reverse primer 10 µm each (2 µl), TaqMan probe 0.5 µl and RNase free water 3.5 µl. The cycling conditions consisted of 45^{o}C for 30 min, followed by 30 s at 95°C, 15 s at 55°C and 45 s at 60°102 C.

Table 3.4. Real-time PCR primer and probe for LChV1 detection(Katsiani et al.,2018)

Primers/ Probe	**Primer Sequences (5'-3')**	**Position (nt)**	**Annealing Tmp (^{o}C)**
LChV1-F	CCAATGCACAAAGCACATATGA	1.068-1.089	62.6
LChV1-R	ACCGCGACGTGGTCCTAATA	1.184-1.203	65.6
LCh-P3	**Taqman probe**	1.156-1.181	69-71
	FAM-TCGAARGGAGCTCTYCATGTTTCGCA-TAMRA		

CHAPTER IV

RESULTS

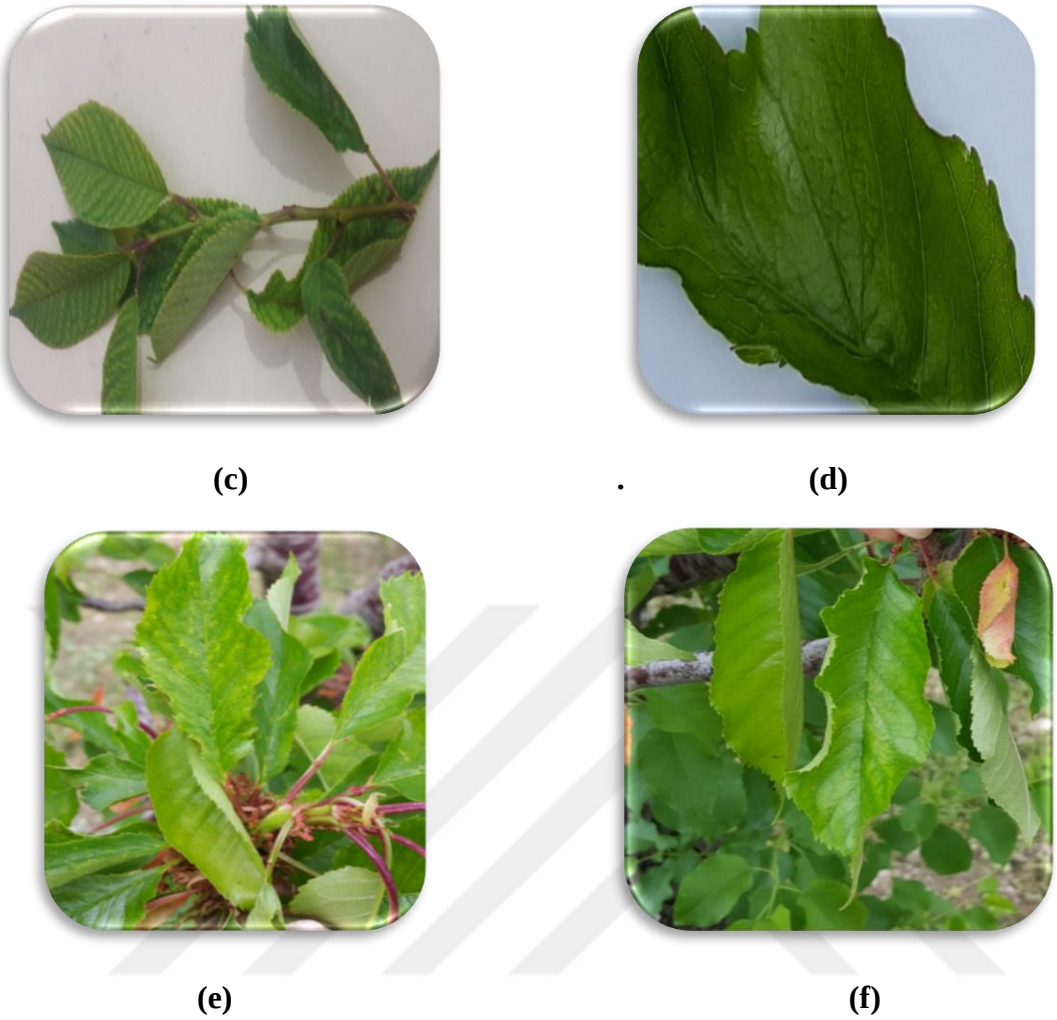
The results of this study based on the investigation of cherry virusesthrough virus specific primers explained in this chapter. The results have been divided into 5 different sections;1) Field observations, 2) molecular screening of viruses infecting cherry plants, 3) PCR analysis for determination genetic diversity of LChV1 isolates, 4) Double stranded RNA analysis, 5) TaqMan Real-Time PCR analysis

4.1Field Observations

Totally ninety (90) leaf samples were collected from sweet and sour cherry trees showing suspicioussymptoms from different locations.The symptoms varied from yellowing,chlorosis and curling of leaves, genetic disorders, and leaf deformation (Figure 4.1).

(a)

(b)



(c) . **(d)**

(e) **(f)**

Figure 4.1. Symptoms observed during sampling yellowing of leaves (a) , deformation (b), yellow veins (c), genetic disorder(d) , leaf curling (e), leaf rolling(f)

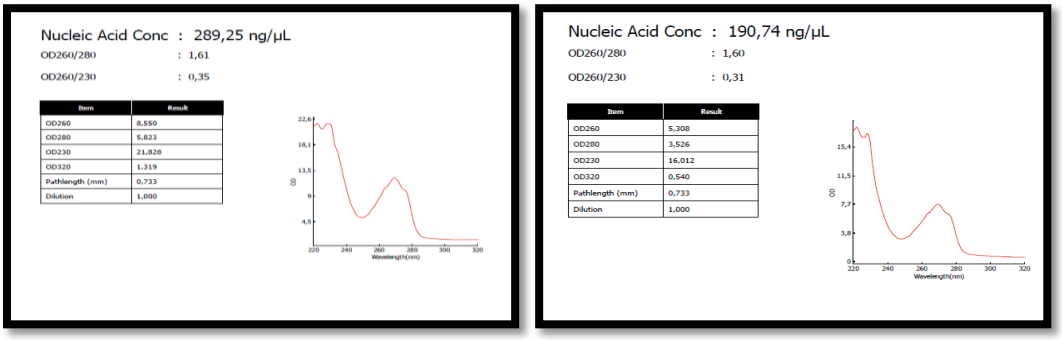
4.2Molecular Screening of the Viruses in Cherry Samples

4.2.1 RNA extraction results

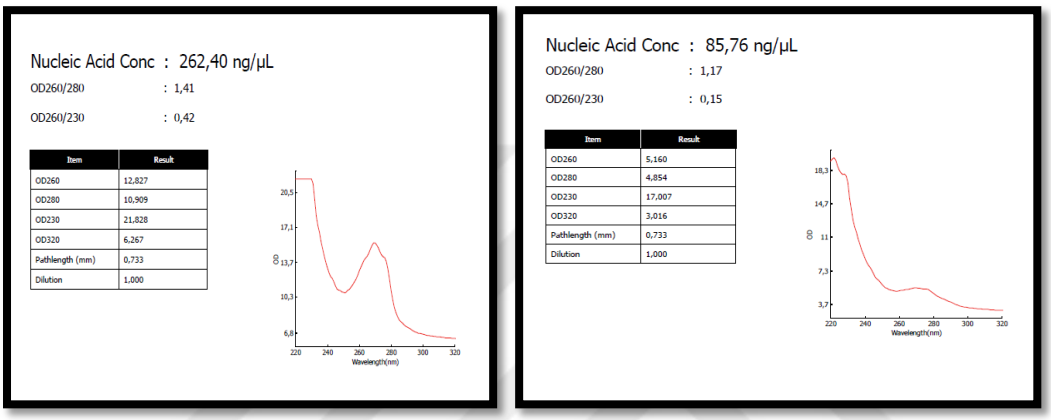
For the optimization of RNA extraction two different methods were performed;RNeasy Plant mini kit -Zymo Research and Guanidium Thiocyanate method. Due to low quality of RNAs obtained by commercial kit, all the RNAs were extracted by Guanidium Thiocyanate method. The concentrations of extracted RNAs were varied from 45 ng/ul to 397 ng/ul. The RNA concentration and quality results showed in Table 4.1 and Figure 4.2.

Table 4.1. The concentration of extracted RNAs with 260/280(abs) ratio values from cherry plants

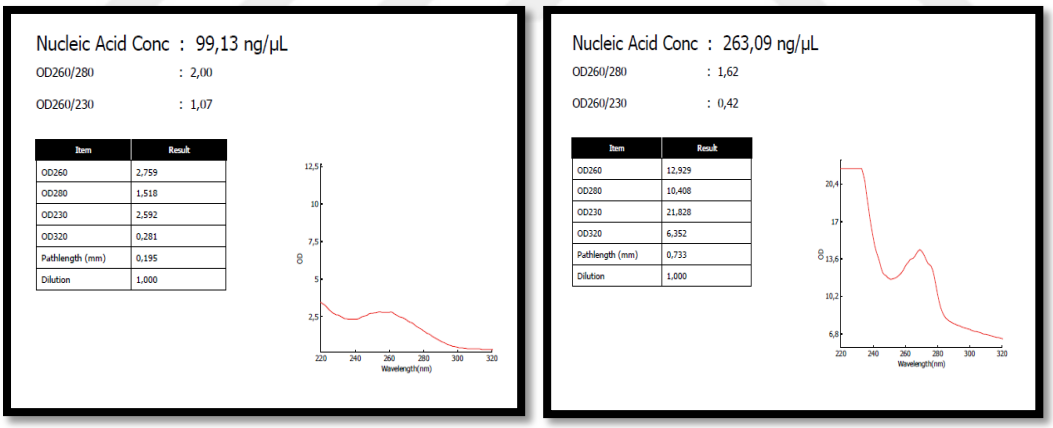
Sample No.	RNA concentration		Sample No.	RNA concentration		Sample No.	RNA concentration	
	ng/µl	260/280		ng/µl	260/280		ng/µl	260/280
1	77.34	2	**11**	157.95	1.86	**21**	119. 59	1.82
2	45.08	1.96	**12**	57.98	2.08	**22**	59.81	2.7
3	69.92	1.93	**13**	108. 55	2.02	**23**	68.75	1.73
4	51.66	1.7	**14**	92.26	2.29	**24**	71.15	1.99
5	61.02	1.99	**15**	159. 52	2	**25**	115.98	2.01
6	57.81	2.16	**16**	71. 50	1.69	**26**	52. 59	2.1
7	110.26	1.76	**17**	155.32	2.26	**27**	69.26	1.9
8	59. 40	2.02	**18**	56.05	1.99	**28**	90.05	2.13
9	88.73	2	**19**	51.28	1.9	**29**	76.09	1.89
10	127.93	2.14	**20**	220.9	2. 50	**30**	50.33	2.05
31	68.9	1.78	**41**	190.98	1.54	**51**	188.06	1.92
32	76.32	2	**42**	189.51	1.69	**52**	176	1.95
33	54.19	1.49	**43**	70. 46	1.82	**53**	173.32	1.68
34	113.13	1.89	**44**	64.27	2.6	**54**	82.98	1.53
35	167.9	1.78	**45**	67	2.52	**55**	90.66	1.61
36	110.34	1.9	**46**	55.84	1.69	**56**	80.85	1.69
37	78.9	2	**47**	69.48	2.11	**57**	72.2	1.5
38	234.12	2.91	**48**	89.52	1.57	**58**	69.9	1.8
39	213	1.98	**49**	93.54	1.92	**59**	99	1.95
40	123.91	1.43	**50**	99.13	2	**60**	102	2
61	245.69	1.69	**71**	219.29	1.59	**81**	182.76	1.59
62	104.36	1.5	**72**	84.33	1.51	**82**	75.64	1.56
63	107.29	1.52	**73**	190.74	1.6	**83**	72.85	1.52
64	85.76	1.5	**74**	353.88	1.54	**84**	44.52	1.56
65	112.13	1.69	**75**	49.19	1.52	**85**	150.68	1.55
66	59.99	1.54	**76**	135.09	1.51	**86**	74.95	1.58
67	151.65	1.3	**77**	289.25	1.61	**87**	275.44	1.51
68	397.75	1.59	**78**	50.85	1.5	**88**	76.2	1.59
69	262.2	1.59	**79**	117.1	1.51	**89**	45.92	1.5
70	361.26	1.46	**80**	58.46	1.53	**90**	263.09	1.62



(a) (b)



(c) (d)



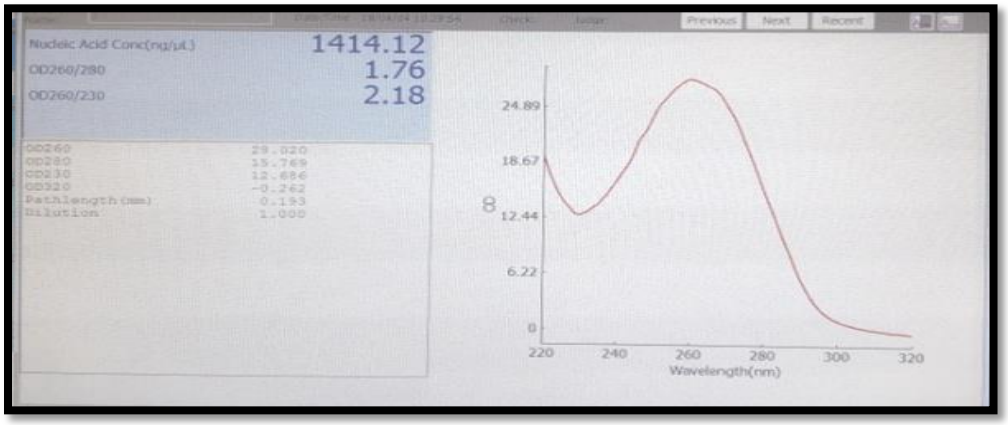
(e) (f)

Figure 4.2. Spectrophotometer results of extracted nucleic acids from Cherriessample 10(a),sample 36(b), sample 80(c), sample 71(d),sample 89(e),sample 12(f).

4.2.2cDNAsynthesis

Template RNA extracted by Guanidium Thiocyanate method were used for cDNA synthesis. After synthesizing of cDNA single stranded RNA was converted to double stranded. The concentration graph shows the quantity and quality of cDNAs are in Figure 4.3.

(a)



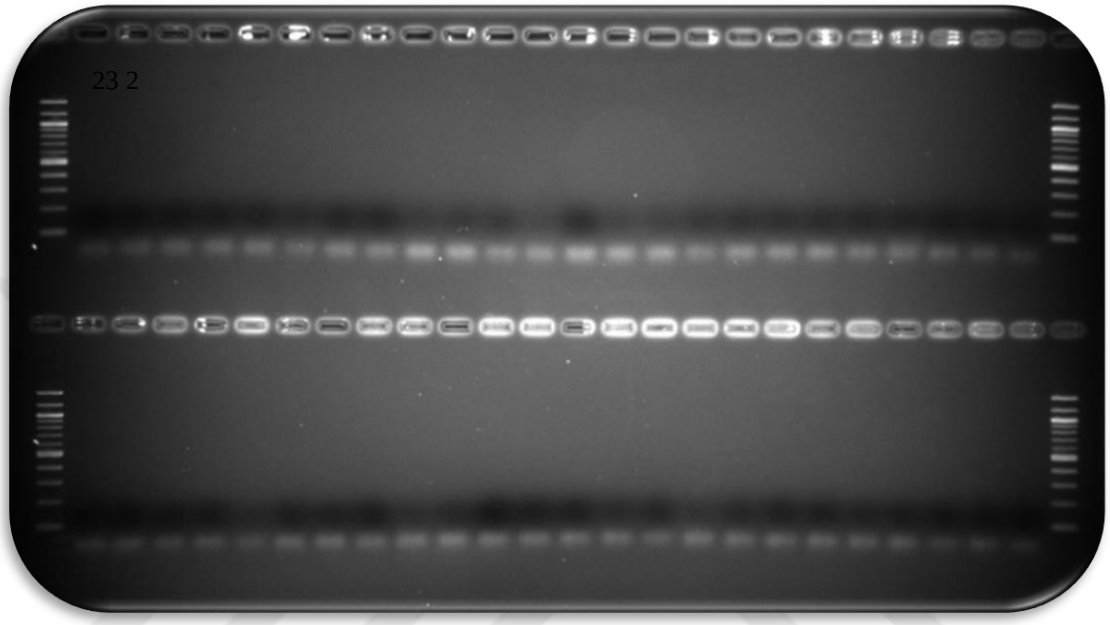
(b)

Figure 4.3. Spectrophotometer results of cDNAs from sample 12(a), sample 63(b).

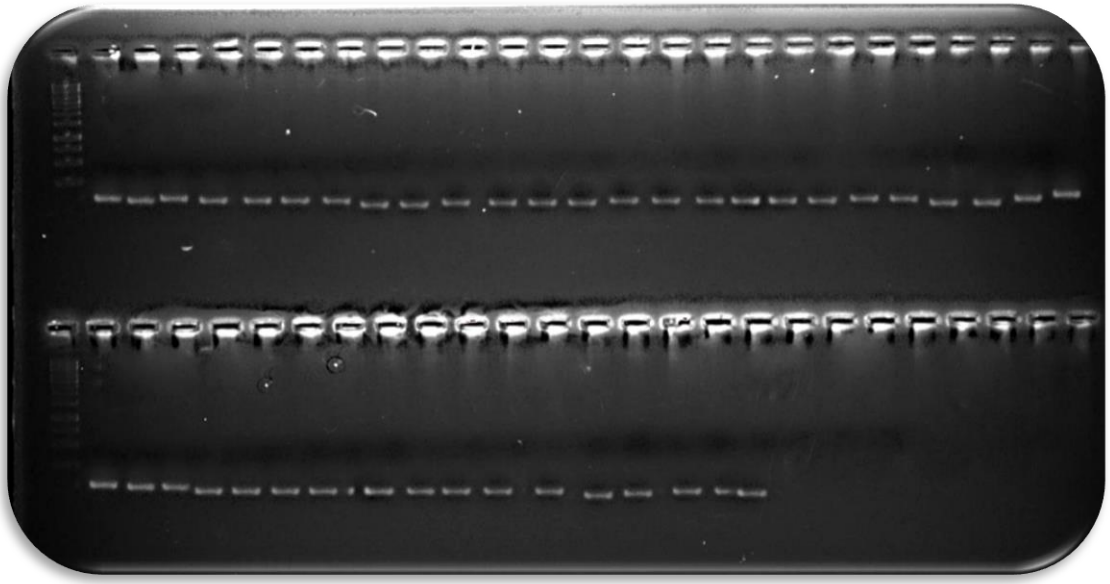
4.2.3PCR Analysis

4.2.3.1*Cherry rusty leaf virus*(CRLV) screening

Cherry samples were screened with CRLV specific primers but no any positive amplificationwas detected. (Figure 4.4).



(a)



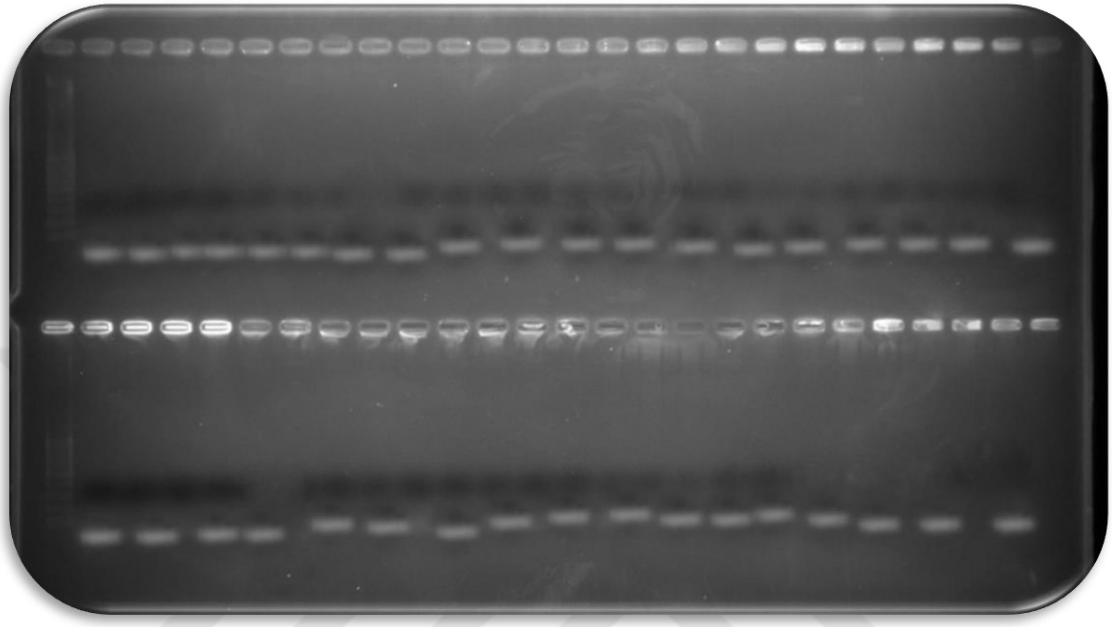
(b)

Figure 4.4.Agarose gel image of CRLV result

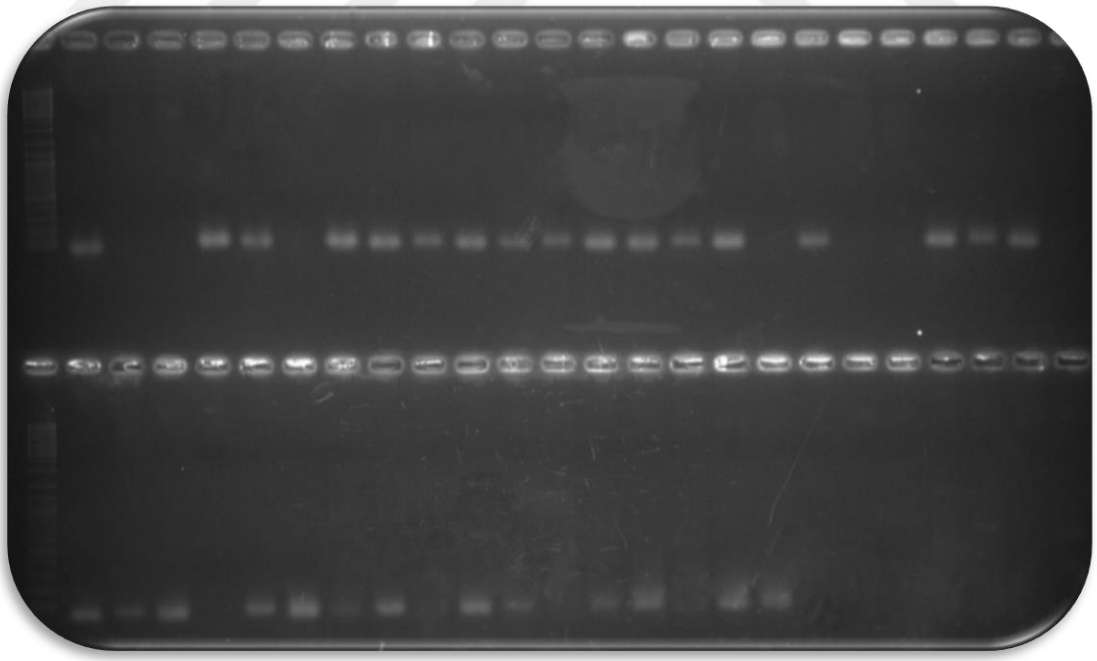
1-48(a), 49-90(b)

4.2.3.2 *Cherry leaf roll virus* (CLRV) screening

Screening of cherry viruses was done with specific primerCLRV but noany useful amplificationwas found(Figure 4.5).



(a)

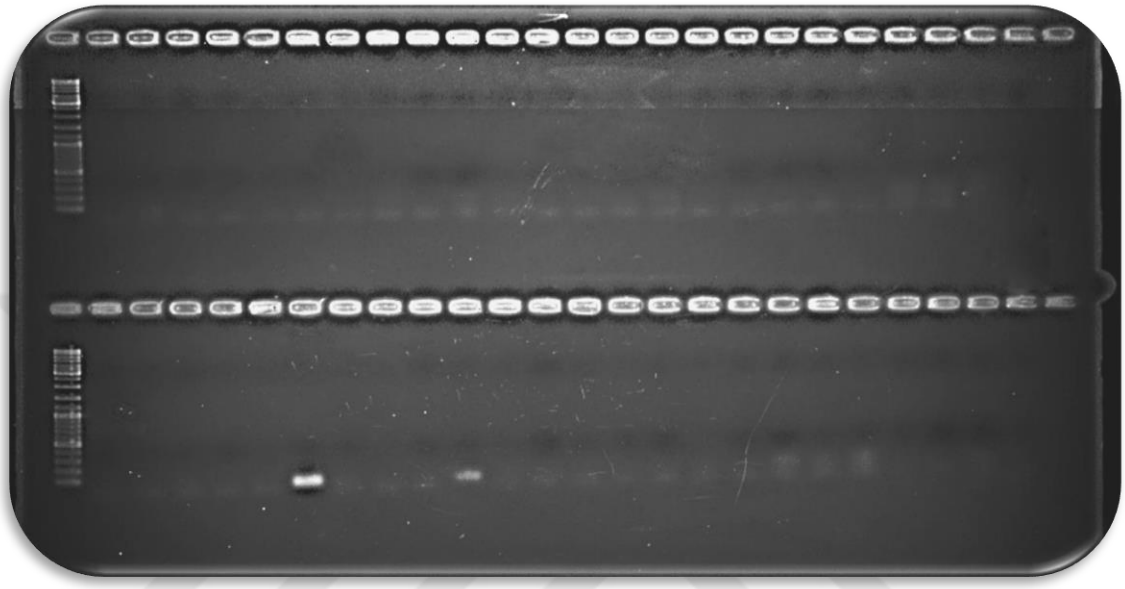


(b)

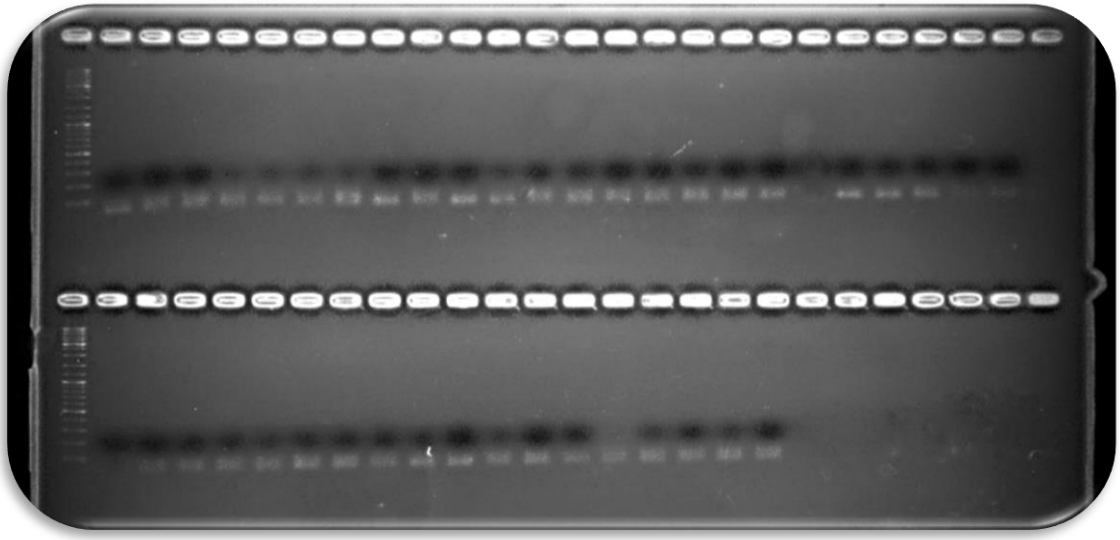
Figure 4.5. Agarose gel image of CLRV result.
1-48(a), 49-90(b)

4.2.3.3 *Prunus* necrotic ring spot virus (PNRSV)screening

Screenings of all samples were done with PNRSV primer but no any amplificationwas found. As a result it wasassumed that there is no any infection of that virus on screened samples because of no amplification (Figure 4.6).



(a)

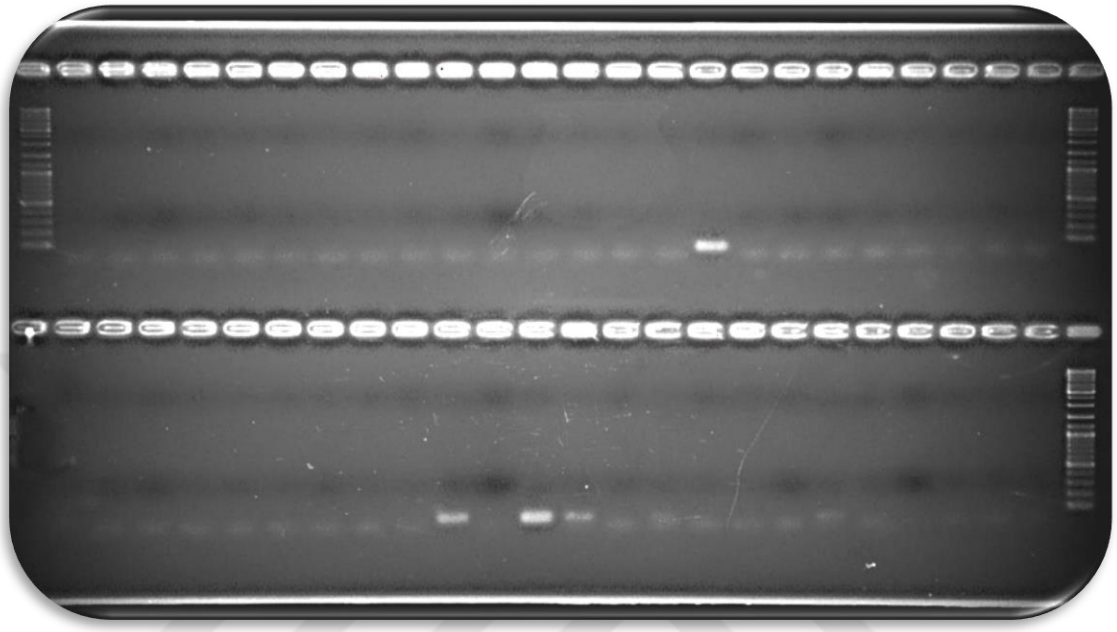


(b)

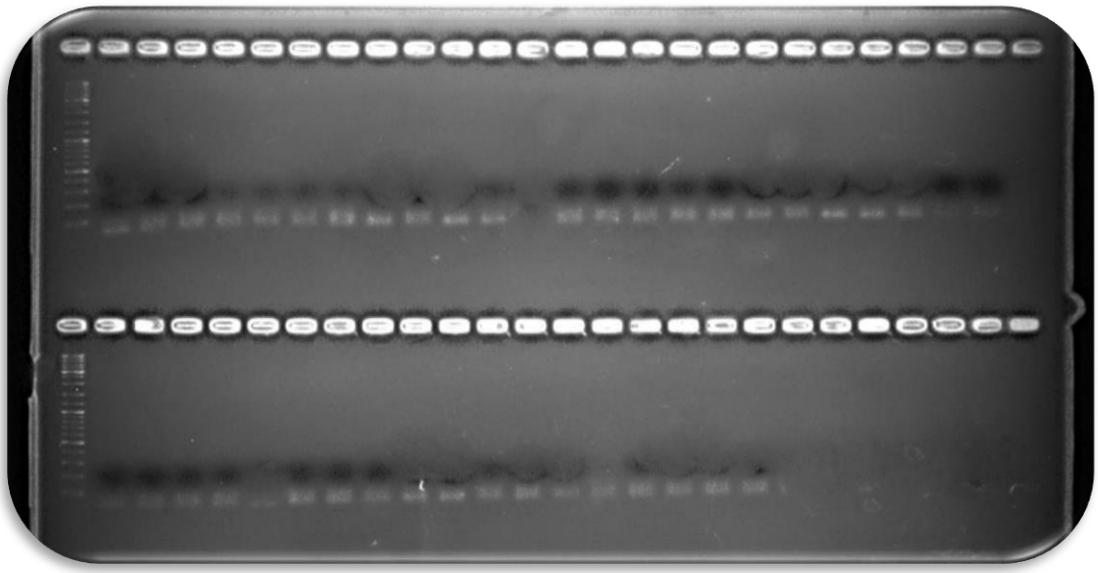
Figure 4.6. Agarose gel image of PNRSV result
1-48(a), 49-90(b)

4.2.3.4 *Plum bark necrotic stem pitting associated virus* (PBNSPaV) screening

In this experiment screening of all suspicious samples were done with PBNSPaV specific primer but no any positive amplifications were found. (Figure. 4.7)



(a)



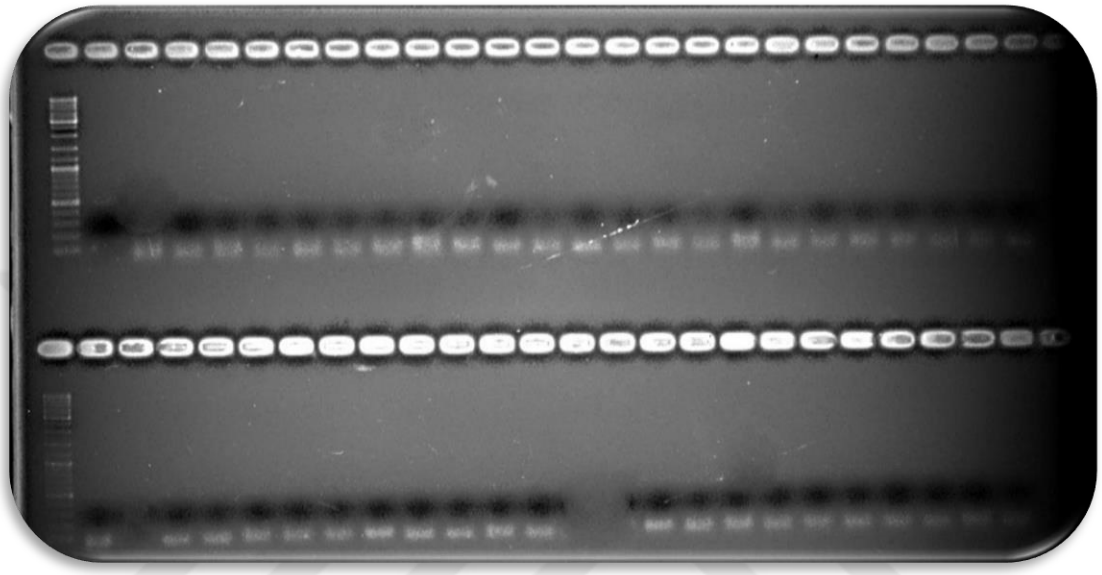
(b)

Figure 4.7. Agarose gel image of PBNSPaV result

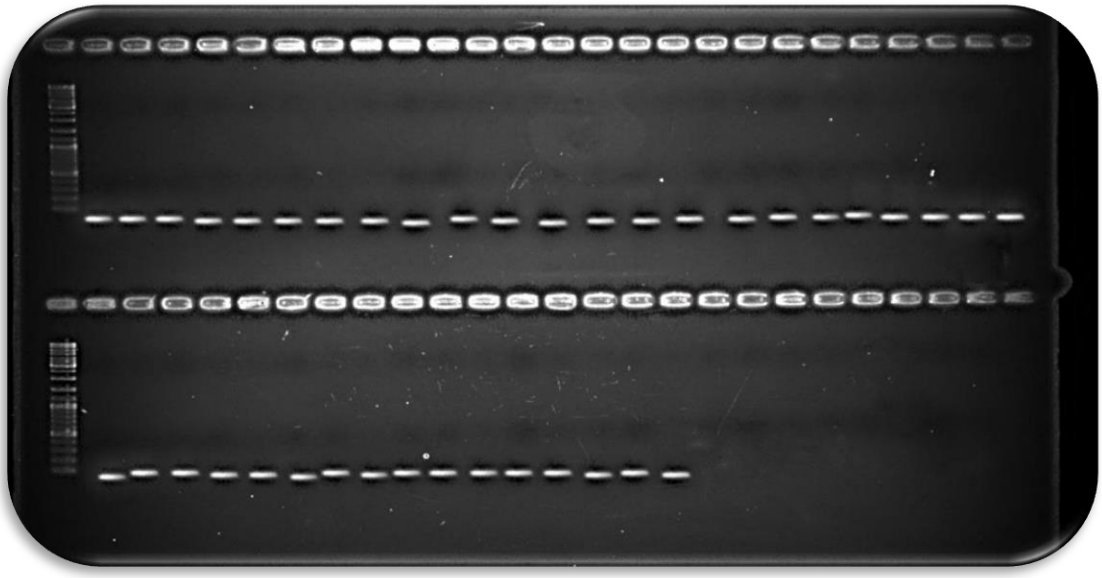
1-48(a), 49-90(b)

4.2.3.5*Apple chlorotic leaf spot virus* (ACLSV) screening

Collected samples were screened in this experiment with ACLSV primer but could notfind any amplification. None of the samples was found to be positive against this virus.(Figure 4.8).



(a)

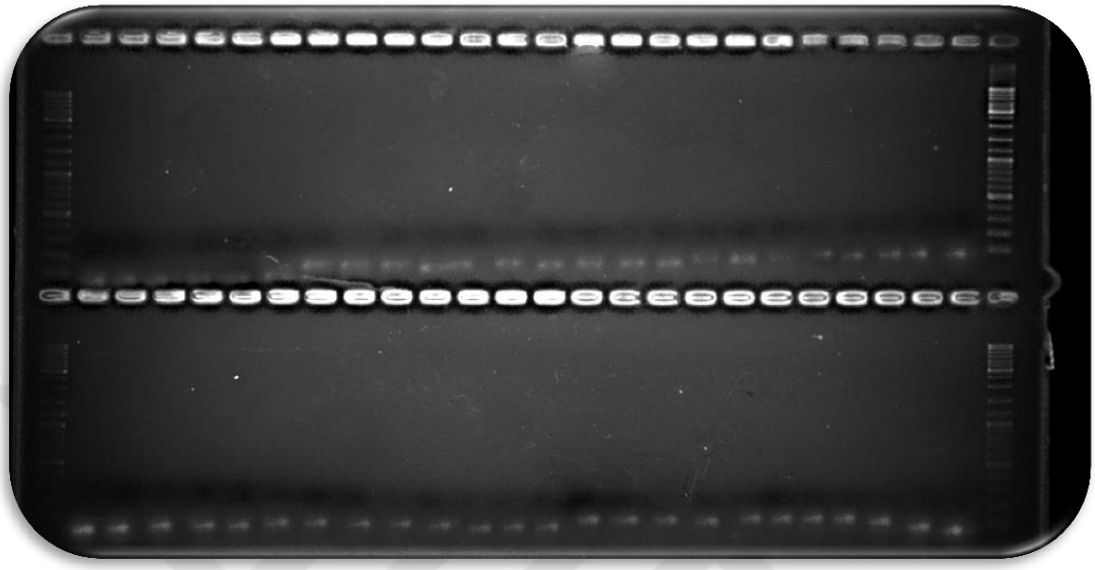


(b)

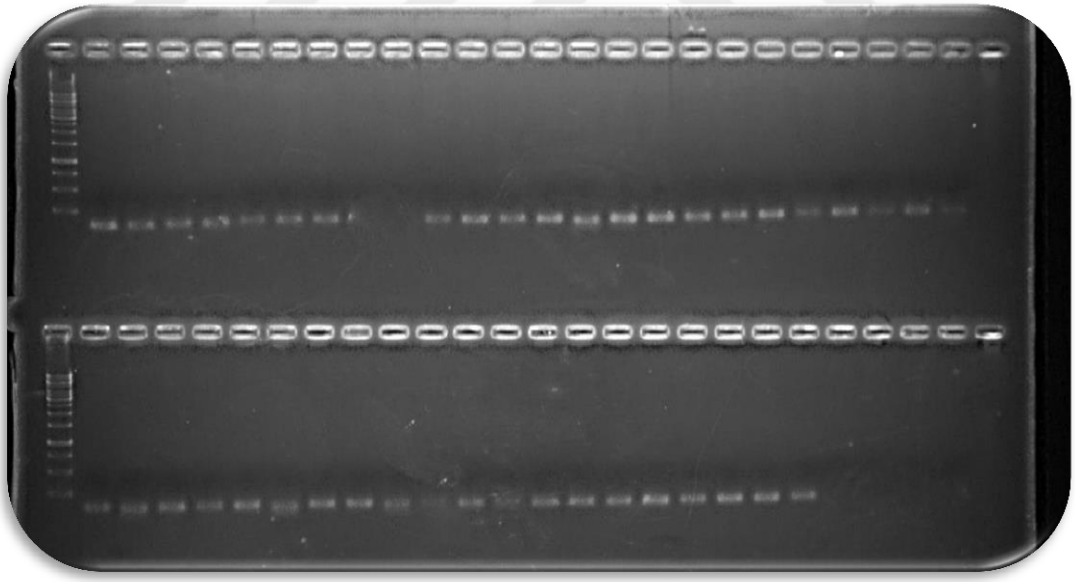
Figure 4.8. Agarose gel image of ACLSV result
1-48(a), 49-90(b)

4.2.3.5.*Apple stem pitting virus* (ASPV) screening

All samples were screened with primer which is specific for ASPV but as a result of this experiment no any amplification wereobserved in Figure 4.9..



(a)

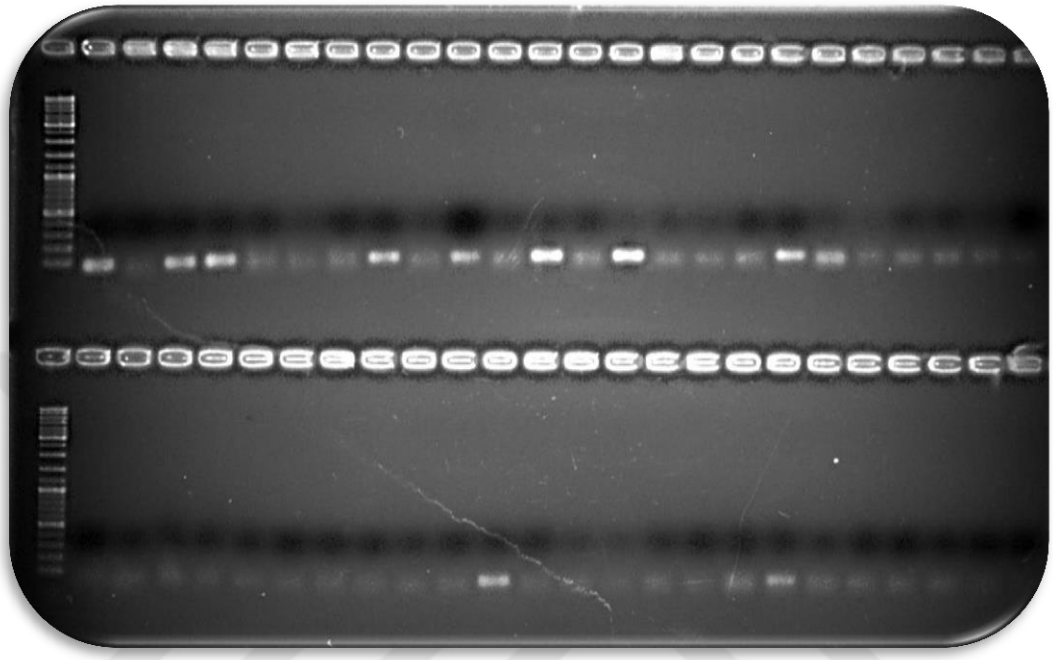


(b)

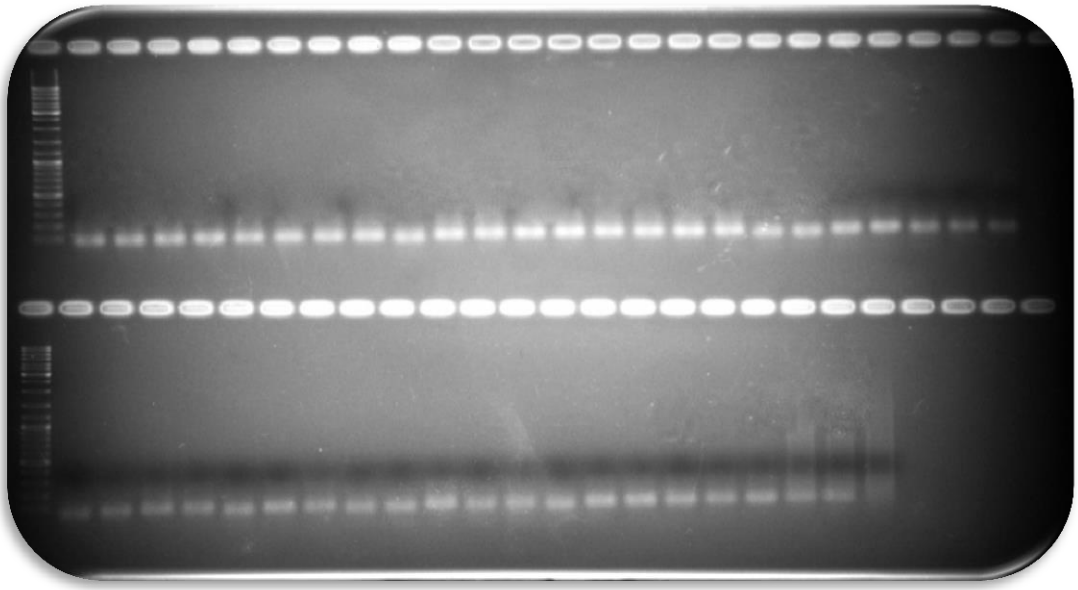
Figure 4.9. Agarose gel image of ASPV results
1-48(a), 49-90(b)

4.2.3.7 *Apple stem grooving virus* (ASGV) screening

Screening of all cherry samples wasdone by primer ASGV that is linked to ASGV CP gene. As a result, no any positive amplification was observed in Figure 4.10.



(a)



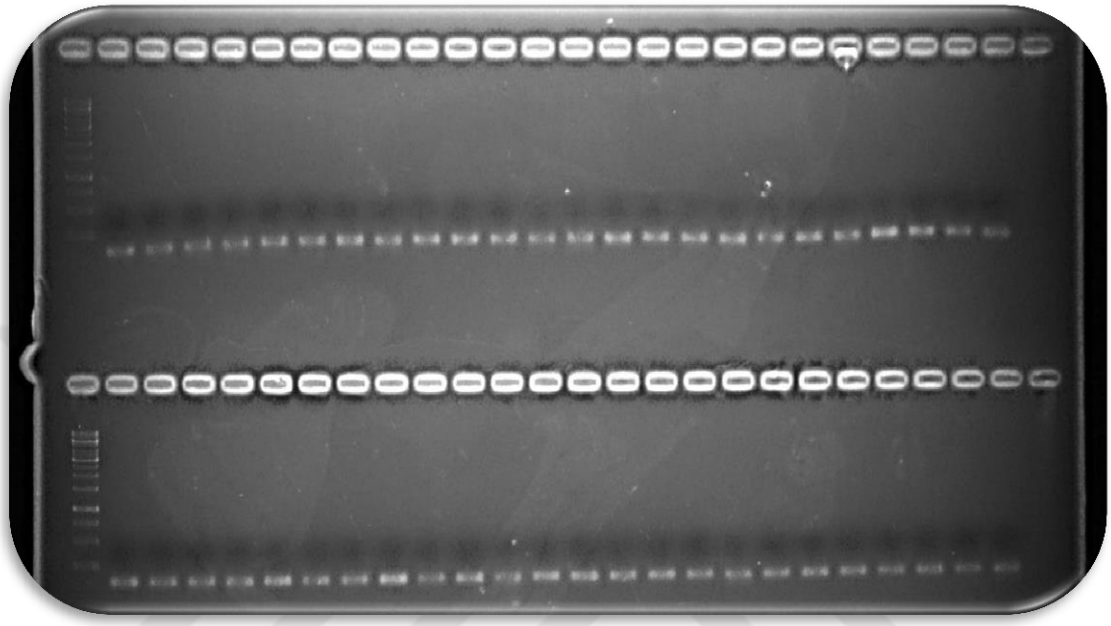
(b)

Figure 4.10. Agarose gel image of ASGV result

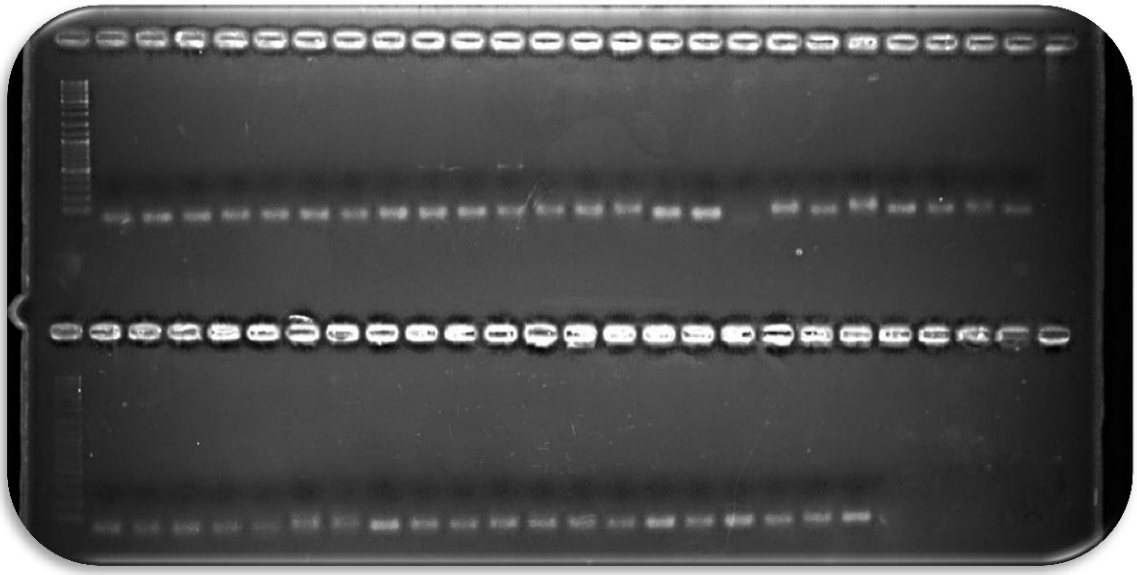
1-48(a), 49-90(b)

4.2.3.8 *Apple mosaic virus* (ApMV) screening

Screening of samples was done with ApMV. No any positive amplification was found(Figure 4.11).



(a)



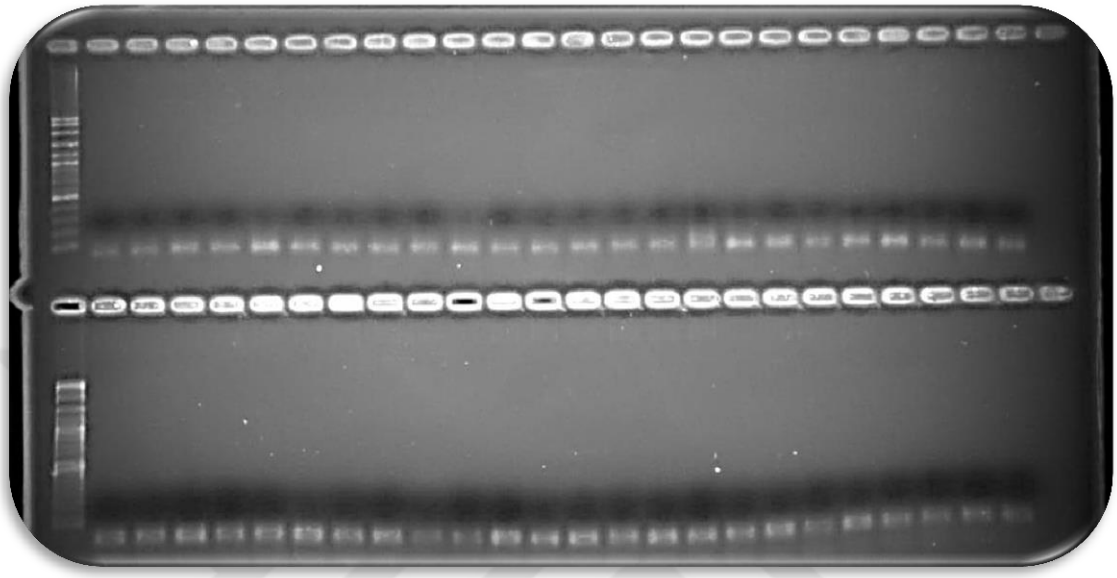
(b)

Figure 4.11. Agarose gel image of ApMV result

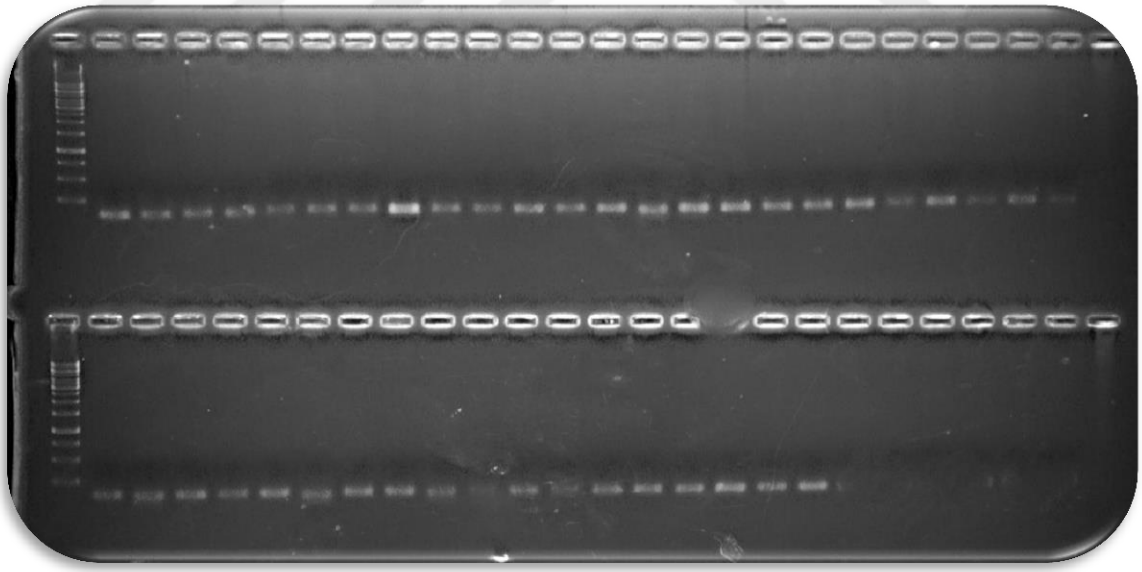
1-48(a), 49-90(b)

4.2.3.9 *Cherry green ring mottle virus* (CGRMV) screening

Screening with CGRMV was done and no anyamplificationwas detectedagainst this primer in Figure 4.12.



(a)

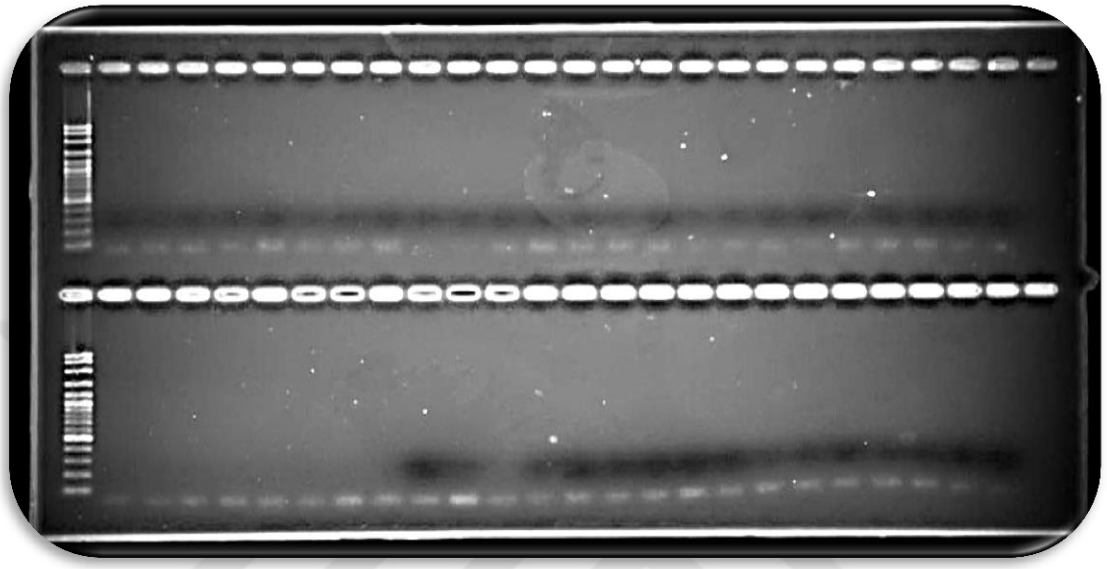


(b)

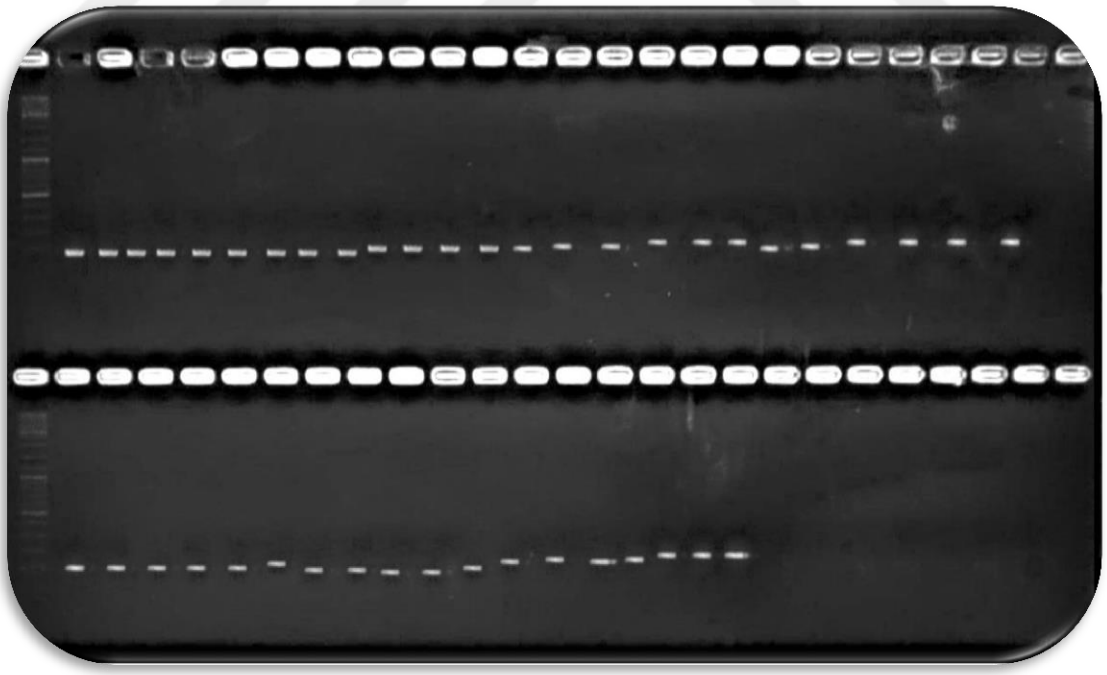
Figure 4.12. Agarose gel image of CGRMV result
1-48(a), 49-90(b)

4.2.3.10 *Cherry necrotic rusty mottle virus*(CNRMV) screening

This experiment was done with CNRMVspecific primers. No amplification detected as a result of this experiment (Figure 4.13).



(a)

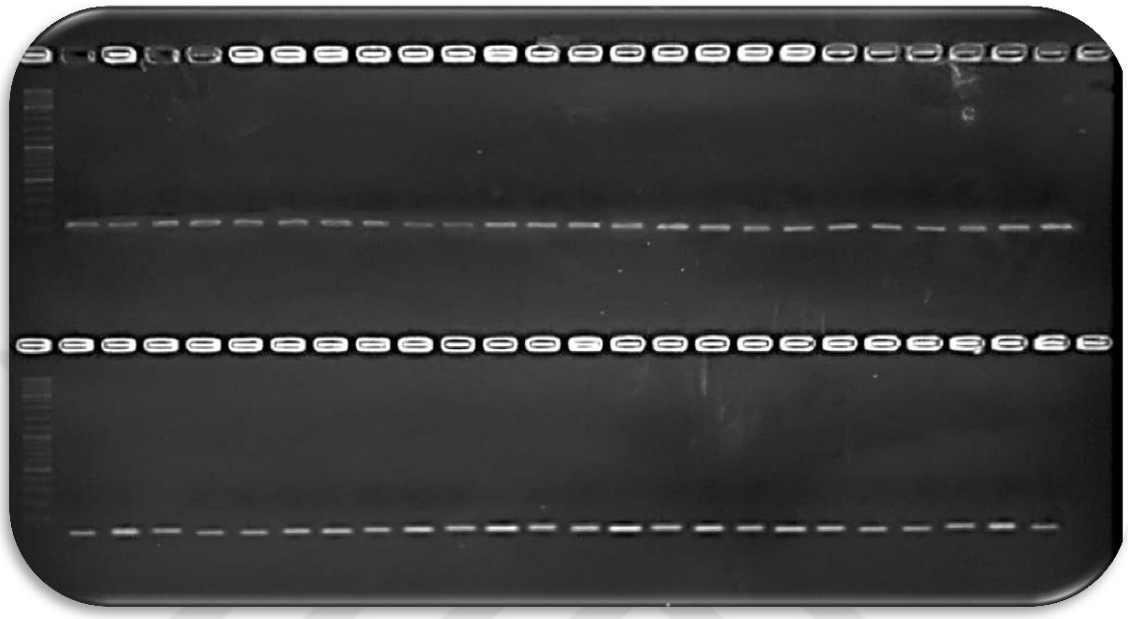


(b)

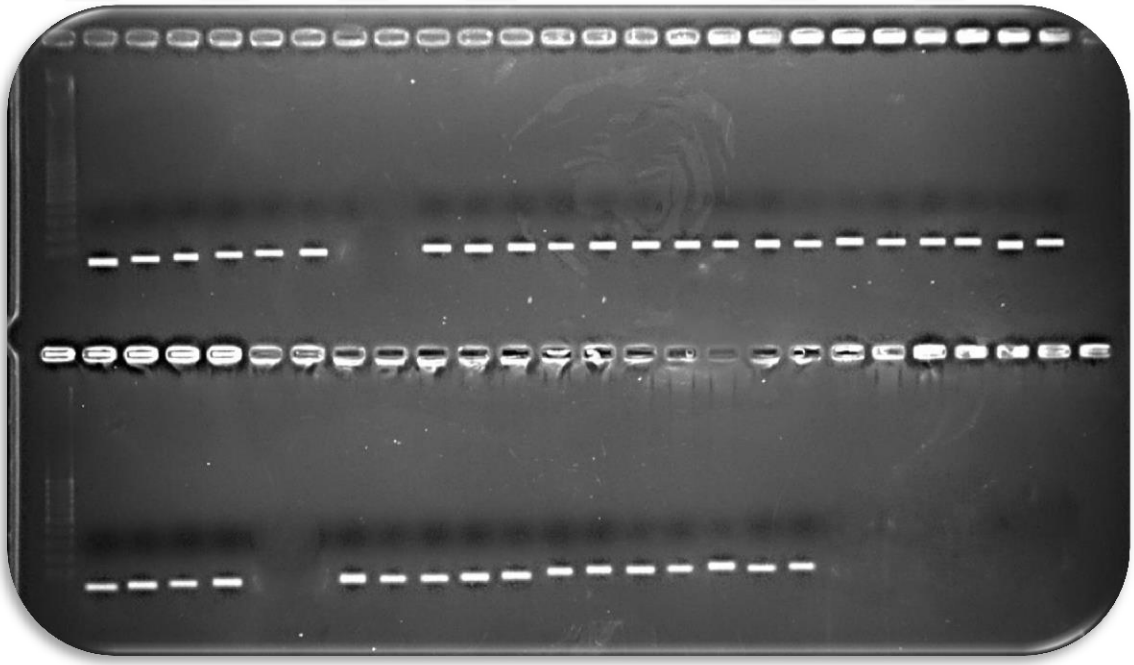
Figure 4.13. Agarose gel image of CNRMV result
1-48(a), 49-90(b)

4.2.3.11 *Cherry mottle leaf virus* (CMLV) screening

All samples were screened in this experiment with CMLV primer but no any positive amplifications were detected(Figure 4. 14).



(a)



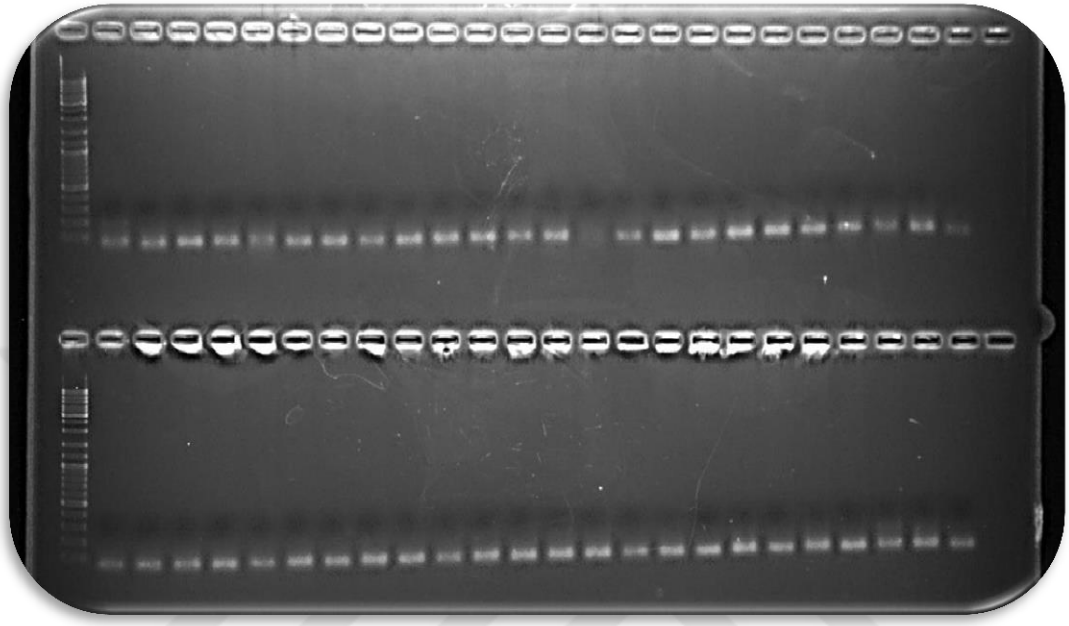
(b)

Figure 4.14.Agarose gel image of CMLV result

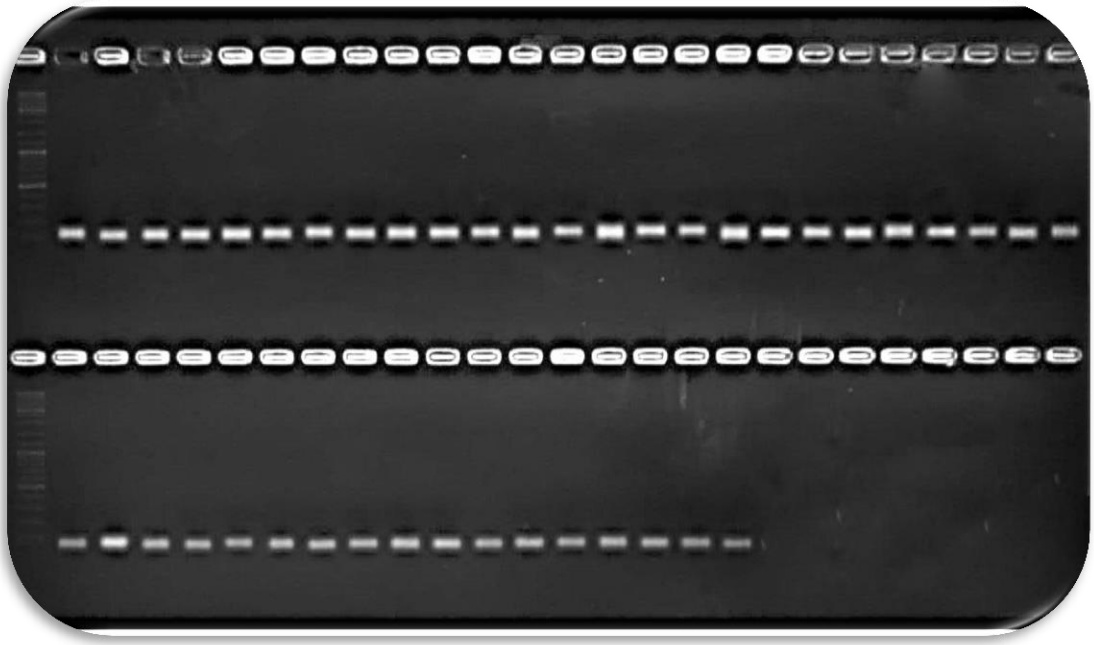
1-48(a), 49-90(b)

4.2.3.12 *Cherry twisted leaf virus* (CTLV) screening

All suspicious samples were screened against CTLV. No any bands were detected as a positive result (Figure 4.15).



(a)



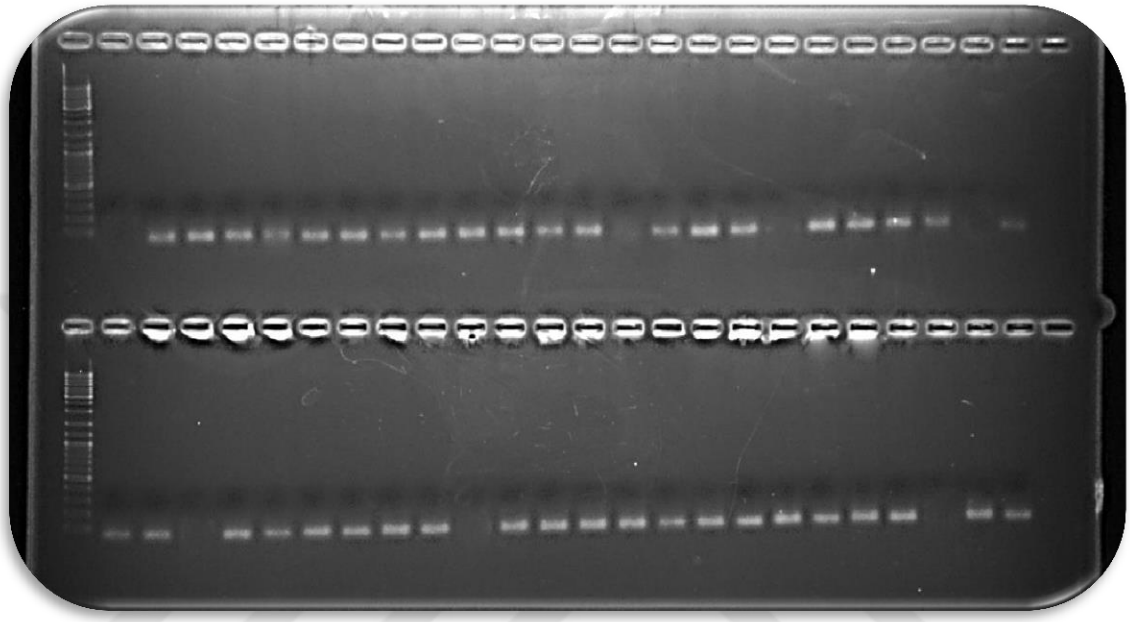
(b)

Figure4.15. Agarose gel image of CTLV result

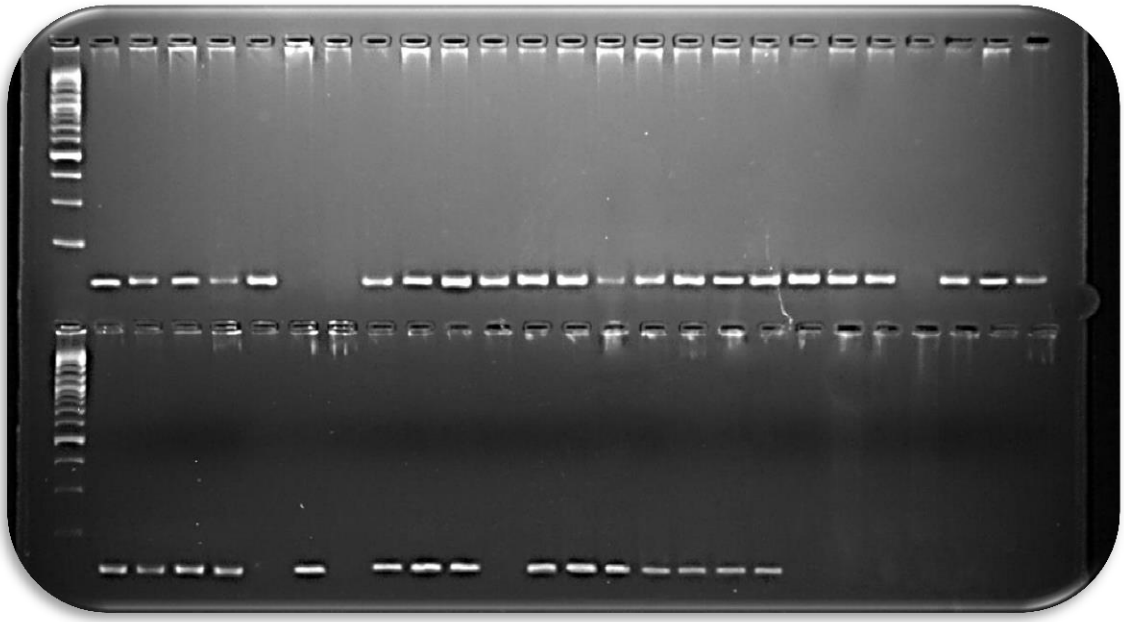
1-48(a), 49-90(b)

4.2.3.13 *Prune dwarf virus* (PDV) screening

PDV was also screened along all samples of cherries both sweet and sour. No any positive amplification wasfound with this primer screening (Figure 4.16).



(a)



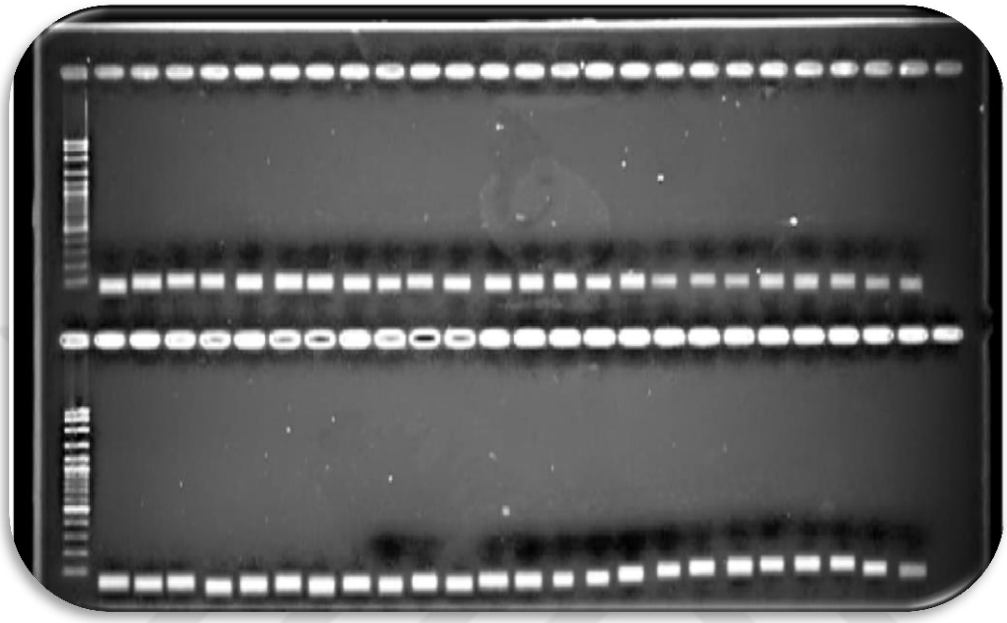
(b)

Figure 4.16. Agarose gel image of PDV

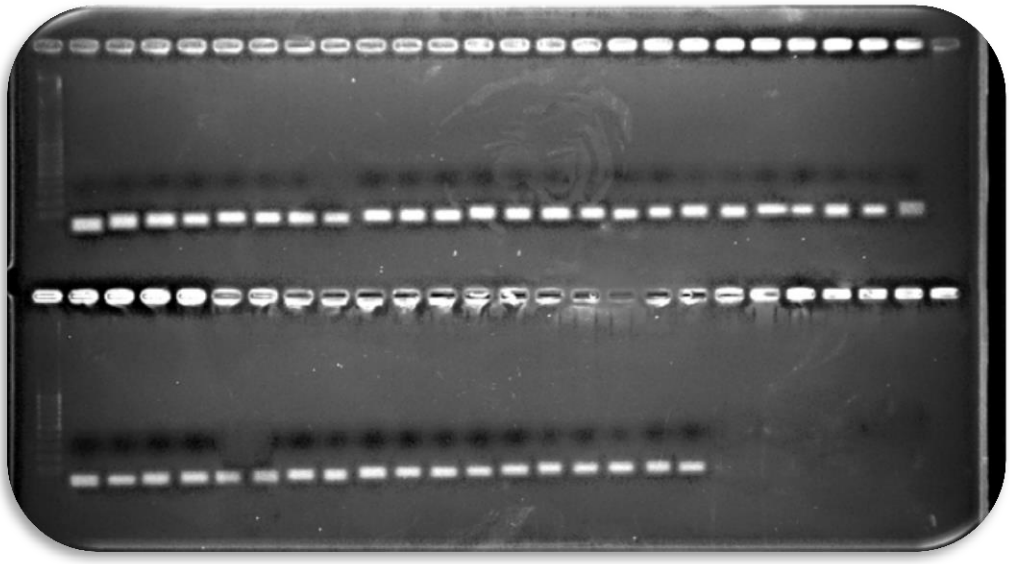
1-48(a), 49-90(b)

4.2.3.14 *Little cherry virus 2* (LChV2) screening

Samples were screened witha CPprimer belongs to this virus but no detection were foundin this result(Figure 4.17).



(a)



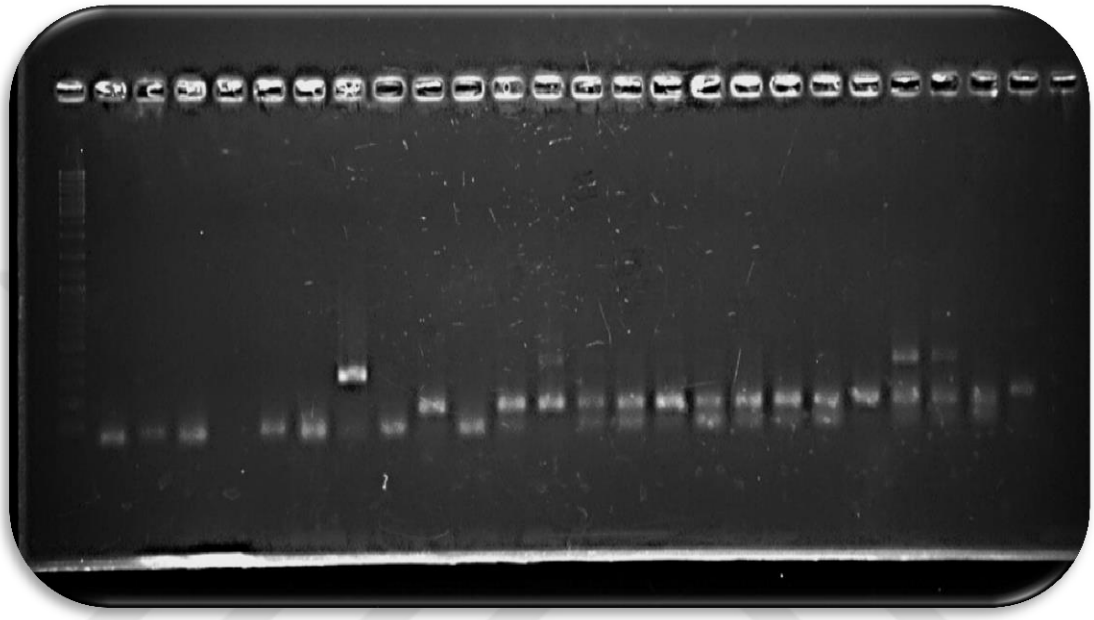
(b)

Figure 4.17. Agarose gel image of LChV2

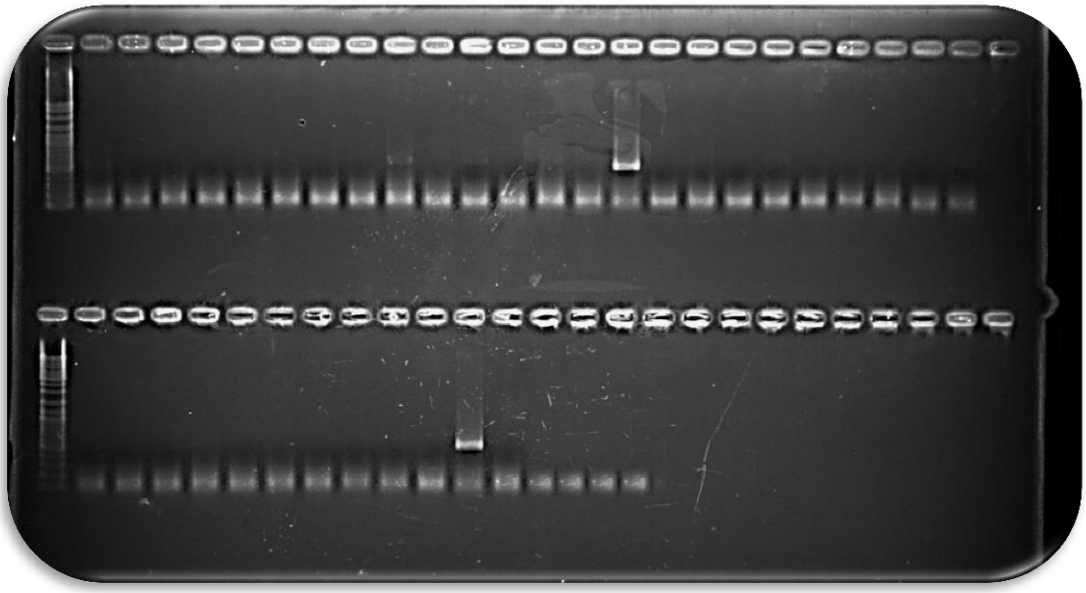
1-48(a), 49-90(b)

4.2.3.15 *Little cherry virus 1* (LChV1) screening

LChV1 primerwas used in this experiment against some cherry plants sample. All samples were screened with LChV1along with internal control nad5.As a result, some plants were found to be positive toLChV1 infections. This experiment was repeated twice and screened same results(Figure 4.18).



(a)



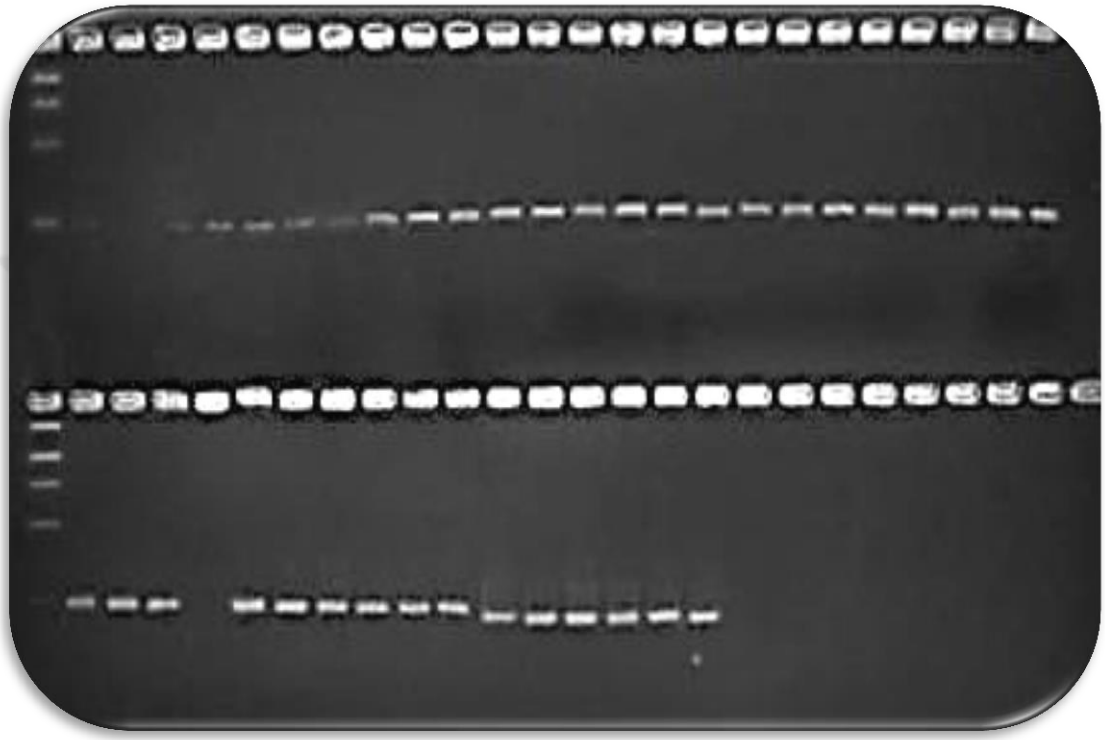
(b)

Figure 4.18. Agarose gel image of LChV1 results

1-48(a), 49-90(b)

4.3 Genetic Diversity Analysis of LChv1

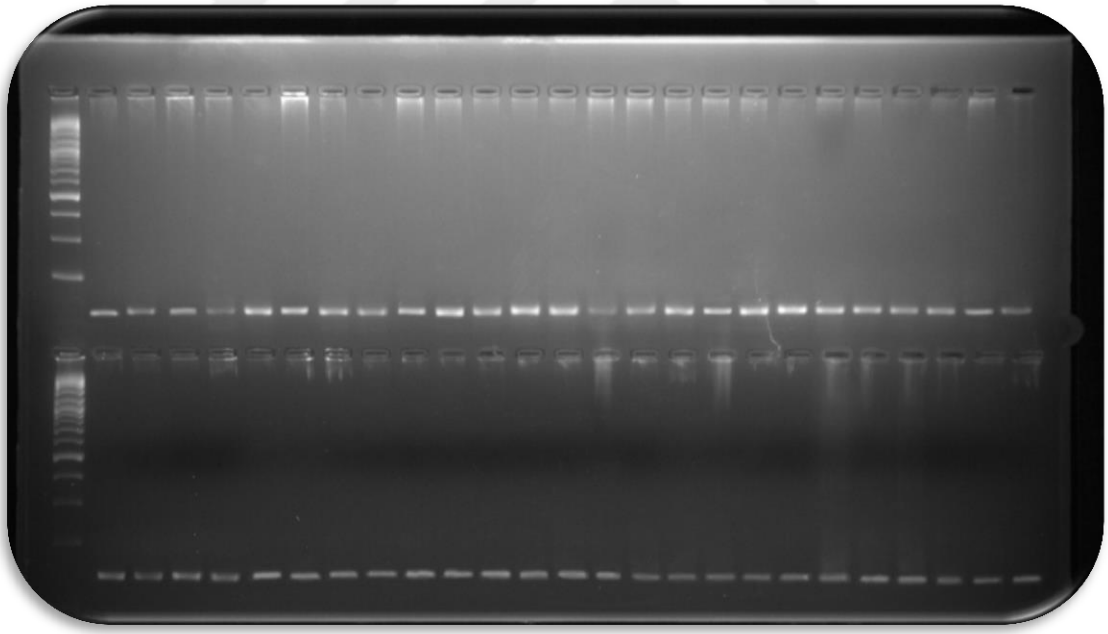
To analyze genetic diversity of LChV1 isolates, four different gene regions screened by PCR analysis. However, none of the primers were gave expected amplification product(Figure 4.19).



(a)

(b)

(c)



(d)

Figure 4.19.Agarose gel image of LchV1 (RdRp) result (a), Screening of LChV1 positive samples with CP primer (b), Screening of LChV1 positive samples with p21 primer (c), Screening of LChV1 positive samples with Hsp70h and p27 primer (d).

All the samples were screened with different primers to check their genetic diversity for the improvement and confirmation of results. But no anyexpected amplification wasdetected by using these primers (Fig. 4.20).

4.4 DoubleStrandedRNAExtraction

Double stranded RNA extraction was used for further analysis of cherry samples.This experiment was used for only 6 suspicious LChV1 samples to confirm the presence or absence of infection. Only one sample was found to have a low quantity of virus shown in Figure 4.20.

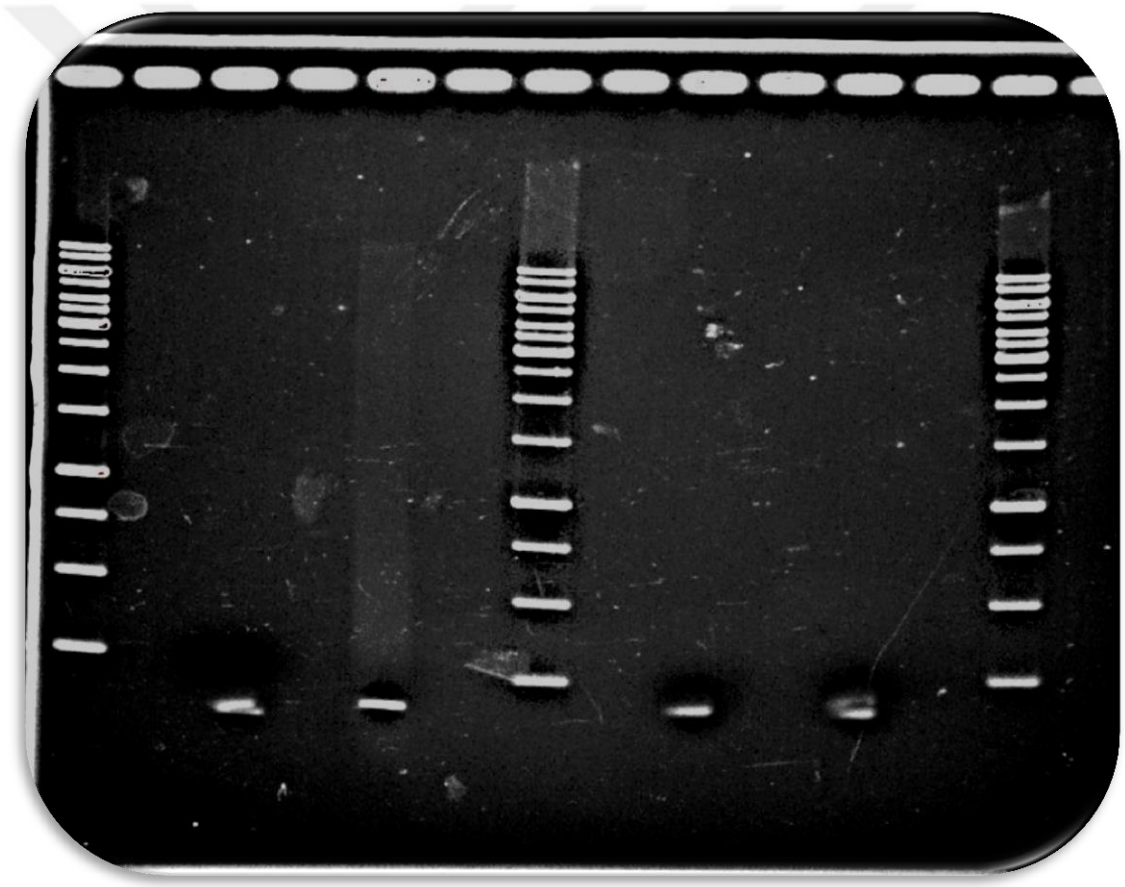
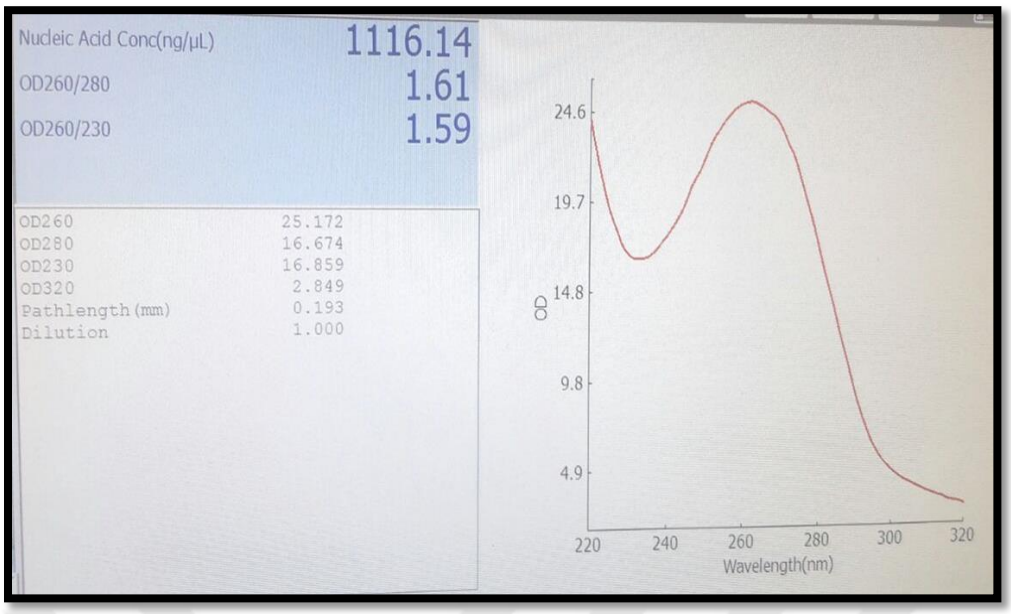


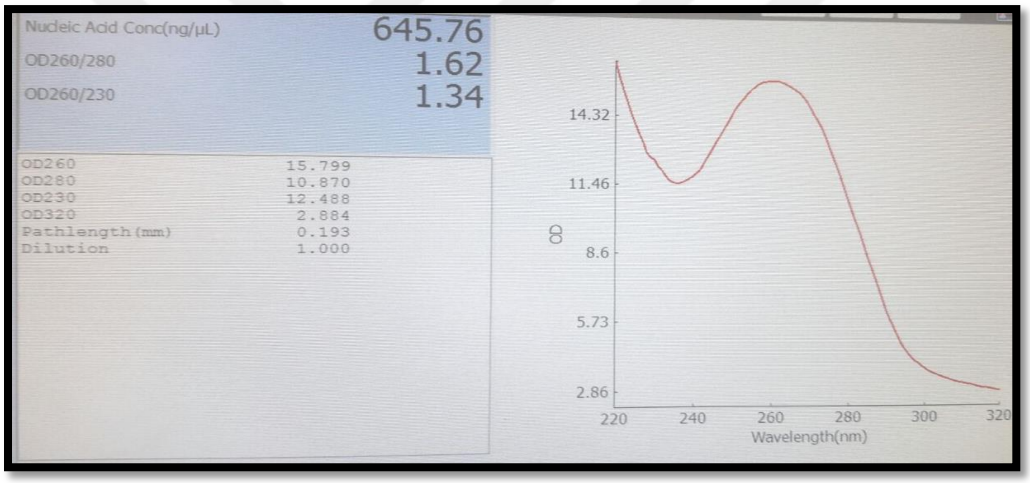
Figure 4.20. Agarose gel image showed result of dsRNA analysis

4.4.1 cDNA synthesis

dsRNAs were used as a template for cDNA synthesis. Both random primers and LChV1 CP specific primers were used for cDNA synthesis. Results of cDNA synthesizedfrom dsRNA were shown in Figure 4.21.



(a)



(b)

Figure 4.21. Spectrophotometer results of cDNAs synthesized from dsRNAs sample no 12(a) ,sample 26(b).

For PCR amplification different kinds of primers were used as random primers which are for cDNA preparation and other are specific primers which are specifically designed for detection.

4.4.2 PCR analysis using cDNA of dsRNA

PCR using cDNA of dsRNA samples were done with only strong positive samples as 8, 12, 21, 31, 33, 57. LChV1 RdRp primer was used, butnoamplification was observed as a result (Figure 4.22)

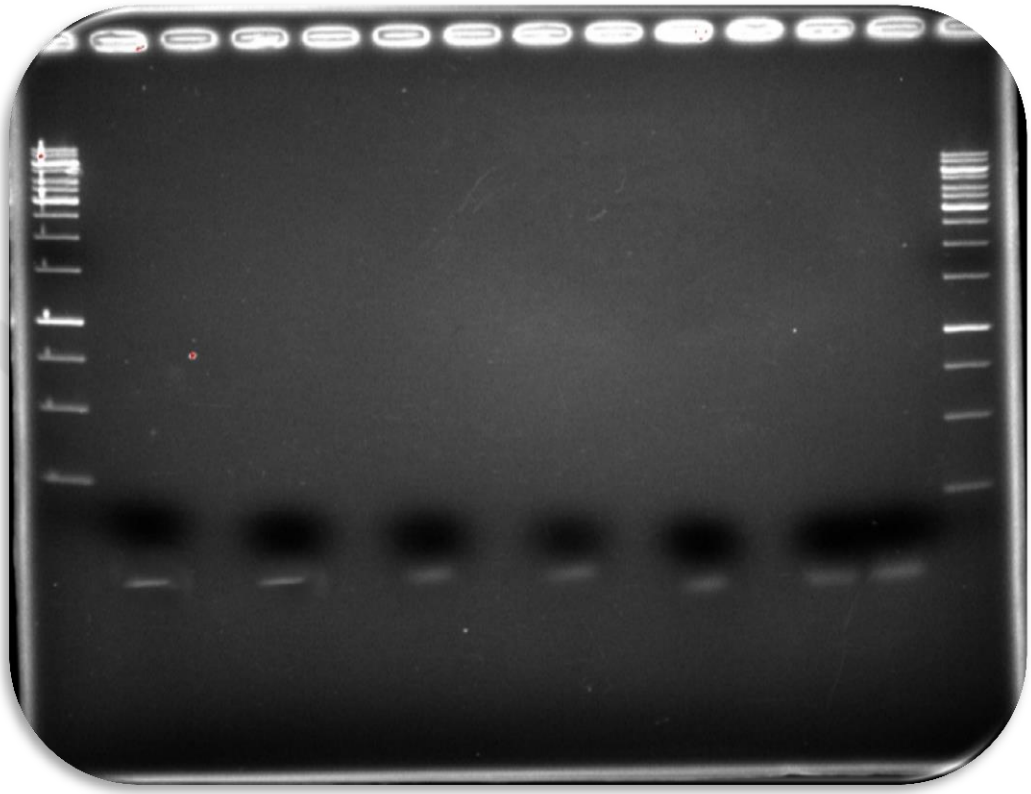
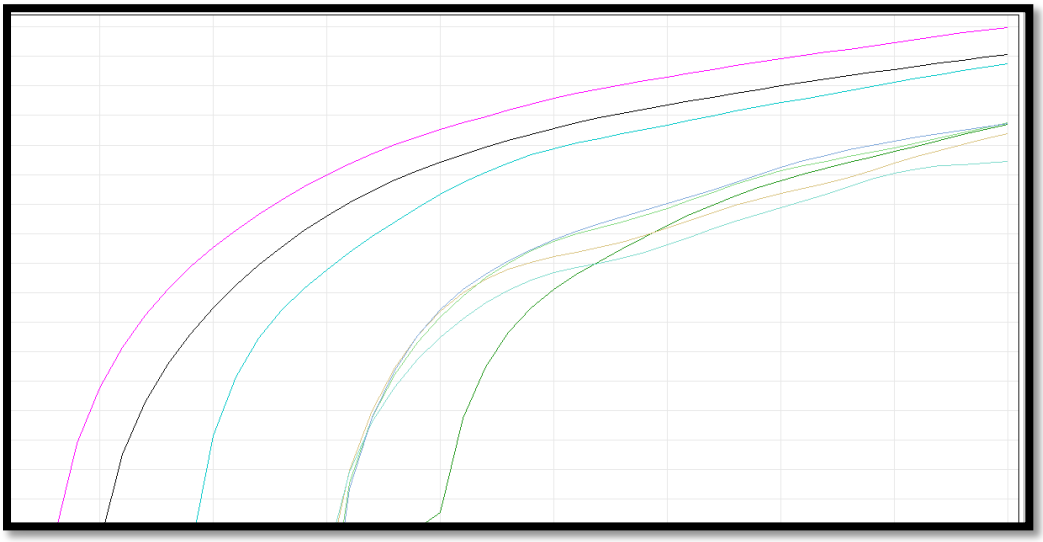


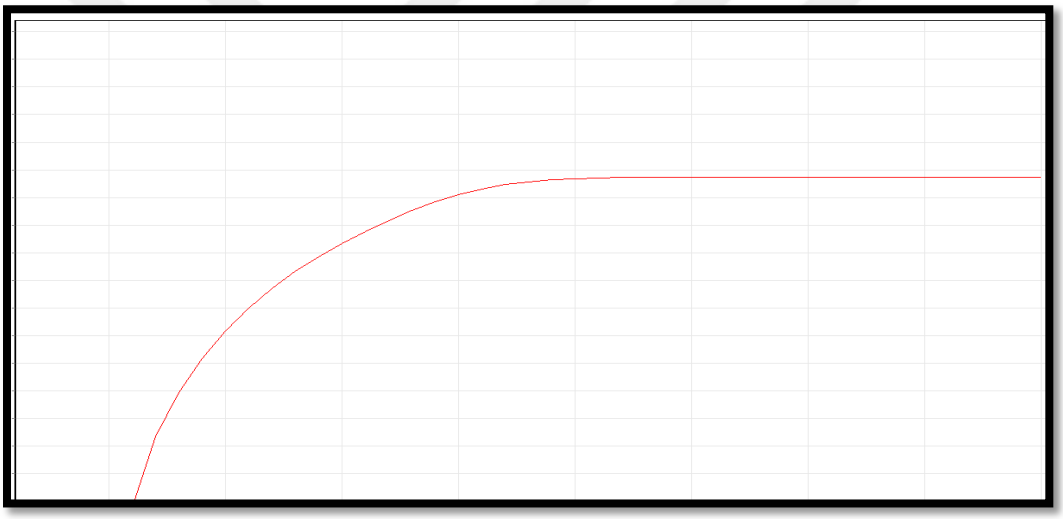
Figure4.22. Agarose gel image of screening of positives with specific CP primers

4.5. TaqMan Real-Time PCR Analysis

TaqMan Real-Time PCR method was also used to confirm virus incidence due to its sensitivity on low quantity viruses.Two different protocols were usedfor optimization of system. In first experiment it showed different curves which were not clear. The second protocol was optimized for detection and the results are shown in Figure 4.24 (a) Real-Time PCR analysis protocol 1 (b) Real-Time PCR analysis protocol 2.



(a)



(b)

Figure4.24. LChV1Real- TimePCR analysis ofsamples 8, 12, 21, 22, 31, 33, 57. Real-Time PCR protocol 1 (a), Real-Time PCR protocol 2. The curve shows only one amplification of sample 8 (b).

CHAPTER V

DISCUSSION

Diseases caused by viruses on cherries are one of the most important and destructive problem on the decreasing the yield and qualityof cherries. Scientist all around the world focuses on molecularmethods to detect these severe diseases and after detection by elimination or limitation of the virus trying to improve the production. One of themost efficient and promising technique is designing of virus specific primer against that specific virus to identify and cope with these problems and with the help of their results we can easily overcome this severe problem to increase yield and for better production.

Therefore, the current study has been accomplished for detection of some cherry viruses with molecular techniques inNiğde, Turkey. Niğde is known for the production of best cherries in Turkey producing about 23.660tons of cherries annually (TUIK., 2017) But till yet no studies have been found for the incidence of viruses in this province which help growers to increase yield and overcome loss. While, these viruses are already identified in many parts of Turkey; however, there are still many provinces which have never been studied to check the presence of these viruses.

A total of 90 cherry samples were selected along the basics of their symptoms. All samples used in this study selected randomly. Fifteenprimer pairs were used for screening of these samples against specific viruses including LChV1, LChV2, CNRMV, ApMV, PDV, CGRMV, CLRV, CMLV, ACLSV, ASPV, ASGV, CRLV, CTLV,PBNSPaV and PNRSV. Some of these viruses are included as a quarantine disease in EPPO list. As apositive controlnad5 (NADH dehydrogenasesubunit 5)181 bp were used as internal control. No any virus specific amplicon were detected as a result of screening of our samples, exceptLChV1.

Double stranded RNA extractionswere also performedto detect LChV1 infections and it showed one low quantity amplification as the smear while observing on the gel. TaqMan Real-Time PCR analysis was doneon that suspicious samples using LChV1

primers and TaqMan probes to confirm its presence of absence. There was only one amplification which observed on one of suspicious cherry samples. One amplification was detected by TaqMan Real-Time PCR. Because of the unavailability of incidence of viruses disease in Niğde province, this will be helpful for growers in this regard because till yet no virus infection had been reported in this province.

The molecular techniques used in this research have tenable data on the comparative occurrence of virusescause infection in different stone fruits. However previously there was no such kind of information. Many of the viruses used in this study were usually disseminated by some insect pest, grafting, pollen or seeds (Nemeth, 1986). Since growers in the province knows much about how viruses and viriods infection and how their dissemination can be controlled.We are confident that the results of this survey will be useful for cherry growers in future.Because, these viruses can cause severe loss in yield and decrease the production.In the end it is concluded that there is not any severe yield loss in Niğde because of the viruses.

CHAPTER VI

CONCLUSION

Cherry is one of the most important stone fruit around the world. Cherry is one of the important fruit used for fresh and dried consumption. Most of the viruses as LChV1, CNRMV, CGRMV, ACLSV, ASGV, ASPV, ApMV, CLRV, PDV, LChV2 and CRLV infecting cherries are including in EPPO list of quarantine diseases having great yield loss in all over the world as well as in Turkey. The current study will be supportive for the growers to produce infection free cherries in Niğde province. The main conclusions of the current study are;

- ✓ The available collected samples are not infected by any targeted viruses in this research.
- ✓ No any amplificationwas detected on cherry samples exceptLChV1.
- ✓ CNRMV, CMLV, CRLV, CTLV, ASPV, ASGV are the viruses which were the first time investigated in this study, have to be surveyed in larger cherry production areas to perform risk analysis of these viruses in our country.

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