MOLECULAR CHARACTERIZATION OF CANNA VIRUS AND DETECTION OF TICK-BORNE VIRUSES

By

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MOLECULAR CHARACTERIZATION OF CANNA

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Abstract: Cannas are the most popular ornamental flowers in *Cannaceae* family. However, cannas are in threat due to various virus infections, especially by *Canna yellow mottle virus* (CaYMV; *Badnavirus*). *Badnavirus* is a plant pararetrovirus (Family: *Caulimoviridae*) or DNA reverse transcribing virus with a genome size of 7.2-9.2 kb. Large scale spread of *Badnavirus* is primarily based on vegetative propagation in perennial hosts. Due to easy exposure to viruses during propagation, the mode of transmission of virus is a key factor to study. The first transmission study was plant-to-plant transmission, where infected and healthy plants were grown in the same pot, followed by soil-borne transmission where healthy plants were grown in the soil where infected plants were grown previously. Also, vector transmission, mechanical transmission and nematodes transmission experiments were performed. A complete genome sequence of CaYMV-AP01 was obtained using massive parallel sequencing. BLAST confirmed the genome of CaYMV-AP01 shares 94% similarity with that of the CaYMV-AP (ginger sequence). There was no transmission of viruses to the healthy plants. Although CaYMV-AP01 was transmitted to healthy cannas in soil-borne transmission experiment. The research studied the infection and transmission of a new virus, CAYMV-AP01.

Additional research on the tick-borne viruses; *Powassan virus* (*Flaviviridae, Flavivirus*) and *Heartland virus* (*Bunyaviridae, Phlebovirus*), were studied which are infectious to humans. Primers were designed to identify the viruses and quality and sensitivity tests were carried out. A plasmid carrying PCR diagnostic inserts was used as a positive control and to mimic infected samples. Similarly, primers designed to detect tick-borne viruses was found sensitive enough to detect even in 1 nanogram quantity of plasmid. A successful mimicking of infection by ticks carrying viruses were observed by PCR and gel electrophoresis. PCR experiments using ticks spiked with plasmids carrying the diagnostic segment specific for each virus were successfully carried out. Hence, the development of primers for detection of viruses causing human disease is significant for future research as well as for rapid detection and analysis in hospitals.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

LITERATURE REVIEW

Canna virus

Family *Caulimoviridae*

Caulimoviridae are unenveloped semi-circular icosahedral or bacilliform double-stranded DNA plant viruses. *Caulimoviridae* are the only double-stranded DNA viruses that infect plants (Geering et al. 2014). According to the international committee on taxonomy of viruses (ICTV), virus particles in *Caulimoviridae* have an average diameter of 35-50 nm for bacilliform and 50-52 nm for icosahedral and a length of 900 nm. *Caulimoviridae* are known as a plant pararetroviruses or DNA reverse-transcribing viruses because during the process of replication reverse transcription is performed by the virus (Geering 2014). This family consists of all plant viruses that have a reverse transcribing phase of the double-stranded DNA genome during its life-cycle. Due to a reverse transcribing capability, *Caulimoviridae* has been compared with retroviruses. However, there is a distinct difference between these two families. Viruses in the *Caulimoviridae* do not encode integrase for the purpose of the integration of the viral genome into the hosts for replication (Fig. I. 2) unlike the retroviruses (Geering 2014).

The genome of *Caulimoviridae* is open circular with 7000-8200 basepairs with discontinuities in both strands.

There are eight genera in the family *Caulimoviridae* which are further classified into 53 species. Each genus defers from the others in the genome (coding region) organization and open reading frames (ORFs). Out of the eight genera, six are icosahedral and contains six or seven ORFs and 10 species (type species *Cauliflower mosaic virus*; CaMV), the *Petuvirus* has one ORF and one species (type species *Petunia vein clearing virus*; PVCV), the *Soymovirus* have eight ORFs and four species (type species *Soybean chlorotic mottle virus*; SbCMV), the *Cavemovirus* contains five ORFs and two species (type species *Cassava vein mosaic virus*; CsVMV), the *Rosadnavirus* has one species (type species *Rose yellow vein virus*; RYVV) and the *Solendovirus* have four ORFs and two species (type species *Tobacco vein clearing virus*; TVCV). The remaining two genera are bacilliform-shaped virions. *Badnavirus* have three ORFs and 32 species (type species *Commelina yellow mottle virus*; CoYMV) and the *Tungrovirus*, has four ORFs and one species (type species rice *Tungro bacilliform virus*; RTBV) (Stavolone et al. 2003; Bhat et al. 2016). Recently, a new genera of *Caulimoviridae* family, *Florendovirus* with one to two ORFs have been reported by Geering at al. (2014).

In 1968, *Cauliflower mosaic virus* (CaMV), a *Caulimovirus,* was recognized as the first plant virus to have a double strand deoxyribonucleotide (DNA) genome. Later in 1982, CaMV was considered a type species for the genus *Caulimovirus* by the International Committee on the taxonomy of viruses (ICTV) (Geering et al. 2014). In 1983, while studying CaMV researchers discovered the involvement of reverse transcription which made CaMV the first pararetrovirus. The *Cauliflower mosaic virus*

(CaMV) genome consists of six to seven ORFs and each ORF and product have their own function. The product of ORF I (37KDa) aids the virus in cell to cell movement. The product of ORF II (18KDa) is an aphid transmission protein (Atf), which is the protein that is involved in virus transmission by aphids. Similarly, the protein from ORF III (15KDa) is involved in DNA genome packaging, which is known as the virion-associated protein (Vap). For transmission of CaMV by aphids, the interaction between both products of ORF II (C-terminal domain) and ORF III (N-terminal domain) are required (Leh et al. 2001). Other ORFs functions are: ORF IV encodes the virus coat protein or precursor of capsid protein (Gag), ORF V (79KDa) encodes a polyprotein with reverse transcriptase, proteases and Ribonuclease H (RNase H) (Pol), and ORF VI encodes a transactivator/viroplasmin protein (Tav) which is a multifunctional protein. In CaMV, the proteins Mov, Atf, and Vap are involved in the movement of virus whereas Gag and Pol proteins are involved in replication. In the same way, Tav is responsible for CaMV protein synthesis, reverse transcription, and assembly of the virion (Kobayashi and Hohn 2003). At the moment the expression and function of ORF VII is uncertain.

Genus *Badnavirus*

*Badnaviruse*s are plant DNA viruses in the family *Caulimoviridae* (Bhat et al. 2016). Currently, there are 32 known species of *Badnavirus* with a genome size of 7.2-9.2 kb (Borah et al. 2013). The virion morphology has a non-enveloped bacilliform structure with circular double-stranded DNA. The particles are 30 nm in diameter and have a length of 120-150 nm (Zhang et al. 2017). A typical *Badnavirus* genome has a GC content of 39.6-44.1% and encodes at least three open reading frames (ORFs), namely ORF I, ORF II, and ORF III (Fig. I. 1) (Siju et al. 2008; Borah et al. 2013). The functions

of polyproteins encoded by ORF I and ORF II have yet to be determined (Jacquot et al. 1996; Zhuang et al. 2011). However, ORF III has been found to encode a 216 kDa polyprotein, which is cleaved by an aspartic protease in the post-translational phase (Siju et al. 2008; Tzafrir et al. 1997). The proteolytic cleavage yields functional products including cell-to-cell movement protein, coat protein, aspartyl proteinase, reverse transcriptase, and ribonuclease H (Jacquot et al. 1996). The distribution of *Badnaviruses* shows a proclivity towards tropical and temperate climate zones of Africa, Asia, Australia, Europe, Pacific regions, and the Americas (Borah et al. 2013; Bhat et al. 2016). The first *Badnavirus* was *Commelina yellow motile virus* (CoYMV) (Medberry, Lockhart, and Olszewski 1990). *Badnavirus* species have been found to affect a wide varieties of crops including yam (*Dioscorea* spp. L.), banana (*Musa* spp. L.), pepper (*Piper* spp. L.), petunia (*Petunia* spp. Juss.), tobacco (*Nicotiana* spp. L.), sugarcanes (*Sachharum* spp. L.), cacao (*Theobroma* spp. L.), and coco yam (*Colocasia* spp. L.) (USDA, NRCS plants database; Borah et al. 2013). Due to most *Badnavirus* disease occurring in tropical regions, *Sugarcane bacilliform virus* (SCBV), *Banana streak virus* (BSV), and *Dioscorea bacilliform virus* (DBV), have been the most studied viruses (Borah et al. 2013). In addition to tropical and sub-tropical crops, other ornamental plants such as spirea (*Spiraea* L.) spp., bougainvillea (*Bougainvillea* Comm. ex Juss.) spp., and canna (*Canna* L.) spp. can be infected by badnaviruses (Rajakaruna et al. 2013). Although most of the *Badnavirus* species infect monocotyledonous plants, they are also known to infection in dicotyledonous plants (Bousalem et al. 2009). The wide host range of these viruses can be detrimental to the agriculture or horticulture industries, causing losses in both crop yields and revenue. Depending upon various factors such as virus

species, host plant, cultivars of host plants, and environmental conditions, infection by *Badnavirus* may exhibit mild to moderate symptoms including chlorotic mottle, necrotic streaks, leaves deformation, and reduced internodes. Badnaviruses primarily spread through vegetative propagation, while some such as *Cacao swollen shoot virus* (CSSV), *Commelina yellow mottle virus* (CoYMV), *Kalanchoe top-spotting virus* (KTSV), are known to be seed-transmitted (Quainoo et al. 2008). Similarly, secondary transmission through biological vectors including mealybugs, aphids, leafhoppers, and nematodes, persistently or semi-persistently, occur for various species of *Badnavirus*. Badnaviruses and other pararetroviruses may form integrated fragmented sequences also known as endogenous sequences in some host genomes. These endogenous badnaviruses may be present as a plant genome component and hence are hard to diagnose and manage. A serological method such as Enzyme Linked Immuno Sorbent Assay (ELISA) has been used to diagnose *Badnavirus,* but the heterogeneity of genomes makes this method less species specific if using broad detection antibodies (Bhat et al. 2016). Polymerase chain reaction (PCR) is as a rapid, sensitive, and reliable method for *Badnavirus* detection. Immunocapture PCR (IC-PCR) or multiplex IC-PCR has been demonstrated to detect the episomal genome (Yang et al. 2005). Similarly, real-time PCR and rolling circle amplification (RCA) are promising methods for detection of various *Badnavirus* (James et al. 2011).

Canna Yellow Mottle virus **(CaYMV)**

Canna yellow mottle virus (CaYMV) is a *Badnavirus* in the *Caulimoviridae* family. *Canna yellow mottle virus* was the first reported in 1985 in Japan as a bacilliform *Badnavirus* virus infecting cannas and then reported in 1988 in Minnesota, North

America (Lockhart 1988). *Canna yellow mottle virus* (CaYMV) has been considered as one of the most threatening disease to the canna industry (Chauhan et al. 2015). Symptoms of CaYMV includes necrotic mottle, foliar chlorotic and veinal yellow streaking in leaves, along with streaking of the flowers as well as the stem (Marino et al. 2008; Momol et al. 2004). However, symptoms vary with the color of the leaves as the green leaf varieties of canna shows different symptoms then red burgundy leaf varieties (Chauhan et al. 2015).

For the confirmation of CaYMV in infected plants, reverse transcriptase – polymerase chain reaction (RT-PCR) is conducted with CaYMV-specific primers, CaYMV-3 and CaYMV-4 by (Momol et al. 2004). *Canna yellow mottle virus* (CaYMV) partial genome is reported to have a size of approximately 565 bp (Momol et al. 2004). However, this length is considered to be CaYMV along with an endogenous viral element associated (CaYMV-AP01). Although the primers CaYMV-3 and CaYMV-4 (Table III. 4.) are widely used for the diagnostic purpose of CaYMV by many researchers, previous research found that these primers can detect CaYMV associated with CaYMV-AP01 and failed to detect the CaYMV genome (Wijayasekara et al. 2017). The primers CaYMV-7 and CaYMV-8 (Table III. 4.) were found to detect the entire genome of CaYMV only (Wijayasekara et al. 2017).

Endogenous pararetrovirus

Viruses have always evolved through various interactions with plants. Studies have shown that virus have conducted numerous horizontal gene transfers in host plants (Hull et al. 2000). Due to this reason, viruses play an important role in the evolution of

living organisms (Hotopp 2012). Viruses that require reverse transcription in replication are known as retroelement or transposable elements. Horizontal gene transfer, a process where a virus modifies the genetic material of the host by being a part of the host, is known as endogenization (Feschotte and Gilbert 2012). During endogenization, the chromosomal viral DNA sequence is integrated in the host germ cells and is referred as endogenous viral elements (EVSs). Endogenous viral elements (EVSs) can contain either partial segments of viral genomic sequence or can be the entire sequence (Staginnus and Richert-Pöggeler 2006). Retrovirus in a plant is known as pararetrovirus.

Caulimovirus was discovered as a pararetrovirus in the late 1990s (Feschotte and Gilbert 2012). *Caulimovirus* replicates through reverse transcription but is different than retroviruses. Pararetroviruses do not integrate into the host chromosome whereas retrovirus do (Staginnus and Richert-Pöggeler 2006; Feschotte and Gilbert 2012). Endogenous pararetroviruses (EPRVs) belongs to the family *Caulimoviridae*. Endogenous pararetroviruses (EPRVs) have been found in many plant genomes including petunia, banana, tomato (*Solanum lycopersicum* L.), *Bambusoideae* spp., dahlia (*Dahlia* spp. Cav.), grape (*Vitis* spp. L.), pineapple (*Ananas* spp. Mill.), rice (*Oryza sativa* L.), potato (*Solanum tuberosum* L.), tobacco, and was recently observed in fig (*Ficus carica* L.) (USDA, NRCS plants database; Staginnus et al. 2006; Chabannes et al. 2013). Endogenization in *Caulimovirus* was observed by chromosomal integration that happened accidentally (Feschotte et al. 2012; Holmes 2011). Very little is known about the endogenization of *Caulimovirus* in terms of molecular mechanisms. Due to whole genome sequencing, advances in bioinformatics, and genomic data analysis, understanding of various aspects of *Pararetrovirus* and their identification increased for

eukaryotic organisms (Chiba et al. 2011). Endogenous pararetroviruses (EPRVs) are found in five genera of *Caulimoviridae* (namely *Cavemovirus*, *Badnavirus*, *Tungrovirus*, *Petuvirus,* and *Caulimovirus*). Chabannes (2013) described that active EPRV have always been present in interspecific hybrid plants. There are three such plant hybrids where plant-virus EPRVs activation has occurred namely, in tobacco *Nicotiana edwardsonii-;* [cross between *N. glutinosa and N. clevelandii* (*N. clevelandii* A. Gray Cleveland's tobacco), infected by *Tobacco vein clearing virus* (TVCV) (Lockhart et al. 2000)], in petunia, *Petunia hybrid-*; [cross between violet flower petunia (*P. integrifolia* (Hook.) Schinz and Thell ssp. *Inflate*) and large white petunia (*P. axillaris* (Lam.) Britton, Sterns and Poggenb spp. *axillaris*), infected by *Petunia vein clearing virus* (PVCV) (Richert-Pöggeler et al. 2003)] and interspecific hybrid of banana between banana-; [B genome (*Musa balbisiana* Colla) and edible banana; A genome (*Musa acuminata* Colla) infected by *Banana streak virus* (BSV) (Chabannes et al. 2013; Chabannes and Iskra-Caruana 2013)].

Pararetrovirus replication and expression

Plant pararetrovirus is composed of double-stranded open circular DNA surrounded with a capsid that is either icosahedral or bacilliform in morphology. Such DNAs are discontinuous (overhangs after reverse transcription). Once viral DNA reaches the nucleus, discontinuities are repaired by ligase and repair polymerases (Hohn and Rothnie 2013). Although pararetroviruses' open reading frame (ORF) comprises Pol (coexpressing along with Gag gene can overcome the translational transactivator function of central mutated strain) genes-reverse transcriptase, protease, and RNase H, unlike

retroviruses, pararetroviruses do not contain integrase gene that assist in integration into the host genome (Kobayashi et al. 2003).

As mentioned before, plant pararetroviruses consist of eight genera. However, additional genera have been proposed for pararetrovirus, i.e. *Orendovirus* (Geering et al. 2010). Linear genome prior to reverse transcription is present (Hohn and Rothnie 2013). Each eukaryotic mRNA is responsible for single function but in case of pararetrovirus, pregenomic transcripts have many functions. In case of *Cauliflower mosaic virus* (CaMV), there are two promoters; the 19S, a monocistronic subgenome that translates transactivator/viroplasmin (TAV) and a second promoter the 35S, polycistronic which is mediated by translated TAV from 19S leading to re-initiation mechanism to produce ORFs (pregenomic RNA transcription) (Park et al. 2001). The 35S promoter consists of various sequence elements that binds to a transcription factor and other factors. Binding of the promoter sequence to these factors leads to different expression patterns resulting in a transgenic plant. Two major expression enhancer domains found in plants was known as region A (expression enhancer in leaves) and region B (in roots) (Hohn and Rothnie 2013).

Splicing or derivation of multiple gene products from a single transcript was observed in CaMV RNAs (Froissart et al. 2004). One donor of splicing is located in 35S (leader sequence) and three other donor sites are in ORF I region. Although splice donors are located in different sites, all of the four donors use the same splice acceptor located in ORF II. The mRNA is produced when splicing occurs between 35S and ORF II (Froissart et al. 2004). Similarly, RNA is produced from the rest of the three splicings that encodes ORF I and ORF II. When splicing occurs in CaMV the protein ORF II reduces the

amount of toxic gene product, which can be toxic if present in an excess amount (Froissart et al. 2004; Hohn and Rothnie 2013). Reverse transcription takes place from pregenomic RNA in presence of methionine initiator tRNA which acts as a primer, because the RNA includes a primer binding site. All plant pararetroviruses (including CaMV) use this primer and primer binding site for transcription, while retroviruses use numerous different tRNAs. In this way, reverse transcription of pararetrovirus is initiated.

For translation to take place, there are two strategies; the first shunting and the second TAV which is used by CaMV. A shunt is a loop structure formed in the 5' untranslated region (UTR) of pregenomic RNA and contains numerous small ORFs. In the shunting strategy, 40S ribosome starts scanning but cannot pass the stem-loop structure (shunt). Then initiation complex (genes; elF3, S6K1, RISP and TOR-target of rapamycin; responsible for cell growth and proliferation) helps to overcome the shunt structure and scanning continues to reach the first ORF. Studies by Ryabova and Hohn (2000) have shown that there are few small open reading frames that are interchangeable before the shunt. However, the interchangeability ceases with re-initiation inhibiting ORFs (Ryabova and Hohn 2000). When small open reading frames or stem-loop structure mutated in CaMV, a delay in infectivity was observed indicating that the shunt structure is of great importance in viruses (Pooggin et al. 2012).

In the TAV strategy, when ORF II was replaced with CAT-reporter in CaMV to test translation by using dicistronic reporter, the translation of ORF II was enabled by expression of ORF VI, which codes for TAV suggesting that translation is polycistronic (Park et al. 2001; Hohn and Rothnie 2013). For the purpose of translation of ORF II,

TAV forms a complex with eukaryotic initiation factors (eIF), re-initiation supporting protein (RISP) and 60S ribosomal subunits (Hohn and Rothnie 2013).

Potyviridae

Potyviridae are monopartite and some are bipartite, unenveloped plant viruses with single stranded sense RNA with filamentous particles. According to the International Committee on the Taxonomy of Viruses (ICTV) 2017, the genome size of monopartite *Potyviridae* is 9.3-10.8 kb in size whereas bipartite are 7.3-7.6 or 3.5-3.7 kb. Virions of monopartite *Potyviridae* are 11-15 nm in diameter and has a length of 650-900 nm. Similarly, bipartite are also 11-15 nm in diameter and the size varies from 250-300 nm or 500-600 nm (Wylie et al. 2017).

Potyviridae consists of eight genera namely *Brambyvirus* (type member *Blackberry virus* Y; BVY), *Bymovirus* (type member; *Barley yellow mosaic virus*; BaYMV), *Ipomovirus* (type member; *Sweet potato mild mottle virus*; SPMMV), *Macluravirus* (type member *Maclura mosaic virus*; MacMV), *Poacevirus* (type member *Triticum mosaic virus*; TriMV), *Potyvirus* (type member *Potato virus* Y; PVY), *Rymovirus* (type member *Ryegrass mosaic virus*; RgMV), and *Tritimovirus* (type member *Wheat streak mosaic virus*; WSMV) (Tatineni et al. 2009). All of the genera in this family are monopartite except members of the *Bymovirus* genus like *Barley yellow mosaic virus* which is bipartite (consists of two positive single-stranded RNA molecule) (Wylie et al. 2017; Description of Plant Viruses).

Potyviridae is the largest group of viruses that cause diseases in plants and all horticultural, agronomic, and additional plants can be infected by one or more species of

virus in this family. Given this ability it is the most important virus family infecting plants from an economic aspect. Some of the *Potyviridae* species with significant economic impact are *Potato virus* Y (PVY), *Lettuce mosaic virus* (LMV), *Bean common mosaic virus* (BCMV) in legumes, *Wheat streak mosaic virus* (WSMV) in cereals, *Plum pox virus* (PPV) in stone fruit, *Zucchini yellow mosaic virus* (ZYMV) and *Maize dwarf mosaic virus* (MDMV) in maize (Lopez-Moy et al. 2009).

Potyvirus

The results of prior research (Wijayasekara et al. 2016; Zhang et al. 2017) indicate that infection by *Canna yellow mottle virus* (CaYMV) always occurred in conjunction with other potyviruses. The name *Potyvirus* is derived from "*Poty*" from the type species *Potato Virus* Y. It is the largest genus in the *Potyviridae* family and hence has been the most studied and causes significant losses in a variety of crops compared to other genera of *Potyviridae*. According to International Committee on Taxonomy of Viruses (ICTV, 2017), *Potyvirus* is unenveloped flexuous filaments virions which are 680-900 nm in length and 11-13 nm wide. Potyviruses consist of almost 200 species, *Potato virus* Y (PVY) being a type member.

Potyvirus has a 5' and 3' untranslated region (UTR) with a single open reading frame (ORF). *Canna Yellow Streak virus* (CaYSV) is a *Potyvirus* causing severe symptoms in cannas and is closely related to *Johnsongrass mosaic virus* (Monger et al. 2007). Symptoms for CaYSV includes yellow streaking, mottling, and discoloration in green leaves of canna (Monger et al. 2007). *Canna Yellow Streak virus* (CaYSV) polyprotein codes for 10 proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, Vpg, NIa, Nib and CP)

(Monger et al. 2010). The genome-linked viral protein (Vpg) is attached to the 5' terminal nucleotide and polyA tail in 3'. P1-Pro is a proteolytic protein that cleaves P1-Pro/HC-Pro. HC-Pro is a proteolytic protein that cleaves HC-Pro/P3. Serine-like proteolytic activity, HC-Pro cleaves at the remaining sites of the polyprotein which is presented in Fig. I. 3 (Lopez-Moya et al. 2009).

Mode of transmission

Plant virus transmission

Plant cells are eukaryotic and structurally are very similar to animal cells. However, plant cell consists of robust walls that prevent direct virus infection or penetration into the cells. For plant virus transmission to occur, there are two mechanisms known as horizontal transmission and vertical transmission. Transmission of viruses among the same generation of plants is known as a horizontal transmission. Plants contain an outer protective layer, the virus needs to penetrate the layer to infect the plant and requires an external source or aid. Typically, plants can be damaged in various ways such as hail, through vectors (fungi, bacteria, nematodes, and insects), or pruning tools and can be a source for virus entry. Plants can also be infected if mishandled during vegetative propagation (cutting and grafting) process by growers, farmers, and horticulturists.

Vertical transmission is a process where viruses are transmitted from a parent plant by reproducing either asexually or sexually. Among plant pathogens, viruses are one of the most important agents being successfully transmitted vertically (Mims 1981). Plants produced from asexual reproduction (vegetative propagation) are genetically

identical and new plants developed from either stems or roots or other parts of the parent plant results in virus transmission. Similarly, in sexual reproduction, transmission of the virus can occur by the infection of seed.

Once a virus enters the plant it can cause a systemic infection, most commonly through the vascular system i.e. xylem and phloem, and move into adjacent cells through plasmodesmata (Fenczik et al. 1996). Viral replication occurs inside plant cells and spreads to adjacent tissues and gradually the whole plant. Hence, to protect the plants the route of infection by viruses needs to be studied in addition to the characterization of viruses. The eradication of viruses in plants has been problematic and often unsuccessful. Many studies have focused on the various modes of transmission (soil, plant, nematodes, pollen, mechanical, and insects) of viruses (Castillo et al. 2017). Knowledge of the modes of virus transmission is crucial to help not only in reducing the occurrence of the viruses but also for developing the strategies to control the spread of viruses.

Plant to plant transmission

After a virus infects healthy plant tissue, the virus moves through the tissue resulting in a systemic infection. Hence, the virus can be found in areas such as leaf and stem hairs. When a healthy plant is in physical contact with an infected plant, a virus can simply move from the infected plant to the healthy plant through damaged cells (Stevens 1983) resulting in plant to plant transmission. However, for infection to take place a large number of viral particles need to be present. Therefore, healthy plants can be vulnerable to disease outbreaks when grown in proximity with infected ones (Stevens 1983).

In the case of ornamental plants such as canna, petunia, and rose (*Rosa* L.), people prefer hybrid varieties because of the flower and leaf colors (Rajakaruna et al. 2014). Canna hybrids are mostly produced by vegetative propagation, which is a significant cause for the increase in infected plants. In such cases, if the mother plant is infected then all the cuttings will also be infected. In case of TMV and other virus diseases, when infected plants in the field are slightly damaged either on the leaf hairs or other outer cells the damaged areas leak sap which can be the source of infection (Mathews 2011).

Soil-borne transmission

Viral particles can be found in most parts of plants with the exception of the shoot apical meristem (Mochizuki et al. 2015). Once roots get infected, viruses usually travel upward through the xylem infecting the whole plant. While in the process of moving through the plant, the plant may or may not show symptoms of infection depending upon the virulence of the virus or interactions of the virus and the host plant (Andika et al. 2016). There are few soil-borne viruses known to cause symptoms in the infected plants roots or organs located underground such as *Beet necrotic yellow vein virus* (BNYVV) (Andika et al. 2016; Biancardi et al. 2016).

When infected plants in the field or greenhouse are removed some plant part can remain in the soil. These remains can act as a reservoir of viruses and be the source of inoculum for the new crop (Mathews 2011). Some viruses such as *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) are considered to be soilborne stable viruses and can be a challenge to completely eradicate from the soil. Soil sterilization or chemical methods used commercially to eradicate viruses have limited effectiveness

because of widespread of virus-carrying underground vector (Andika et al. 2016). Few changes such as the use of sand/peat substrates instead of soil have been a significant development in combating the problem of soil-borne transmission (Mathews 2011).

Vector transmission

Insects that have pierce-sucking type mouth parts are the most important vectors of viruses and pose an economic threat to horticultural crops (Blanc et al. 2011). Many pierce-sucking type insects have been extensively studied and include aphids, whiteflies, mealybugs, and leafhoppers (Blanc et al. 2011). Insect vectored viruses have four specific methods for transmission; (Table I. 1) (Fig. I. 4.): a) Non-persistent transmission, b) Semi-persistent transmission, c) Circulative non-propagative transmission and d) Circulative propagative transmission, which can vary depending on the time required for the vector to acquire, have the virus circulate in the vector and then transmit (Roossinck 2015).

a) Non-persistent transmission. Viruses get attached to the external mouthpart of the vector; stylet, (Fig. I. 4b) while feeding on the infected plants. Later when the vector feeds on the healthy plant, these viruses are released (Blanc, Uzest, and Drucker 2011).
b) Semi-persistent transmission. Similar to non-persistent transmission. Viruses do not internalize in the gut of the vector but do stay in the stylet of the vector for longer time than non-persistent transmission (Dietzgen, Mann, and Johnson 2016).

c) Circulative non-propagative transmission. Viruses move through the food canal to the gut (midgut than hindgut) of the vector and gradually enter hemolymph. When vector feeds on the plant, viruses come out of the salivary glands of the vector and are released into the plant (Blanc, Uzest, and Drucker 2011).

d) Circulative propagative transmission. Same as circulative non-propagative but with one exception. During the movement of viruses in the inner body of the vector, viruses replicate infecting vector cells as well.

Nematode transmission

Most plant parasitic nematodes feed on plant roots in the soil. In 1958, the first scientific evidence documenting the plant to plant transmission of viruses by nematodes was provided by Hewitt, W. B., Raski D. J., and Goheen, A. C. Additional evidence for nematode transmission was for the *Grapevine fanleaf virus* that could be transmitted by *Xiphinema index* (Thorne and Allen, 1951) from infected plants to a healthy plant (Raski et al. 1983; Wyss 2000). Nematodes are known to transmit some of the viruses in the genera *Nepovirus* and *Tobravirus* by acquiring and feeding on infected roots. Generally, there are two families of nematodes that transmit plant viruses the *Longidoridae* that consists of three genera *Longidorus, Paralongidorus* and *Xiphinema*, and *Trichodoridae* consisting of two genera *Trichodorus* and *Paratrichodorus*. *Longidoridae* are vectors for the isometric or polyhedral-shaped virus known as *Nepovirus,* whereas *Trichodoridae* are vectors for straight or tubular shaped virus known as *Tobravirus*. Some of the symptoms in plants caused by root feeding nematodes are necrosis of the root and meristematic discoloration (O'Bannon et. al 1990; Brow, Robertson, and Trudgill 1995).

During a survey of fields in Pakistan, five new hosts for root-knot nematodes namely Santa Maria feverfew *(Parthenium hysterophorus* L.), olive (*Olea europaea* L.), ceylon spinach (*Basella rubra* L.), Indian shot (*Canna indica* L.) and star jasmine (*Jasminium multiflorum* (Burm. f.) Andrews) were identified (Shahina et al. 2012). A fact sheet provided by Plantwise Knowledge Bank, indicates that *Meloidogyne incognita* is cable of infecting canna (CABI, 2018). There are five primary species of root-knot nematodes (*Meloidogyne*) that are of significance *M. incognita, M. javanica, M. arenaria, M. graminicola* and *M. hapla* as these nematodes have wide host ranges and cause large economic losses (Shahina et al. 2012). This research is focused on identifying the 47 nematodes from the soil where cannas are grown to find out the possibility of virus transmission by nematodes in healthy cannas from infected plants.

Mechanical transmission

Mechanical transmission of viruses from infected plants to virus-free cannas can be done either accidentally or intentionally regarding animals yet no specific biological interaction has been confirmed (Stevens 1983). Humans can be one of the major causes of mechanical transmission of viruses. For instance, transmission of viruses through tools, clothing, worker hands, or any devices (where virus such as *Potato Virus* X can survive) used in infected plants when introduced to virus-free plantings. *Badnavirus* generally are detected infecting perennial hosts that are vegetatively propagated. Therefore, an outbreak of *Badnavirus* is primarily based on vegetative propagation (Bhat, Hohn, and Selvarajan 2016). Transmission of the virus can also take place from the knives used for cutting infected as well healthy propagative material (Stevens 1983). Typically, people prefer canna varities because of the vibrant color flowers and variable

leaves. However, for hybrids, farmers solely depend on vegetative propagation (Rajakaruna et al. 2014). Some viruses can also spread while harvesting by hand. Although, viruses can be transmitted mechanically not all are transmissible (Stevens 1983).

When animals come in contact with an infected plant, those animals can act as vector or carrier of a virus. When animals rub against infected plants, animals pick up viruses on their coat and enter in fields of healthy crops, subsequently transferring the virus. There is a study by Broadbent (1963) that shows the possibility of transfer of TMV (*Tobacco Mosaic Virus*) by birds using a similar method (Stevens 1983).

Ticks and associated human viruses

Tick

Ticks are external parasites, in the phylum Arthropoda with insects, and in the subphylum Chelicerata, Arachnida class, and Acari subclass. There are two families of ticks that are of significance to humans and animals, *Ixodidae* also known as hard tick and *Argasidae* known as soft ticks (Soneshine et al. 2013). Hard ticks have long been known to transmit animal and human pathogens. Tickborne diseases are of major medical importance around the world with human and livestock diseases costing millions of dollars and lives annually (Donovan et al. 2002). Ticks are present in large numbers in Oklahoma (Wright et al. 2017) and throughout the United States. Oklahoma State University boasts a facility for rearing ticks for the purpose of study and research. When this facility was established in the mid-1980s, only Oklahoma State University could use the ticks for research purpose but late by the mid-1990s ticks were sold to industries and

other universities. Some important tick-borne pathogens include *Ehrlichia chaffeensis; Francisella tularensis; Rickettsia rickettsiax; Borrelia burgdorferiy; Anaplasma phagocytophilum* that are important among others.

Ixodes scapularis

(Common name: Blacklegged tick/Deer tick)

According to Illinois department of public health (IDPH), there are three active stages of *I. scapularis* and has the tick broad host range for feeding (Keirans et al. 1996). Once an egg is hatched (in spring), larvae feeds on white-footed mice (*Peromyscus leucopus*) as well as other small warm-blooded organisms. Larvae further molt (brown nymph) that feeds on large mammals (human) as well as mice. Once molted into adults (in fall) ticks feed on deer. Adults are dark brown to black in color (Fig. I. 5b). Their habitat (Fig. I. 5a) lies on the wooded areas. Beside *Powassan virus* blacklegged ticks actively transmit Lyme disease and babesiosis. A unique "questing" behavior is observed.

Dermacentor variabilis

(Common name: American dog ticks)

Dermacentor variabilis is native to areas without tree cover (Sonenshine 2013). The ticks are commonly found around trails, feed on variety of host, and in the absence of a host can survive for up to 2 years without feeding. Adult looks like dark brown to reddish brown in color (Fig. I. 6). According to the TickEncounter Resource Center (www.tickencounter.org), these tick have several active stages including the nymphs that are present in summer and feed on rabbits, mice, skunks, and raccoons. The larvae are

found in late spring to early fall and feed on opossums, mice, voles, and rodents. The adults are usually observed in late spring to early fall and found on twigs. The adults feed on skunks, coyotes, dog, raccoons and also humans. In addition to the *Powassan virus*, this tick can also transmit Rocky Mountain Spotted Fever disease and Tularemia (Brites-Neto et al., 2015).

Amblyomma americanum

(Common name: Lone start tick)

The habitat of the lone star tick is dense woodlands and around the resting areas of animals (Cooley and Kohls 1944, Bishopp and Trembley 1945, Kollars et al. 2000). There are three active life stages of lone star tick. The larvae that are active in summer can feed on a variety of host such as deer, squirrels, small birds, and turkeys. After approximately 4 days of feeding larvae molt into nymphs and are often found in leaf litter. Nymphs are active in summer to early fall and feed on raccoons, squirrels, turkey, cat, dogs, as well as humans. Nymphs feed for 5-6 days then fall off the host and molt into adults. Adults are active in late spring to early fall and feed on large animals such as coyotes, deer, cattle, and can also feed on humans. The female has a characteristic white dot in the center of its body and the male has black streak around the outer body (Fig. I. 7). *Amblyomma americanum* can transmit the *Heartland virus* to human but also transmits diseases like 'stari' borreliosis, ehrlichiosis and rocky mountain spotted fever.

Powassan Virus

Powassan virus is in the family *Falviviridae* and in the genus *Flavivirus*. *Powassan virus* is also known as the *Deer tick virus*. *Flaviviridae* consists of positive single stranded RNA enveloped viruses. There are four genera under *Flaviviridae*, namely *Flavivirus, Hepacivirus, Pegivirus*, and *Pestivirus*. Many viruses in this family are pathogenic and also host specific such as *Hepacivirus* genus consisting of the Hepatitis C virus (Simmonds 2017). *Flavivirus* is derived from latin word ("flavus" which means "yellow") while investigating epidemic yellow fever (Huang et al. 2014). *Flavivirus* can infect mosquitos and ticks and the primary animal hosts are birds and mammals. Most all of the known viruses in genus *Flavivirus* are arthropod-borne (arbovirus) (Balasuriya et al. 2017). *Powassan virus* cases are mostly observed in Wisconsin and Minnesota (Fig. I. 8).

The *Powassan virus* causes a neuro-invasive disease similar to West Nile disease in Russia and North America. *Powassan virus* is tick-borne and found to be present in *Ixodes cookie*, *Dermacentor variabilis*, but mostly by *Ixodes scapularis* (Birge and Sonnesyn 2012). In humans the *Powassan virus* causes the disease encephalitis (brain tissue inflammation). Not all humans will experience the disease if fed on by a tick with the virus and a feeding time of 12-48 hours is required for transmission of the pathogen (Ebel and Kramer 2004). Studies has revealed that *Powassan virus* consist of two lineages; Lineage I, transmission by *Ixodes cookie* and *Ixodes marxi* and Lineage II, *Deer tick virus* (DTV) lineage, where transmission is by *Ixodes scapularis* (Brackney et al. 2010). Some of the factors for two different lineages are distinct transmission cycle and

genetic variation; however, the reason for the adaptation of viruses to two different transmission cycles is not known (Brackney et al. 2010).

Heartland Virus (HRTV)

Heartland virus (HRTV) is in the family *Bunyaviridae* and in the genus *Phlebovirus* and Bhanja group. *Bunyaviridae* is a family of enveloped single stranded RNA viruses consisting of five genera: *Phlebovirus, Nairovirus, Orthobunyavirus, Hantavirus* and *Orthotospovirus* (only plant infecting virus: *Orthotospovirus*) (Balasuriya 2017, Boyes et al. 2017). *Phlebovirus, Nairovirus* and *Orthobunyavirus* can replicate alternatively in arthropods and vertebrates (arthropod vector and vertebrate host). Transmission of *Orthotospovirus* is through thrips into a plant and the replication of the virus takes place in both thrips as well as plants (King 2012). In the case of *Hantavirus*, the viruses are maintained in a cycle of vertebrate-vertebrate transmission and no arthropod vectors are found yet (King 2012).

According to International Committee on Taxonomy of Viruses (ICTV), *Phlebovirus* members are differentiated into 10 different complex species groups, type species, *Rift valley fever virus* (RVFV), *Chilibre virus* (CHIV), *Bujaru virus* (BUJV), *Sandfly fever Naples virus* (SFNV), *Uukuniemi virus* (UUKV), *Frijoles virus* (FRIV), *Severe fever with thrombocytopenia syndrome virus* (SFTSV), *Candiru virus* (CDUV), *Punta Toro virus* (PTV) and *Salehabad virus* (SALV) (Wuerth et al. 2016). Transmissions of viruses that belong to *Phlebovirus* is by mosquito, ticks, and sandflies. In 2015 through genetic analysis, the tick-borne *Phlebovirus* was further divided into three groups, Bhanja group, SFTS group, and Uukuniemi group (Matsuno et al. 2015).

The *Heartland virus* was first identified in two infected patients bitten by ticks in June 2009 from Heartland Regional Medical Center in St. Joseph, Missouri from where the name of the virus originated (McMullan et al. 2012). According to Centers for Disease Control and Prevention (CDC, 2017), the *Heartland virus* is transmitted by the tick, *Amblyomma americanum* (common name: Lone Star tick) and other species of ticks responsible for *Heartland virus* are yet to be known.

Table I. 1: Major characteristics of vector transmission.

Types of transmission	**Location of virus**	**Acquisition time** [(a)]	**Retention time** [(b)]	**Transmission time** [(c)]	**Vector**
Non-persistent	Stylet	Minutes	Minutes to hours	Minutes	Aphids
Semi-persistent	Stylet, food canal	Minutes to hours	Minutes to hours	Minutes to hours	Aphids, beetle, mites, leafhopper, thrips, whiteflies
Circulative non-propagative	Midgut, hindgut	Hours to days	Hours to days or to life	Hours to days	Aphids, leafhoppers, treehoppers, whiteflies
Circulative-propagative	Midgut, hindgut, Excretion of the infected vector.	Hours to days	Days to life or to generations	Days to life	Aphids, mites, thrips, leafhopper, planthopper

(Roossinck 2015)

(a) Acquisition time. Time required for acquisition of virus by the insect.

(b) Retention time. Time till when the insect possess virus.

(c) Transmission time. Time when insect feeds on the new host and virus gets transmitted.

Fig. I. 1. *Badnavirus* species with linear representation of genome organization.

The *Badnavirus* genome consists of three open reading frames (ORFs). Each ORF encodes for a product. The function of a product encoded by ORF I and ORF II are yet to be determined (Zhuang et al. 2011). However, ORF III encodes for a polyprotein to include, MP (movement protein), ZnF (Zinc finger binding domain), Pro (Aspartic acid protease) domain, RT (Reverse transcriptase), and RNase H (Ribonuclease H) domain. The gap between each ORF represents a non-coding region.

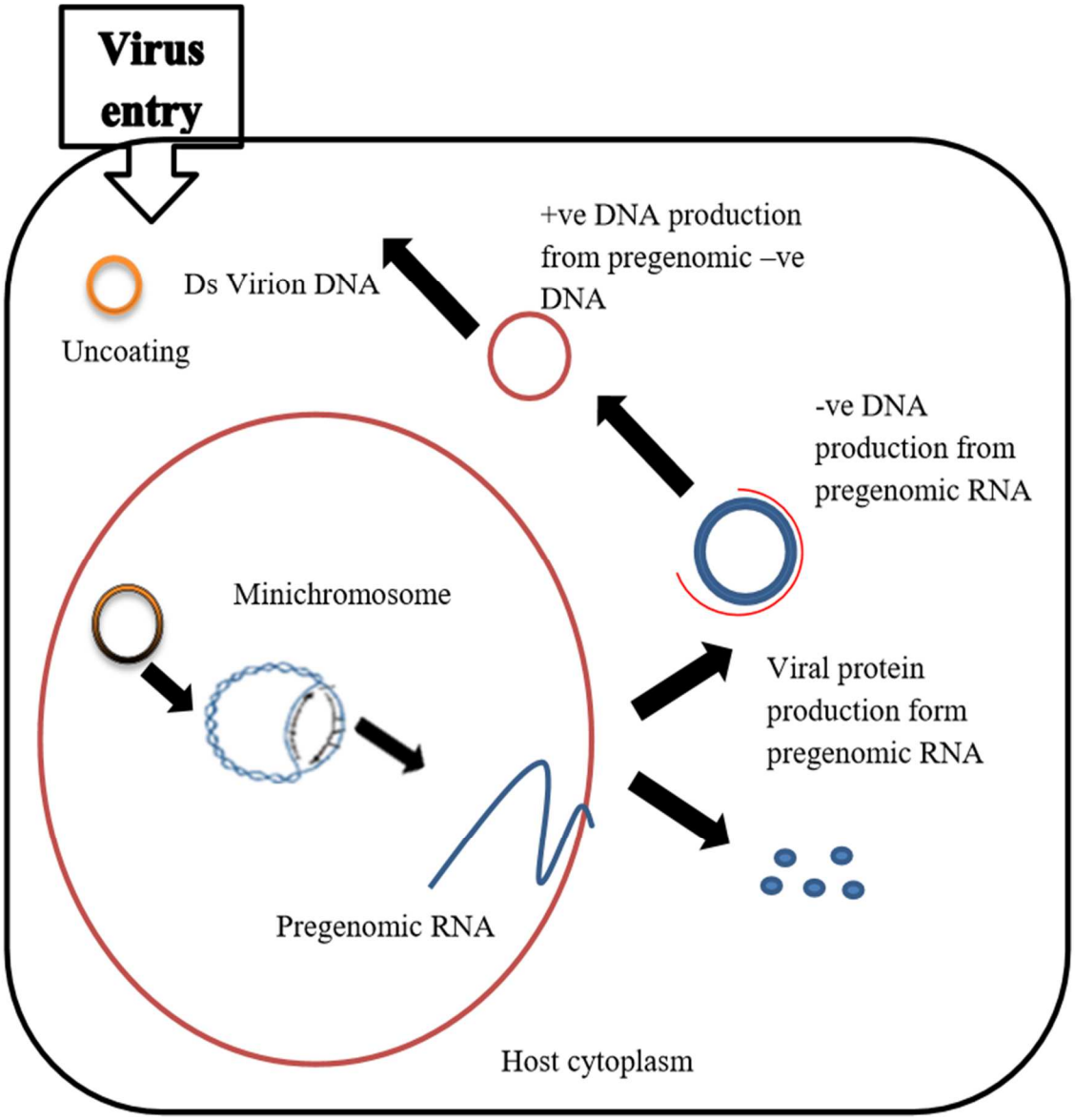

Fig. I. 2. Replication cycle of a virus.

(Pfeiffer and Hohn 1983; Dulanjani Wijayasekara 2017)

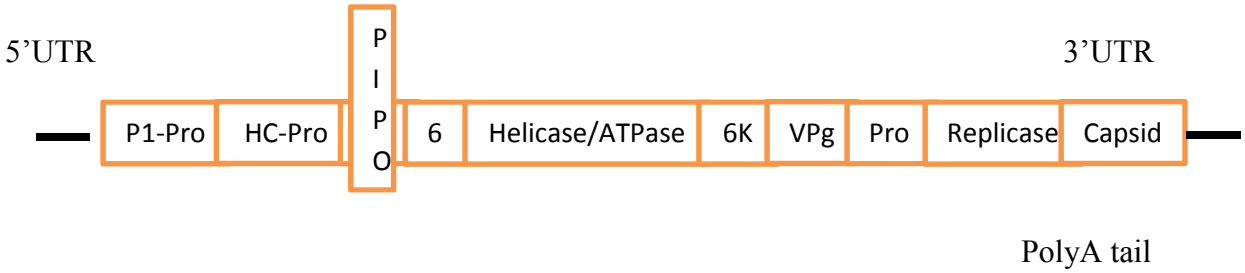

Fig. I. 3. *Potyvirus* genome organization (Lopez-Moya, Valli, and Garcia 2009).

RNA genome is shown from 5'UTR to 3'UTR. Box indicates translated element of ssRNA. P1-Pro protein is responsible for cleavage at P1-Pro and HC-Pro. HC-Pro protein is responsible for cleavage at HC-Pro and P3 and also acts as helper component for aphid transmission factor. Pro protein is responsible for cleavage at the remaining site.

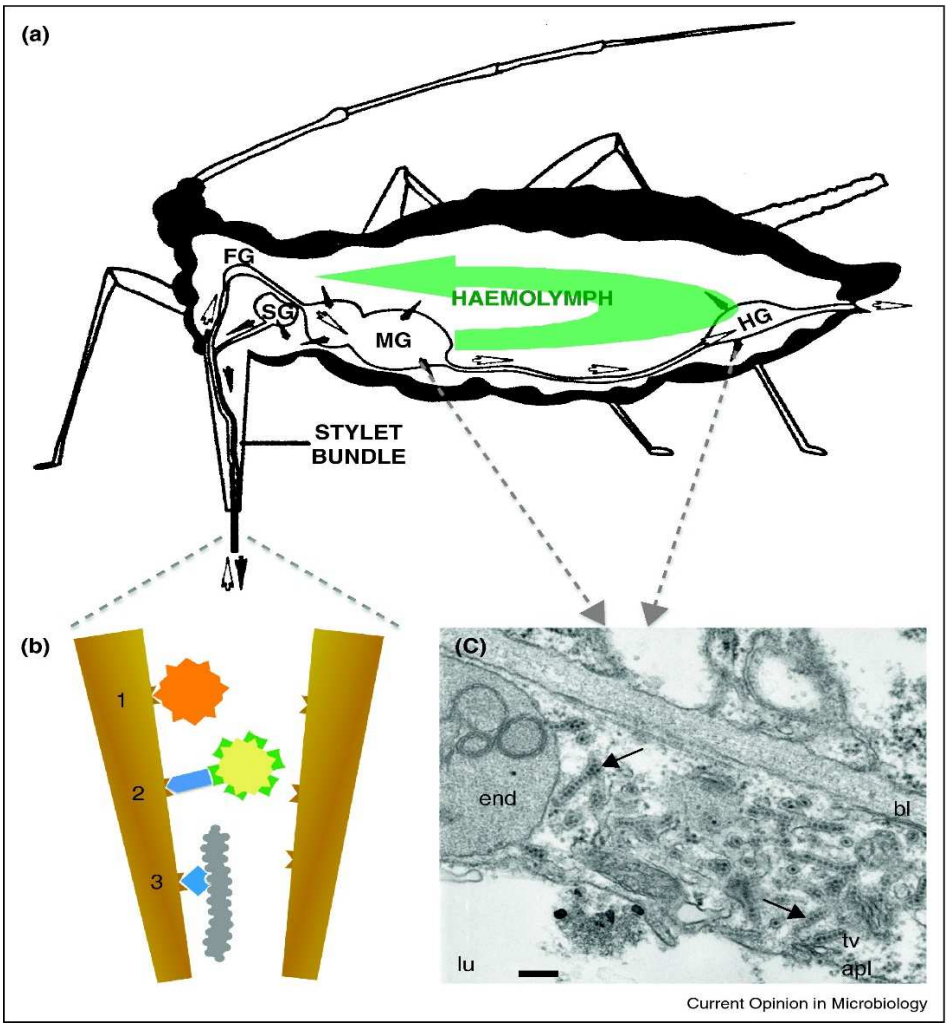

Fig. I. 4. Routes of virus transmission by an aphid. (Blanc, Uzest, and Drucker 2011) (FG, foregut; SG, salivary gland; MG, midgut; HG, hindgut)

a) In a circulative transmission of the virus in the aphid, the virus travels through the gut (green arrow). Some virus can replicate during passing through the body of a vector; circulative propagative transmission.

b) Non-persistent transmission. Virus found in the stylet of the aphid

c) micrograph of *Cucurbit aphid-borne yellow virus* in the hindgut of the aphid.

(a)

(b)

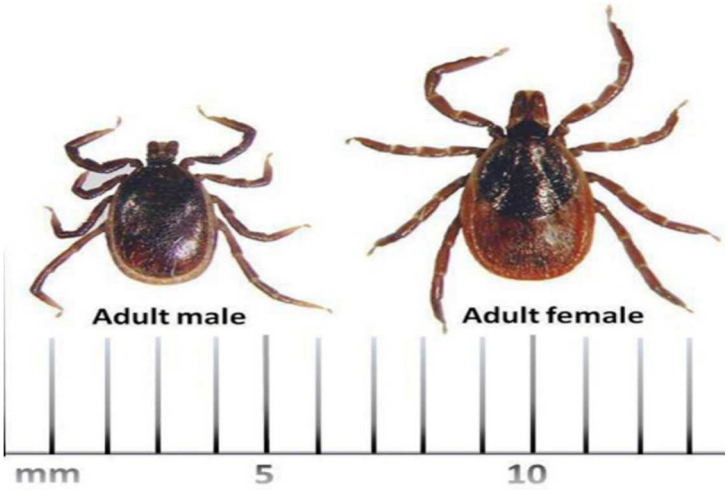

Fig. I. 5. *Ixodes scapularis*. (a) distribution (b) Male and Female

Source: (a) CDC (b) (Schattenberg 2016)

https://www.cdc.gov/ticks/maps/blacklegged_tick.html

(a)

(b)

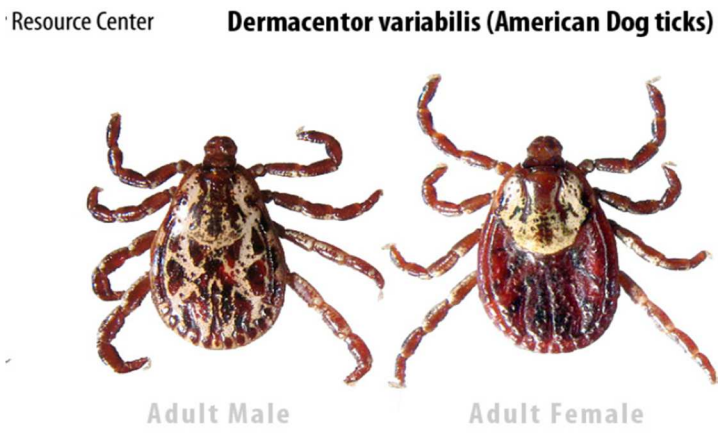

Fig. I. 6. *Dermacentor variabilis*. (a) distribution (b) Male and Female

(Source: (a) CDC (b) http://www.tickencounter.org/tick_identification/dog_tick)

(a)

(b)

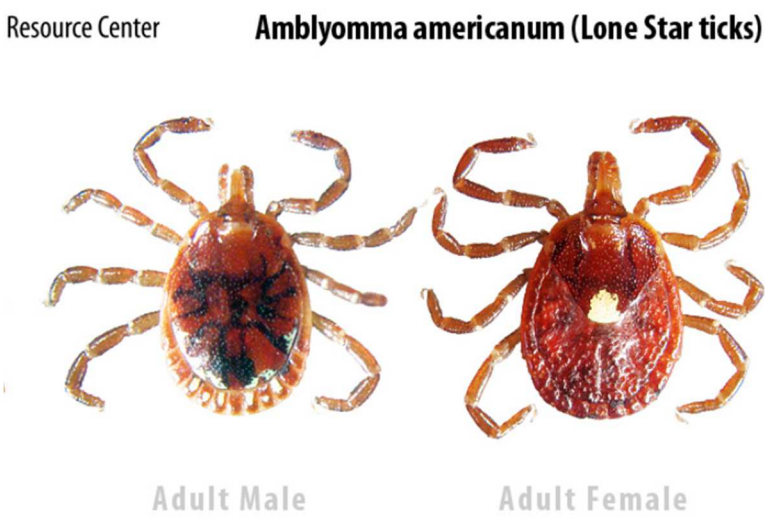

Fig. I. 7. *Amblyomma americanum*. (a) distribution (b) Male and Female

(Source: (a) CDC (b) http://www.tickencounter.org/tick_identification/lone_star_tick)

https://www.cdc.gov/ticks/maps/lone_star_tick.pdf

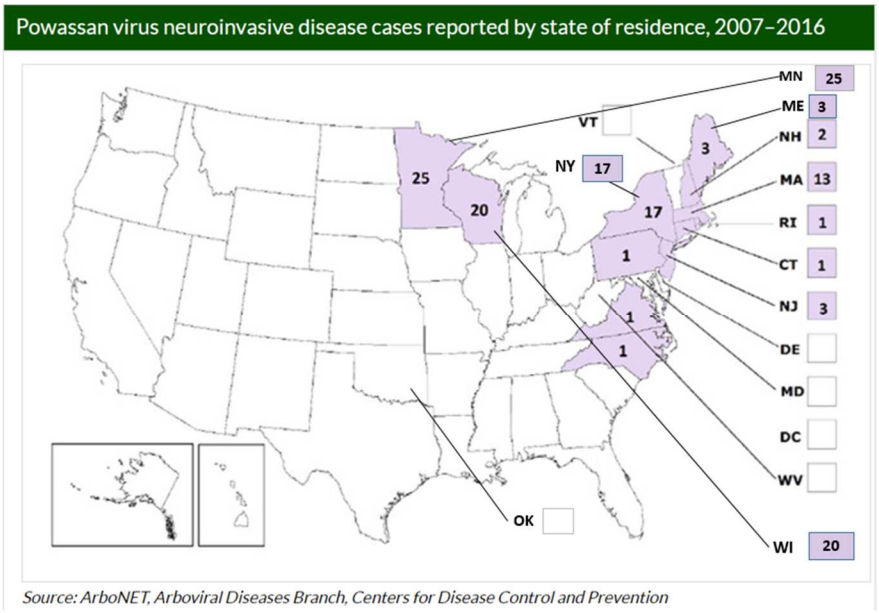

Fig. I. 8. *Powassan virus* cases in United States (2007-2016) CDC

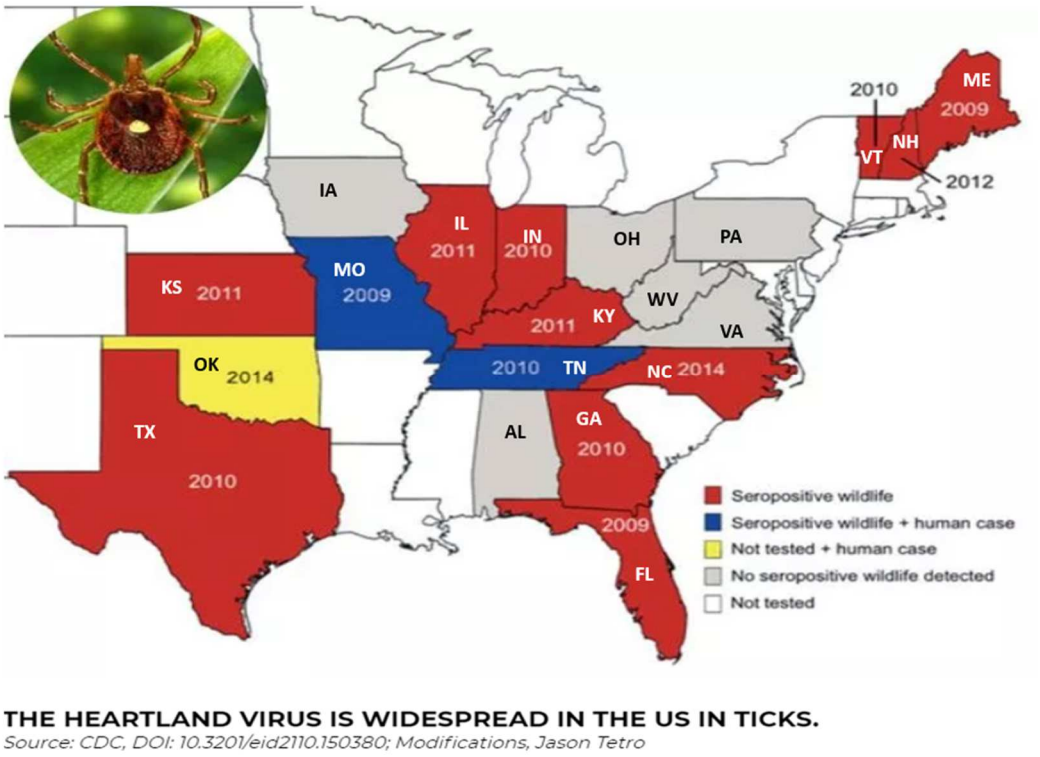

Fig. I. 9. Distribution of *Heartland virus* (2009-2014) in different states.

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CHAPTER II

The complete genome sequence of *Canna Yellow Mottle Virus* isolate CaYMV-AP01

Abstract

Canna (*Canna* L.) species are the most popular ornamental flowers in the *Cannaceae* family but are threatened by viral diseases. *Badnavirus Canna yellow mottle virus* (CaYMV), is considered the most important disease of canna due to the economic losses the virus can cause. The main objective of this study was to determine the complete genome sequence of CaYMV-AP01, a new *Badnavirus* of canna. Canna plants (*Canna* variety 'Red Futurity') were screened to identify virus infected plants, using RT-PCR and a primer pair that amplified a 1288 bp product from virus CaYMV-AP01. Positive 'Red Futurity' plants were selected for CaYMV-AP01 genome sequencing. RT-PCR was performed using 10 different primer pairs that amplifyied overlapping segments of CaYMV-AP01 using an annealing temperature of 52°C for 45 seconds. The amplicons were cloned using pGEM-T easy vectors and the obtained inserts sequenced using massive parallel sequencing and aligned sequences were assembled and annotated. The obtained sequences, matched those of CaYMV from *Alpinia pupurata* (ginger) (CaYMV-AP) which has been recently isolated in Hawaii. This suggests that CaYMV that infectes ginger can potentially infect canna.

Introduction

According to the Interagency Taxonomic Information System (ITIS), *Canna Linnaeus* is in the *Cannaceae* family, is the only described genus, and consists of more than 30 species (Verchot et. al 2015). Cannas are in the *Zingiberales* order and are closely related to *Musaceae* (bananas) and *Zingiberaceae* (gingers) based on morphological as well as molecular and phylogenetic analysis (Kress 1990; Kress et al. 2001; Prince 2010). Cannas are also referred to as canna lily; however, cannas are not true lilies. Cannas are perennial plants that grow from a rhizome in tropical and subtropical climates and are usually planted in April or May, so that vibrantly colored leaves and flowers can be produce in spring and summer (Khoshoo et al. 1976).

Canna production has been threatened by several viral diseases (Rajakaruna et al. 2013). There are five viruses that are known to cause disease of canna namely, *Canna yellow streak virus* (CaYSV; *Potyvirus*), *Canna yellow mottle virus* (CaYMV; *Badnavirus*), *Tomato aspermy virus* (TAV; *Cucumovirus*), *Bean yellow mosaic virus* (BYMV; *Potyvirus*) and *Cucumber mosaic virus* (CMV; *Cucumovirus*). *Badnavirus* (pararetrovirus) has been reported to infect a numbers of crops that have significant economic values throughout the world. Various species of *Badnavirus* have caused huge economic losses of several crops ranging between 10% to 90%. Cannas produced by a grower in Oklahoma were believe to be infected by several viruses and possibly one not previously characterized.

The overall goal of this study was to determine the whole genome sequence of a potentially new *Badnavirus* of cannas cultivar by analysis of plants that were infected with CaYMV-AP01. Various bioinformatic tools were used to design primers for the

whole genome sequencing of CaYMV-AP01 and to carry out the annotation of the raw sequences.

Material and methods

Plant samples

Healthy as well as virus infected cannas (cultivars 'Pretoria', 'Red Futurity', 'City of Portland', 'Richard Wallace', 'Stripe Beauty' (Horn Canna Farm Inc.) were grown in SUNSHINE® LC1 growing mix in the Department of Horticulture and Landscape Architecture greenhouse (Stillwater, OK) at 30℃ with a 14 hour light cycled for 13 weeks. 'Red Futurity' leaves samples from the greenhouse were tested against CaYMV-3/4 primers (Fig. II. 3.b). Twelve 'Red Futurity' plants positive for CaYMV-3/4 primer set were taken as samples.

Ribo Nucleic Acid (RNA) extraction using Sigma-Aldrich Kit

A single leaf was punctured at two sites with a cork-borer (13 mm). The removed leaf samples collectively weighed approximately 100 mg and were placed in pre-sterilized XXtuff® reinforced microvials (BioSpec Products, Bartlesville, OK) containing 2.0 mm Zirconia beads (Biospec Products, Bartlesville OK) and immediately submerged in liquid nitrogen. The leaf discs were taken out of liquid nitrogen and stored at -80℃. The frozen leaf tissues were ground in BeadBug® microtube homogenizer (Benchmark Scientific, Edison, NJ) at 16,000 g for 2 min prior to the RNA extraction process using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Darmstadt, Germany). During the RNA extraction process, the samples were constantly maintained at -4℃ unless otherwise stated. To each tube, 500 µl Lysis/2-mercaptoethanol solution provided in the kit was added. Tubes with leaf samples and solution were then ground using BeadBug®

microtube homogenizer (Benchmark Scientific, Edison, NJ) at 16,000 g for 2 min. Once ground, the samples were incubated at 56℃ for 5 min followed by centrifugation at 13,000 rpm for 6 min. Following centrifugation, 500 µl of supernatant was transferred to filtrate column (in the kit) in a 2 ml centrifugation tube provided in the kit. The protocol provided by the manufacturer for the plant total RNA extraction kit was followed. For the last step of the extraction of RNA, pre-chilled nuclease-free water provided in the kit was used to elute the column. Once the RNA was extracted, checked for quantity and quality using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA, USA). For the analysis of RNA quality, the samples were separated by electrophoresis in an 0.8% agarose gel and stained with 2% ethidium bromide. The RNA was immediately transferred to -80 ℃ to prevent degradation until the sample was further processed.

After the RNA isolation, was precipitated in ethanol. For the ethanol precipitation process, 3M DEPC-treated sodium acetate (pH 5.2) was added in $(1/10)^{th}$ of total volume and 100 % ethanol that is 2.5 times more than a sample was added. Centrifugation at 16,000 g for 15 min at 4□ was carried out. Further, supernatant was discarded and the pellet was washed with a mixture of 75% of ethanol and 25% 0.1 M sodium acetate, pH 5.2, and mixed by inversion of the tube. Supernatant was discarded and the tube was allowed to dry. Finally, RNAse free water was added and the RNA was stored at -80□ for further processing. The RNA was treated with RNA clean and concentrator™ - 5 (Zymo research, Irvine, CA, 1994) to purify RNA. Once purified, rRNA from mitochondria, cytoplasm and chloroplast were removed using Ribo-Zero rRNA Removal Kit (Illumina; San Diego, CA; 1998). The quality of rRNA removed RNA was evaluated using Nanodrop-1000. Furthermore, Ribogreen analysis was conducted to determine the

concentration of RNA in the total solution prior to sequencing (HBRC; Oklahoma State University).

Identification and complete sequence of CaYMV-AP01

Following sequencing, the RNA sequences were submitted for pairwise alignment of obtained sequences (comparison to known samples) using BLAST search algorithm at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The CaYMV-AP01 sequence was identified to be consistent with a viral sequence and were similar to the recently identified CaYMV-AP virus in ginger (accession: MF074075) (Zhang et al. 2017). Various sets of primers (Table II. 1) were further developed by using the CaYMV-AP virus genome sequence to identify the complete genome structure of CaYMV-AP01.

RT-PCR

Reverse transcription of RNA was done using a High Capacity DNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 500 ng RNA per the manufacturer's protocol. Once cDNA (Complimentary DNA) was synthesized the quality of cDNA was determined using a NanoDrop ND-1000 spectrophotometer. Multiplex RT-PCR reaction was carried out to detect the presence of BYMV, CaYSV, and CaYMV, and single RT-PCR for the detection of CaYMV-AP01. For both, 2 µg of cDNA was used along with Go Taq Flexi DNA polymerase (Promega Corp, Madison WI, USA) and 5 µM of primers listed in Table III. 4. Conditions of thermal cycling for multiplex RT-PCR were one cycle at 95℃ for 2 min followed by 25 cycles at 95℃ for 45 sec, 52℃ for 45 sec, 72℃ for 45 sec and a final extension at 72℃ for 7 min (Chauhan et al. 2015). The expected amplicon size of CaYSV, BYMV, CaYMV were 695, 382, and 565 bp, respectively (Chauhan et al. 2015; Wijayasekara et al. 2017). For single RT-PCR,

thermal cycling conditions were one cycle at 95℃ for 2 min, followed by 35 cycles at 95℃ for 1 min, 56.1℃ for 1 min, 72℃ for 1 min and a final extension at 72℃ for 7 min. The expected amplicon size for CaYMV-AP01 was 1,296 bp.

A plasmid control, pYM0036P1, containing a fragment of 0036 DNA sequence (recently CaYMV-AP01) was inserted into the p-GEMT Easy vector (Promega Corporation, Madison, WI, USA). The pYSOK7672 control used for CaYSV contained a CaYSV genome (OK isolate) fragment of 1,827 bp, extending from nucleotide 7,672 to 9,502 and was inserted in a pCR2.1 vector (ThermoFisher scientific, Waltham, MA, USA). The pBYMV382 control for BYMV contained a coat protein fragment of BYMV that was 382 bp in size located from nucleotide 8,949 to 9,329 and was inserted into pGEM-T Easy vector. All these controls were used for RT-PCR throughout the experiment while using the same thermal cycle conditions. Nuclease free water was used to dilute 1 ng of each plasmid which was used for each experiment (Chauhan et al. 2015; Rajakaruna et al. 2014). Products of the PCR were further analyzed and separated by electrophoresis in a 1% agarose gel and stained with 2% ethidium bromide.

Cloning and plasmid isolation

The PCR products obtained using 10 sets of primers (Table II. 2) were then cleaned using Wizard® SV Gel and PCR Clean-Up System (Promega Corp, Madison WI, USA). The pGEM-T Easy vector systems (Promega Corp) cloning protocol was followed for ligation and transformation into high efficiency competent cells; JM 109 (Promega Corp, L2005). The transformed cells were plated in Luria-Bertani (LB) agar (by streak plate method) containing 100 µg/ml ampicillin, 50 µg/ml X-gal, and 50 µg/ml IPTG (Sigma-Aldrich, St. Louis, MO) and incubated at 37℃ for 16 to 18 hours to screen the

transformants for inserts. Five white colonies were streaked in LB media containing 100 µg/ml ampicillin and incubated at 37℃ overnight. Single colonies were then allowed to grow in 5 ml LB media containing 100 µg/ml of ampicillin and incubated at 37℃ overnight.

The plasmid DNA was isolated using Wizard MiniPrep kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA was subjected to restriction digestion (Promega, R6011) using EcoR I using the manufacturers protocol to verify the ligation. To verify the digested product was similar to the size expected, the DNA was separated by electrophoresis in a 1% agarose gel. Those plasmids with the expected amplicon size (Table II. 1) were sent for massive parallel sequencing using Roche 454-Junior™ at the OSU Biochemistry and Molecular Biology Array and Bioinformatics Core Facility.

Next generation sequencing (NGS) and data analysis using bioinformatics tools

Once the DNA was purified, sequencing was conducted using a Roche 454-Junior™ genomic sequencer at OSU Biochemistry and Molecular Biology Core facility. The software, Roche Genome Assembly version 2.7 was used to assemble the read. The raw sequences were then cleaned using the software FastQC and timmomatic to eliminate contamination by the vector or primer. Using BLASTn and MEGAN6 (University of Tubingen, Germany) software contigs were identified to be of viral origin and cleaned contigs were used for further study of the sequence and characterization using SDSC Biology Workbench (SDSC, UC San Diego, CA, USA). When the full genome was obtained and edited, the sequences were compared with other *Badnavirus* sequences using BLAST. A phylogenetic tree with maximum likelihood (ML) of *Badnavirus* species (Table II. 2) was generated using Muscle in MEGA6.

Results

RNA isolation from CaYMV-AP01 positive plant leaves

Although quality of RNA extracted was good, the yield was not sufficient (Table II. 3; Fig. II. 1.) therefore, RNA samples 1-6 and 7-12 were combined to increase the concentration. The groups of RNA sample were given sample IDs as S1 and S2. After RNA precipitation and treatment with Ribo-zero magnetic kit, rRNA was removed and better yield was observed when nanodrop was taken (Table II. 4). S1 and S2 were treated using ribogreen before sequencing (Table II. 5) (Dr. Hwang, HBRC, Oklahoma State University) which depicts the usable concentration of RNA (200 ng) for sequencing.

RT-PCR of 'Red Futurity' positive samples

Reverse Transcriptase (RT) PCR was conducted for six samples positive for the CaYMV-AP01 virus from the cultivar 'Red Futurity' using the CBV primers. 2,000 ng of cDNA were used for PCR.

Annotation and molecular characterization of CaYMV-AP01 genome

The sequenced DNA from CaYMV-AP01 infected canna 'Red Futurity' leaves revealed in 163,870 reads along with an average read length of 359 bp. The CaYMV-AP01 genome numbering was started with $tRNA^{met}$ binding site which is a characteristic genome for *Badnavirus* (Xu et al. 2011). The first 18 sequence of the $tRNA^{met}$ site for CaYMV-AP01, TGGTATCAGAGCTGAGTT (underlined- mismatches) was highly conserved when compared with other badnaviruses (Laney et al. 2012). The TATA box sequence was located upstream of $tRNA^{met}$ binding site and Polyadenyaltion signal (AATAAA) was located in 7,291 bp. Annotation of CaYMV-AP01 indicated the

presence of two small ORFs (ORF1 and ORF2) and one longer ORF (ORF3) that encode polyproteins (Fig. II. 3).

CaYMV-AP01 ORF1 was located between 544 to 1,060 nucleotide positions and was found to encode a 172 amino acid protein sequence, whose function is yet to be known. Open reading frame 2 was located between 1,059 to 1,463 nucleotide positions and encodes protein sequence of 134 amino acids. Stop/start sites for ORF1 and ORF2 resided in TGATGA common element. Both ORF1 and ORF2 sequences were highly similar with that of CaYMV-AP sequences (Fig. II. 4).

Open reading frame 3 was located between 1,463 to 6,848 nucleotide positions. When BLASTn was conducted, ORF3 region was found to be similar to that of CaYMV-AP. Open reading frame 3 encodes for polyproteins that have several functions and contained 1,795 amino acids. The polyprotein found was aspartic preotease, zinc finger motif and reverse transcriptase and RNase H which matched with the characteristic features of badnaviruses. Although the exact function of zinc finger motif is not clear, it has been reported that in retroviral replication, the zinc finger acts as a RNA binding motif and is necessary for CaMV viability (Hohn and Rothnie 2013). The aspartic protease domain aids in polyprotein maturation and is a required structural enzyme. Reverse Transcriptase and RNase H motifs is believed to be the major polyproteins required during the replication of pararetoviruses and is considered to be highly conserved among pararetroviruses (Hohn and Rothnie 2013).

Phylogenetic analysis of CaYMV-AP01

A maximum likelihood (ML) analysis of the CaYMV-AP01 complete genome along with 39 badnavirus related in three clades (Fig. II. 5). Clade 1 contains 19 species of badnaviruses. According to the tree, CaYMV-AP01 genome shares 94% similarity

which is above the standard threshold (80%) for species demarcation (King et al. 2011). Hence, CaYMV-AP01 genome isolated from *Canna* 'Red Futurity' appears to be variant of CaYMV-AP genome isolated from ginger (*A. purpurata*) (Dulanjani et al. 2018; Zhang et al. 2017).

Conclusion and Discussion

Badnavirus have a circular dsDNA genome consisting of RT (reverse transcription) replication. Reverse transcription consists of three elements that are host biased. The first element is RNA containing retroviruses only found in vertebrates. The second element is retrotransposons in many hosts such as fungi, insects, vertebrates, and plants. Similarly, the third element is a DNA containing pararetrovirus in plants and vertebrates (Hohn, Futterer and Hull 1997; Will and Hull 1990). Bacilliform badnaviruses are one of the two main classes of plant pararetroviruses. Both plant pararetroviruses and animal retroviruses utilize polyprotein processing to enhance the protein numbers per RNA and hence share the same evolution that is branched from retroviruses (Xiong and Eickbush 1990; Hohn et al. 1997).

This study was conducted to obtain the full genome sequence of CaYMV-AP01 (accession MF074075), which was considered as a sub-viral element (EVS-0036). The complete genome of CaYMV-AP01 virus from canna, which was previously known as EVS 0036 (accession KX255723.1), was determined using next generation sequencing (NGS) (Wijayasekara et al. 2016). Due to the short sequence of EVS-0036 was assumed to be catalytic in nature for a short period of time and also due to the absence of an ORF region (three-seven ORFs for *Badnavirus*), EVS-0036 was considered as a sub-viral element. However, after sequencing, CaYMV-AP01 was found to be 7,385 bp in length

and to be circular DNA (standard *Badnavirus* genome: 7,200-9,200 bp in length) having 3 ORFs (Wijayasekara et al. 2018). The sequences were further subjected to BLASTn analysis which revealed that CaYMV-AP01 shares high similarity with partial CaYMV sequence of canna (*Canna indica*) and betel pepper (*Piper betel* L.) (Kumari et al. 2014; Agneroh et al. 2015). CaYMV-AP01 was also identified as a variant of CaYMV in ginger (*Alpinia purpurata*) (accession: MF074075) (Wijayasekara et al. 2018). This implies that the genome sequence of *Canna yellow mottle virus* can share a high degree of similarity regardless of infecting different hosts.

Further studies on CaYMV-AP01 are required to determine the interaction with the host. As this is a new virus strain in canna, its possible host range in the future can be known by further research. This study indicates that CaYMV-AP01 is a new virus isolate of canna and has also been observed infecting other hosts such as betel and recently ginger. Further research is required to identify if there is a larger host range and the host range is expanding.

Table II. 1. Primers set used for amplification of CaYMV-AP01.

Primers	Sequence	Primers	Sequence	Amplicon size (bp)
CBV-F1	TGG TAT CAG AGC TGA GTT CAG G	CBV-R1	CTT CCT TCC AGT TGG GGG C	1-1336
CBV-F2	GCG GCC ACT GGC TTT ACA GC	CBV-R2	TGT CTT GCT GGT CAT TTG CGA G	1132-1824
CBV-F2	GCG GCC ACT GGC TTT ACA GC	CBV-R2B	CTG CAT GAC TCC TAA ATG CAC G	1132-1901
CBV-F3	GGC GCG CTA TGA AGC TCA AC	CBV-R3	TTG CTT GGA CTC GTC AAG TCT TG	1568-2674
CBV-F4	GCT TAC CGA ATA GAA CAA GTA GCA GA	CBV-R4	CGG CTT CAT AAG CCA TCC TCC	2202-3138
CBV-F5	GAA AGA TGG GAG TCA GTT GTG CT	CBV-R5	GGG CAT ATA TCT CTT CAA TTC CTC CC	2997-3912
CBV-F6	CGC TAG GGA CTG CCG TAA C	CBV-R6	TCC GAA CTC CAC CTT CGA GAC TTC	3740-4929
CBV-F7	AGG TTA AGA GCC GGC CCA AG	CBV-R7	CTG TTT TCC TTT CTT TTC CAG GCC TG	4578-5396
CBV-F8	GAC ATG CAG AAG CAC ATA GAC CAA T	CBV-R8	GGA ACA TGA CTA CTA ACC TTG ACA GTC	5256-6531
CBV-F9	CAC AGG TAG ATT TCC TTG GTG CAA	CBV-R9	GAC CTG CAG TCT TTT GCT TGC A	6158-7300

CBV = Canna Badnavirus

Table II. 2. Species of *Badnavirus* used for developing phylogenetic tree. (Wijayasekara et al. 2017).

Name	Acronym	Accession
Banana streak GF virus	BSGFV	NC_007002.1
Banana streak IM virus	BSIMV	NC_015507.1
Banana streak MY virus	BSMYV	NC_006955.1
Banana streak OL virus	BSOLV	NC_003381.1
Banana streak UA virus	BSUAV	NC_015502.1
Banana streak UI virus	BSUIV	NC_015503.1
Banana streak UL virus	BSULV	NC_015504.1
Banana streak UM virus	BSUMV	NC_015505.1
Banana streak VN virus	BSVNV	NC_007003.1
Bougainvillea chlorotic vein banding virus	BSCVB	NC_011592.1
Cacao mild mosaic virus	CMMV	NC_033738.1
Cacao swollen shoot virus	CSSV	NC_001574.1
Canna yellow mottle virus	CaYMV	MF074075.1
Citrus yellow mosaic virus	CiMV	NC_003382.1
Dioscorea bacilliform AL virus	DiBALV	KX008573.1
Dioscorea bacilliform RT virus 2	DibrtV	KY827393.1
Dioscorea bacilliform SN virus	DiBSNV	KT160426.1
Fig badnavirus 1	FBV-1	NC_017830.1
Gooseberry vein banding associated virus	GVBaV	NC_018105.1
Grapevine vein clearing virus	GVCV	NC_015784.2
Kalanchoe top-spotting virus	KTSV	NC_004540.1
Pagoda yellow mosaic associated virus	PYMAV	NC_024301.1
Pineapple bacilliform CO virus	PBCOV	NC_014648.1
Piper yellow mottle virus	PYMoV	NC_022365.1
Rubus yellow net virus	RYNV	NC_026238.1
Sugarcane bacilliform IM virus	SCBIMV	NC_003031.1
Sugarcane bacilliform MO virus	SCBMOV	NC_008017.1
Sweet potato pakakuy virus	SPPV	NC_015655.1
Taro baciliform CH virus	TaBCHV	NC_026819.1
Taro bacilliform virus	TaBV	NC_004450.1
Yacon necrotic mottle virus	YNMoV	NC_026472.1

Table II. 3. Nanodrop of *Canna* 'Red Futurity' samples positive for CaYMV-AP01.

Sample ID ('Red Futurity')	ng/µl	260/280	260/230
1	136.3	2.07	1.89
2	136.3	2.03	1.93
3	138.6	2.10	2.11
4	131.1	2.06	2.09
5	172.4	2.01	1.99
6	118.9	2.03	1.95
7	171.6	2.04	1.95
8	101.9	2.09	2.20
9	85	2.01	2.04
10	80	2.06	2.15
11	98.7	2.06	2.10
12	107.2	2.04	1.95

Table II. 4. Nanodrop reading after the removal of rRNA from RNA of canna 'Red Futurity'.

Sample ID	ng/µl	260/280	260/230
S1	55.1	1.98	1.38
S2	56.8	1.85	1.32

Table II. 5. Ribogreen analysis before sequencing.

Sample ID	ng/µl	ng/µl (average)	Volume (µl) required for 200 ng
S1	26.93	26	7.7
	25.02		
S2	24.50	24.3	8.2
	24.00		

Table II. 6. Amplicon size obtained using CBV primers.

Primers	Result obtained (bp)	Primers	Result obtained (bp)
CBV-F1/R1	1336	CBV-F6/R6	1190
CBV-F2/R2	692	CBV-F7/R7	819
CBV-F3/R3	1078	CBV-F8/R8	1276
CBV-F4/R4	937	CBV-F9/R9	-
CBV-F5/R5	916	CBV-F2/R2B	770

CBV= Canna *Badnavirus,* designed on November 2016

All primers successfully amplified DNA with the expected product except CBV-F9/R9 primer sets because these primers had a base that was not in the expected sequence.

Table II. 7. NCBI BLASTn results for CaYMV-AP01 complete genome.

Best Match	NCBI accession no.	Max score	% query cover	% identity	E value
CaYMV-AP	KU168312.1	6802	96	96	0
Canna Yellow Mottle Virus – *Alpinia Purpurata*					
DBSNV	DQ822073.1	128	4	74	3e-24
Dioscorea bacilliform SN virus clone B39-4					

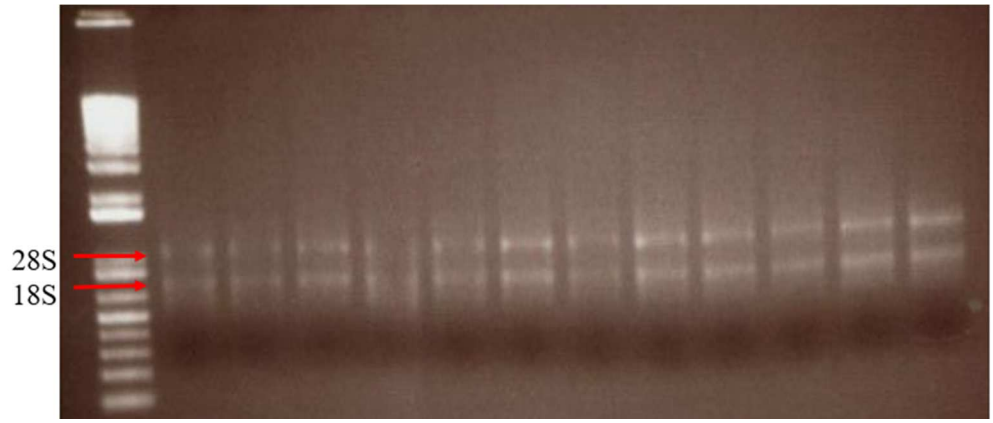
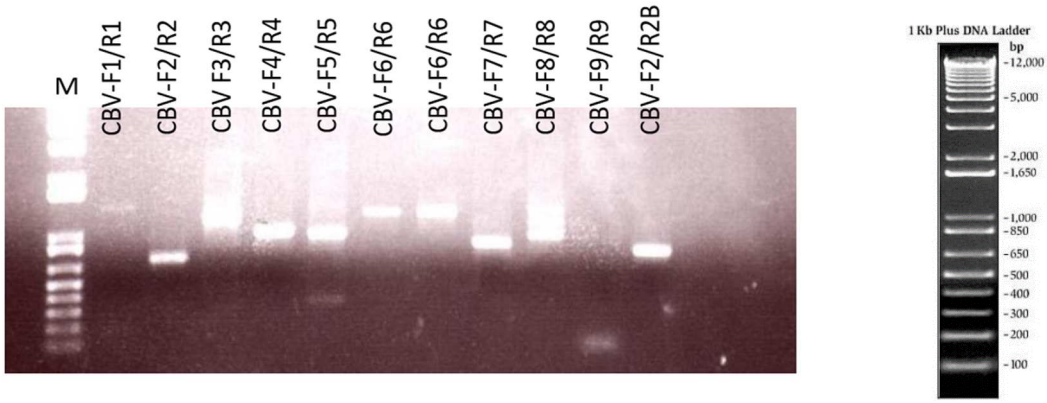


Fig. II. 1. Gel picture showing a clear intact RNA (28S and 18S) in 1% agarose gel. RNA was isolated using Sigma-aldrich kit.

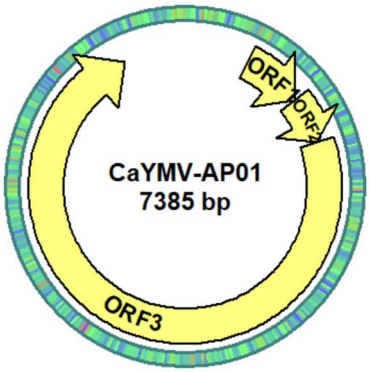


Ladder: *Invitrogen 1 kb Plus DNA Ladder No. 10787-018*

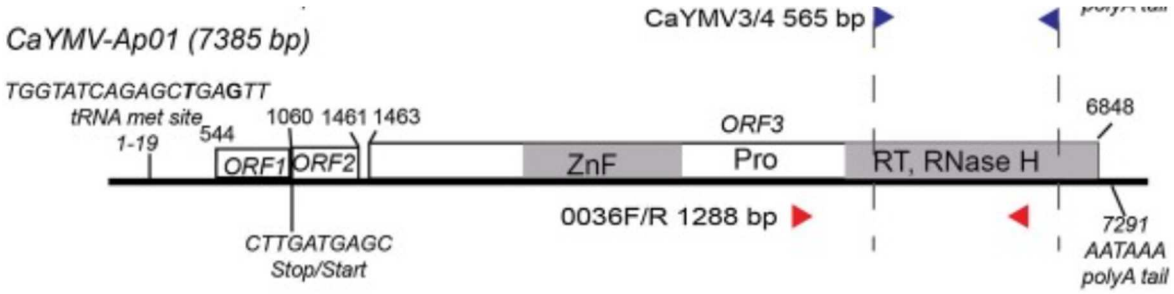
Fig. II. 2. Gel electrophoresis showing CBV primers binding.

First lane is ladder (M). CBV primers binding to the CaYMV-AP01 positive leaf samples to find complete genome.

(a)



(b)

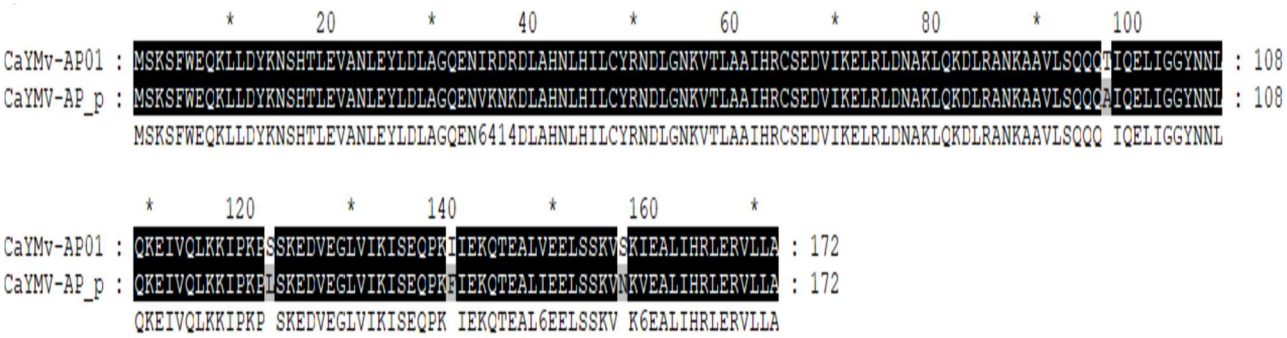


Source: Wijayasekara et al. 2018

Fig. II. 3. Properties of CaYMV-AP01.

(a) Circular view of the sequence showing ORF1, ORF2, and ORF3
(b) Linear orientation of the 7,385 bp genome. Based on the $tRNA^{met}$ binding site, nucleotide position 1 is determined. Nucleotide position of ORF1, ORF2 and ORF3 polyproteins are indicated. ORF3 consists of zinc finger binding domain (ZnF), aspartic acid protease domain (Pro) and Reverse transcriptase (RT) and RNase H domain and their position is shown. Stop/start site for ORF1/2 and PolyA tail position is depicted.

(a)



(b)

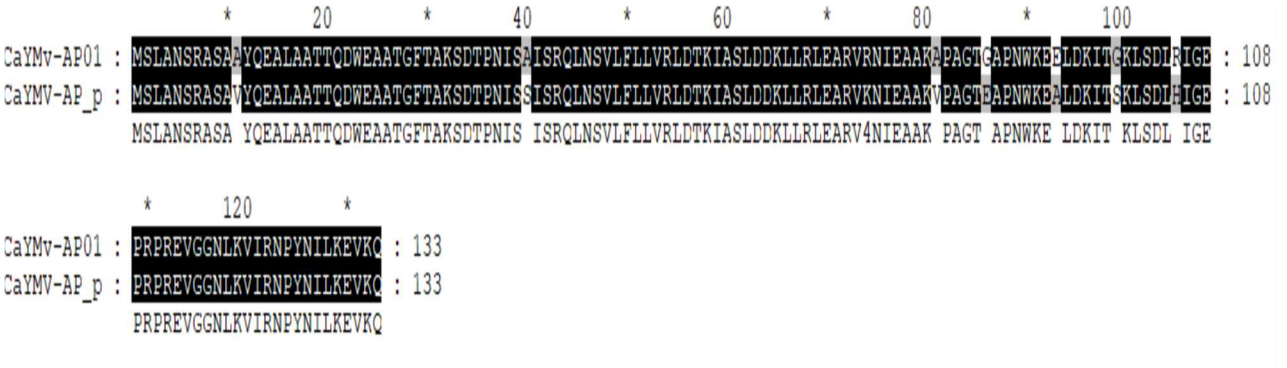


Fig. II. 4. Multiple sequence alignment of CaYMV-AP01 and CaYMV-AP. (highly similar sequence) protein sequences using GeneDoc. Black shade represents identical amino acid. Region where there is no shade is the dissimilar amino acid between two protein sequences. All upper letter consensus sequence represents conserved region.

((a) ORF 1 (b) ORF 2)

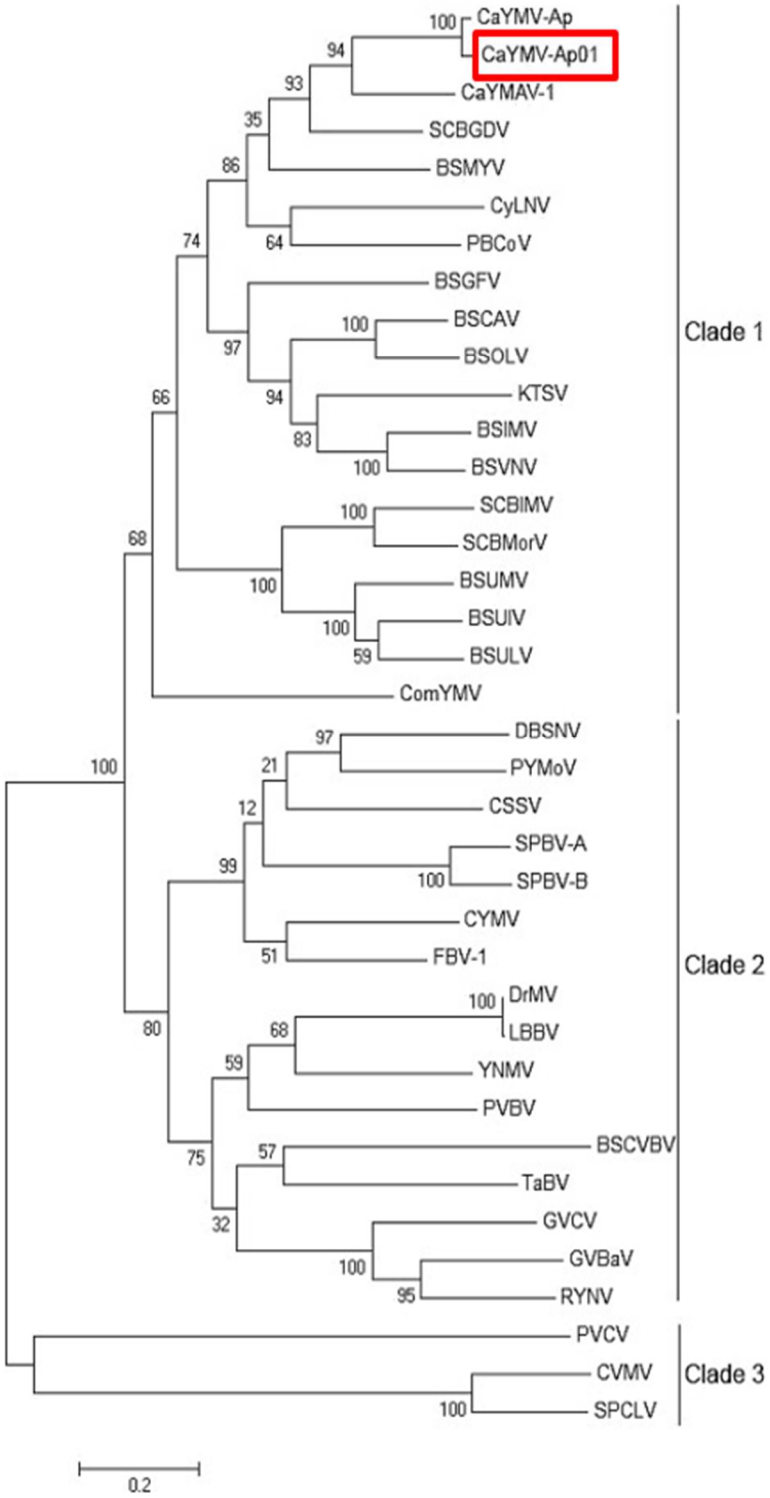


Fig. II. 5. Maximum likelihood (ML) tree of badnavirus species. Badnaviruses of 30 species' complete nucleotide sequence used to create ML tree with MEGA 6.0 software, selecting Kimura model with 1000 bootstraps (Wijayasekara et al. 2018).

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CHAPTER III

Investigations into the potential modes of Canna CaYMV-AP01 virus transmission

Abstract

Most of the species of *Badnaviruses* are limited to small host range but can infect both monocots as well as dicots. Large scale dissemination of *Badnavirus* is primarily through the movement of vegetative organs from perennial hosts. The canna industry is largely based on vegetative propagation of new plants and hence are vulnerable to *Badnavirus* infection. In this study, various modes of transmission (via plant to plant, soil-borne, nematodes, vector and mechanical) of CaYMV-AP01 were studied. For plant to plant transmission, infected and healthy cannas were grown in the same pot in close contact with each other. For soil-borne transmission, healthy plants were grown in a potentially virus infested soil (soil in which infected plants had grown previously) and maintained in greenhouse and in field sites. Numerous potential vectors were introduced to feed on infected plants and transferred to healthy plants to observed and determine if vector based transmission could occur. Additional studies with pruning equipment was conducted to determine if transmission from infected plants to healthy plants could occur through mechanical transmission. The only transmission observed was for plants grown in soil prior to which infect canna plants were grown.

Introduction

Among all nursery growers, cannas are one of the most popular ornamental plants, not only in Oklahoma but also in Texas and Arkansas. Canna is popular because of its vibrant color and giant leaves and flowers. The popularity of canna has resulted in some producers that are solely found on production and shipping of cannas, to both national and international markets. Among the canna industries, canna hybrids (two or more cross species) are the most important because of their variable height and color. For the production of hybrids, vegetative propagation is required and is usually done by cutting the rhizome into pieces. However, vegetative propagation is thought to be the primary route for viral disease proliferation in the industry. Hence, the viruses that are infecting cannas such as *Canna yellow mottle virus* (CaYMV, *badnavirus*), *Canna yellow streak virus* (CaYSV, *potyvirus*), and *Bean yellow mosaic virus* (BYMV, *potyvirus*) among others are responsible for the challenges facing the industry (Verchot n.d.). Plant pathologist at Oklahoma State University have been working with growers to evaluate and analyze the various viral diseases of *Canna* (Wijayasekara et al. 2017).

The canna industry is threatened by the potential for widespread viral diseases. The canna industry in Oklahoma is significant with the production and the sales in the national and international markets (Rajakaruna et al. 2014). The overall goal of this study was to determine the potential modes of transmission of canna viruses. For example, transmission could occur through plant to plant, soil-borne, mechanical, nematodes and insect vectors (in a greenhouse and in the open fields). Secondly, research was conducted to determine the presence of viruses and plant symptoms associated with infection.

Material and methods

Plant samples

Healthy as well as naturally infected cannas (cultivars 'Pretoria', 'Red Futurity', 'City of Portland', 'Richard Wallace', 'Stripe Beauty' were taken from Horn Canna Farm Inc.) were grown in SUNSHINE® LC1 growing mix (Sun Gro Horticulture, Agawam, MA) in the greenhouse at 30℃ with 14 hours of supplemental lighting for 8-10 weeks. For plant to plant transmission, healthy and infected cannas were grown in the same pot. For every test plant, 'Cannova' was used as a clean control. For infected cannas, various cannas with different viruses or combination of viruses were used (Table III. 1.). In the case of soil-borne transmission, various cultivars of canna screened to be positive for virus infection were grown in different pots. Once grown, these infected cannas were removed and 50% of the soil from each pot was discarded. The remaining half of the soil was then mixed with same amount of fresh 902 mix soils in separate individual pots (Table III. 1.). The healthy 'Cannovas' were then grown in the mixed soil to facilitate transmission of viruses from infected plant residues in the soil. This experiment was done on June 2016 at three different places, (a) Oklahoma State University (OSU) greenhouse, (b) OSU botanical garden, and (c) OSU research station. These sites were chosen to evaluate any difference in plants when are exposed to viruses in control conditions and in the open field. At the OSU botanical garden and research station, plants were grown in a row, experiment set up was similar to that of the greenhouse but was conducted in open field.

The insect transmission experiment was conducted in a greenhouse inside confined cages (1.2m X 0.92m) where the different combination of viruses infected plants were put along with healthy 'Cannovas' and different vectors were introduced (Table III. 3.).

To check if viruses were transmitted by the nematodes, soil samples from 10 different locations at Horn canna farm (Carnegie, OK) were collected. Soil samples were collected from the depth of at least 15 cm. Nematodes was isolated using bucket decanting and centrifugal flotation (Coolen and D'Herde method; 1972) and identified using a microscope.

For mechanical transmission, healthy 'Cannovas' leaves and rhizomes were cut down with the same tool used to cut infected *Cannas*. Further, these healthy cannovas were grown in the greenhouse and tested for virus transmission from a tool using RT-PCR.

TNA (Total Nucleic Acid) extraction

A single leaf was punctured at two sites with a cork-borer (13 mm). The removed leaf samples collectively weighed approximately 100 mg and were placed in pre-sterilized XXtuff® reinforced micro vials containing 2.0 mm Zirconia beads (Biospec Products, Bartlesville OK) and immediately submerged in liquid nitrogen. The leaf discs were taken out of liquid nitrogen and stored at -80℃. The frozen leaf tissues were ground in BeadBug® microtube homogenizer (Benchmark Scientific, Edison, NJ) at 16,000 g for 2 min prior to the TNA extraction process in Maxwell® 16 instrument using Maxwell® 16 LEV SimplyRNA Tissue Kit (Promega Corp, Madison WI, USA). During the TNA extraction process, the samples were constantly maintained at -4℃ if otherwise stated. TNA was isolated by following LEV SimplyRNA Tissue Kit protocol except for the

addition of DNAse I solution provided by the manufacturer. The protocol was followed according to the manufacturer of the SimplyRNA Tissue Kit. RNA was extracted and was checked for quantity and quality using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA, USA). For the analysis of RNA quality, the samples were separated by electrophoresis in an 0.8% agarose gel and stained with 2% ethidium bromide. Sample RNA was immediately transferred to -80℃ to prevent degradation until it was further processed.

Single and Multiplex RT-PCR

Reverse transcription of total nucleic acid (TNA) was done using a High Capacity DNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 500 ng TNA per the manufacturer's protocol. Once cDNA (Complimentary DNA) was synthesized the quality of cDNA was determined using a NanoDrop ND-1000 spectrophotometer. Multiplex RT-PCR reaction was carried out to detect the presence of BYMV, CaYSV, and CaYMV, and singleplex RT-PCR for the detection of CaYMV-AP01. For both, 2 µg of cDNA was used along with Go Taq Flexi DNA polymerase (Promega Corp, Madison WI, USA) and 5 µM of primers listed in Table II. 1. Conditions of thermal cycling for multiplex RT-PCR were one cycle at 95℃ for 2 min followed by 25 cycles at 95℃ for 45 sec, 52℃ for 45 sec, 72℃ for 45 sec and a final extension at 72℃ for 7 min (Chauhan et al. 2015). The expected amplicon size of CaYSV, BYMV, CaYMV were 695, 382, and 565 bp, respectively (Chauhan et al. 2015; Wijayasekara et al. 2017). For singleplex RT-PCR, thermal cycling conditions were one cycle at 95℃ for 2 min, followed by 35 cycles at 95℃ for 1 min, 56.1℃ for 1 min, 72℃ for 1 min and a final extension at 72℃ for 7 min. The expected amplicon size for CaYMV-AP01 was 1,296 bp.

A control plasmid, pYM0036P1, containing a fragment of 0036 DNA sequence (CaYMV-AP01) was inserted into the p-GEMT Easy vector. The pYSOK7672 control used for CaYSV contained a CaYSV genome (OK isolate) fragment of 1,827 bp extending from 7,672-9,502 bp and was inserted in a pCR2.1 vector. The pBYMV382 control for BYMV contained a coat protein fragment of BYMV that was 382 bp in size located from 8,949-9,329 bp and was inserted into pGEM-T Easy vector. All these controls were used for RT-PCR throughout the experiment while using the same thermal cycle conditions. Nuclease free water was used to dilute 1 ng of each plasmid which was used for each experiment (Chauhan et al. 2015; Rajakaruna et al. 2014). Products of the PCR were further analyzed and separated by electrophoresis in a 1% agarose gel and stained with 2% ethidium bromide.

Results

Plant to plant transmission

After 8 week and 12 weeks when RT-PCR was conducted for the identification of virus, none were found to be present in the non-infect host plants. It was as expected that the healthy plants may have disease once the rhizomes and leaves came in close contact with infected plants by growing them together; however, no symptoms of disease were observed.

Soil-borne transmission

Infected canna plants with various combinations of viruses were grown in prior to healthy canna plants growth in the same soil. Experiment was carried out in three different places (Table III. 2., III. 5., III. 6., III.7.).

Vector transmission

Four different vectors namely, bird cherry-oat aphid, green peach aphid, green bug and sugarcane aphid were introduced in a controlled environment. Aphids were exposed to infected and healthy canna plants (different plants tested) inside a cage to feed and transmit the disease.

Similarly, green peach aphid, green bug and sugarcane aphid were also introduced individually. None of the insects survived on canna plants. It was determined with the assistance of Dr. J. Scott Armstrong (Research Entomologist in USDA ARS) that the insects died due to starvation (insects did not feed on canna) before the aphids could transmit the virus.

Nematode transmission

None of the nematodes; (Stunt) *Tylenchorhynchus* , (Lesion) *Pratylenchus*, (Spiral) *Helicotylenchus*, (Ring) *Bursaphelenchus*, (Lance) *Hoplalaimus* present in soil samples collected from Horn Canna Farm were known vectors for plant viruses.

Conclusion and Discussion

Badnaviruses are found frequently infecting vegetatively propagated plants, which makes canna a suitable host for multiple infection and transmission. Some of the host commonly known for *Badnavirus* seed transmission to occur are *Piper yellow mottle virus* (PYMoV), *Cacao swollen shoot virus* (CSSV) and *Commelina yellow mottle virus* (ComYMV) (Bhat et al. 2016). Bhat (2016) conducted a study that showed the opportunities for *Badnavirus* mechanical transmission are very low.

Plant to plant transmission was conducted and no evidence of infection of healthy plants was found when these plants were growing next to infected plants. This finding suggests that growers can store canna plants at high density for the purpose of vegetative propagation because viruses will not be transmitted plant to plant by contact (leaf to leaf or rhizome to rhizome). Mechanical transmission assay was conducted. Cutting tools were contaminated by cutting infected plants before inflicting cuts on healthy cannas; however, no disease symptoms developed and tested negative after applying molecular methods (data not shown).

Badnaviruses are typically transmitted by a mealybug in the *Pseudococcidae* family (Harper et al. 2004). However, the insect responsible for the transmission of *Canna Yellow Mottle Virus* is unknown (Momol et al. 2004). Result obtained from the insect transmission assay showed the bird-cherry oat, the green peach aphid, and the sugarcane aphid did not transmit the virus from infected plant to healthy plant. Mealybug and whiteflies were observed colonizing canna plants in the greenhouse, but none of the cannas plants showed symptoms of infection even after a few months. This result suggests aphids may not be play a role in the canna virus transmission. Regarding the nematode transmission experiment, none of the recovered nematodes are identified as a vectors of plant viruses and nematodes are unlikely to aid in virus movement in this case.

Since farmers use the same land for growing canna during multiple generations, debris infected with viruses and the fungi such as Plasmodiophorales or Chytridiales which are known capable of virus transmission may be virus vector (Verchot 2002). During the study, positive transmission of CaYMV-AP01 to healthy plants through contaminated soil where infected plants were previously growing was determined. The

soilborne transmission result suggested that CaYMV-AP01 transcripts were present as a major infectious virus. Symptoms related to CaYMV-AP01 virus are yet to be studied in canna. CaYMV-AP01 did not show any visible symptoms in canna cultivar 'Australia' which has burgundy leaves. However, when CaYMV-AP01 is mix infected with CaYSV and CaYMAV-1, necrosis and yellowing symptoms are expressed in canna cultivar 'Striped Beauty' (Dulanjani et al. 2018). Infected plants not showing symptoms or masking the symptoms during certain time is common in *Badnavirus* (Bhat et al. 2016). It is important to study the various modes of transmission of the CaYMV-AP01 virus as the disease may increase in importance because the reports of *Badnavirus* have increased in recent years.

Questions that remain to be considered for future research of soilborne transmission is whether CaYMV-AP01 virus is suppressed in the presence of CaYSV in *Canna* variety 'City of Portland' and whether water might be responsible for cannas' virus transmission?

Table III. 1. Experimental set up for plant to plant and soil-borne transmission in canna.

Experiments	Test plants (healthy)	Infected Canna cultivar	Virus associated
Plant to plant Transmission	Cannova	Stripe Beauty	CaYSV+CaYMV+CaYMV-AP01
	Cannova	City of Portland	CaYSV
	Cannova	Richard Wallace	CaYSV+ CaYMV-AP01
	Cannova	Pretoria	CaYMV+CaYMV-AP01
	Cannova	Cannova	CaYMV-AP01
	Cannova	Red Futurity	CaYSV+CaYMV+ByMV+CaYMV-AP01
Soil-borne	Cannova	Stripe Beauty	CaYSV+CaYMV+CaYMV-AP01
	Cannova	City of Portland	CaYSV
	Cannova	Richard Wallace	CaYSV+ CaYMV-AP01
	Cannova	Pretoria	CaYMV+CaYMV-AP01
	Cannova	Cannova	CaYMV-AP01
	Cannova	Red Futurity	CaYSV+CaYMV+ByMV+CaYMV-AP01

Table III. 2. Experiment set up for soil transmission in canna.

Plant samples from	Address	Number of samples (healthy cannova)
Greenhouse	1301 N. Western Rd. Stillwater, OK	60
OSU botanical garden 1	3300 W. 6th Street, Stillwater, OK	35
Research station	10820 S Jardot Rd, Perkins, OK	13

Table III. 3. Vector transmission experiment set up in canna.

Cage	healthy plant (n)	Infected plant (n)	Infected plants	Virus associated	Vectors	Vectors introduced (M/D/Y)
1	10	3	Stripe Beauty	SV + MV + MV-AP01	Bird cherry-oat aphid	10/22/16
2	10	3	City of Portland	SV	Bird cherry-oat aphid	10/22/16
3	10	3	Richard Wallace	SV + MV-AP01	Bird cherry-oat aphid	10/22/16
4	10	3	Pretoria	MV + MV-AP01	Bird cherry-oat aphid	10/22/16
5	10	3	Cannova	MV-AP01	Bird cherry-oat aphid	10/22/16
6	10	3	Red Futurity	MV + MV-AP01 + SV + BYMV	Bird cherry-oat aphid	10/22/16
7	4	1	Stripe Beauty	SV + MV + MV-AP01	Green peach aphid	1/19/17
8	4	1	Stripe Beauty	SV + MV + MV-AP01	Green bug	2/17/17
9	4	1	Stripe Beauty	SV + MV + MV-AP01	Sugarcane aphid	2/17/17
10	4	1	Stripe Beauty	SV + MV + MV-AP01	Bird cherry-oat aphid	2/17/17
11	4	1	Stripe Beauty	SV + MV + MV-AP01	Green peach aphid	2/17/17

(n) = numbers of plants taken
SV = CaYSV; MV= CaYMV; MV-AP01 = CaYMV-AP01
M/D/Y = Month/Date/Year

Table III. 4. List of primers used in PCR and RT-PCR for diagnosing the listed viruses infecting canna.

Virus	Primers	Amplicon size (bp)	Primer Sequences (5'-3')	Melting Temperature	References
CaYSV	Canna-F1	695	GAT CTA ACG CAA CAT ATT CGA AAG	52.2	Monger et al. 2007
	Canna-F2		GAT CAT CCA TCA TTA CCC AAT AC	51	
CaYMV	CaYMV-3F	565	GAC TTC CTG GGT GCA ACA AT	55.6	Momo et al. 2004
	CaYMV-4R		TCT GTG CAA TCT TGG CGT AG	55.2	
BYMV	BYMV-F	382	GTG AAT GGA CAA TGA TGG ATG	51.6	Chauhan et al. 2015
	BYMV-R		CAC CAT TGA CAT CTC CTG CTG	55.8	
CaYMV-AP01	301-F	1296	ATG GAC GAG GAG AAG GGC TA		
	1621-R		GGA TGG CTT GTT CTG TGC AA		

Table III. 5. Soil-borne experiment conducted in a greenhouse.

Previously grown plants in soil	Virus associated	Healthy cannova grown in same soil afterwards infected with				CaYMV-AP01 infection
		CaYSV	CaYMV	CaYMV-AP01	BYMV	
'Stripe Beauty' n=9	SV+MV+MV-AP01	-	-	8	-	89%
'City of Portland' n=10	SV	-	-	10	-	100%
'Richard Wallace' n=11	SV+MV-AP01	-	-	10	-	91%
'Ptoria' n=9	MV+MV-AP01	-	-	9	-	100%
'Cannova' n=12	MV-AP01	-	-	11	-	92%
'Red Futurity' n=9	SV+MV+MV-AP01+BYMV	-	-	8	-	89%

Table III. 6. Soil-borne experiment carried out in OSU botanical garden.

Previously grown plants in soil	Virus associated	Healthy cannova grown in same soil afterwards infected with				CaYMV-AP01 infection
		CaYSV	CaYMV	CaYMV-AP01	BYMV	
Row-1 n=8	SV+MV+MV-AP01+BYMV	-	-	6	-	75%
Row-2 n=5	SV+MV+MV-AP01+BYMV	-	-	5	-	100%
Row-3 n=5	SV+MV+MV-AP01+BYMV	-	-	5	-	100%
Row-4 n=5	SV+MV+MV-AP01+BYMV	-	-	5	-	100%
Row-5 n=6	SV+MV+MV-AP01+BYMV	-	-	6	-	100%
Row-6 n=6	SV+MV+MV-AP01+BYMV	-	-	6	-	100%

Table III. 7. Soil-borne experiment carried out in OSU research station.

Previously grown plants in soil	Virus associated	Healthy cannova grown in same soil afterwards infected with				CaYMV-AP01 infection
		CaYSV	CaYMV	CaYMV-AP01	BYMV	
n=13	SV+MV+MV-AP01+BYMV	-	-	13	-	100%

CaYMV-AP01 was transmitted from infected to healthy plants in high rate.

Table III. 8. Vector transmission experiment.

	Vector Information			
Vector	Date introduced	Observation date of death	No. of aphid introduced	Comments
Bird cherry-oat aphid (*Rhopalosiphum padi*)	02/21/017	02/24/017	15 per plant (13 plants per cage)	In 10/22/2016, around 200 aphids per plant were introduced. Within a week almost all aphids were dead. Plants were kept for 2 months, no symptoms were observed.
Green peach aphid (*Myzus persicae*)	01/09/017	01/16/017	10 per plant (5 plants per cage)	All aphids were dead. Another batch was exposed to plants from 02/28/017–03/06/017 but all were dead.
Green bug aphid	02/17/017	02/21/017	10 per plant (5 plants per cage)	All aphids were dead.
Sugarcane aphid (*Melanaphis sacchari)*	02/17/017	02/21/017	10 per plant (5 plants per cage)	All aphids were dead.

Table III. 9. Nematodes present in soil samples collected from Horn canna farm.

Soil samples number	Different types of nematodes and its number present in soil samples				
	Stunt	Lesion	Spiral	Ring	Lance
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	7	-	1	-	1
6	5	20	1	-	2
7	15	29	-	-	-
8	7	9	-	1	-
9	3	4	1	-	-
10	17	21	1	-	2

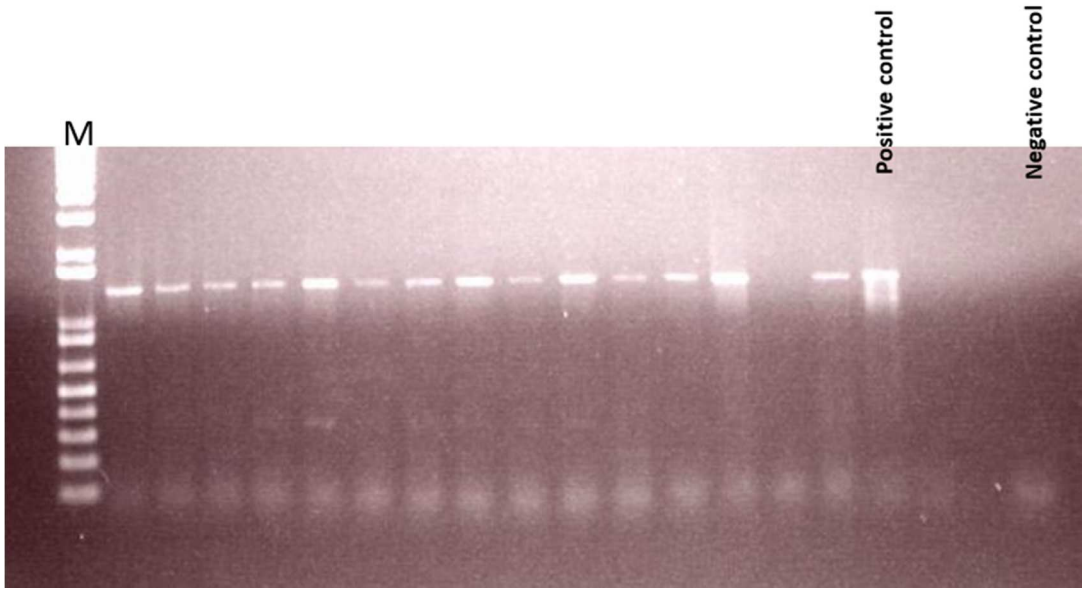

Fig. III. 1. Gel electrophoresis of CaYMV-AP01 positive samples from soil-borne transmission.
First lane is a Ladder (1 Kb plus DNA ladder, Invitrogen). Other lane in a continuous row shows positive samples of CaYMV-AP01 except for one.
For positive control, pYM0036P1 plasmid is used.
For negative control, Nuclease Free water is used.

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CHAPTER IV

Evaluation of various PCR primers for detection of two human viruses associated with ticks

Abstract

Powassan virus (POWV) is a positive single stranded RNA virus transmitted to humans by *Ixodes scapularis*, *Ixodes cookie,* and *Dermacentor variabilis*. *Heartland virus* (HRTV) is an enveloped single stranded RNA virus transmitted to humans by *Amblyomma americanum*. POWV and HRTV virus infection in human is increasing so a proper technique for the detection is important. For this purpose, a set of primers for the identification of POWV and HRTV were developed. Three sets of primers were developed as an internal control for three ticks studied (*Ixodes scapularis, Dermacentor variabilis,* and *Amblyomma americanum*). Trial for mimicking the situation where a tick is infected and virus primers are used for the identification was carried out by introducing a plasmid. Qualitative and quantitative analysis of primers were done using a bioinformatics tool. After suitable thermodynamic check, optimal sets of primers were developed. Further, gradient PCR was carried out through which an optimal annealing temperature of primers was found to be 59□. Endpoint PCR was carried out at 94□ for 4 min followed by 37 cycles of denaturation at 94□, 20 sec followed by annealing at 59□, 20 sec and extension at 72□, 20 sec. For this study we followed three objectives. First, PCR of virus primers with tick DNA was carried out for a negative control and PCR with virus primers, tick DNA along with its appropriate plasmid was done for positive control. Second, sensitivity of the primers was checked by doing PCR of gradient concentration of virus plasmid with respective primers. Hence, the result depicts that the primers are very sensitive (1 femtogram is enough) for virus detection. Third, PCR with virus primer, gradient concentration (10^{-9} to 10^{-15}) of plasmid and tick DNA was carried out to mimick the infection at various concentrations. Hence, it was found that infection can be detected even in small amounts.

Introduction

Ticks are arthropods, along with insects, and are well known vectors of animal and human diseases. Disease transmission by ticks has been well studied because of the social and economic importance as the disease agents (Bowman and Nuttall 2008). There are two well-known families of ticks, *Ixodidae* (hard tick) and *Argasidae* (soft ticks) (Soneshine and Roe 2013).

The *Powassan virus* (POWV) is in the *Flaviviridae* family and *Flavivirus* genus. Since *Powassan virus* can infect the *Ixodes scapularis* tick (common name: Deer tick) this virus is also known as deer tick virus (DTV). Lyme disease is a common tick-borne (carried by *Ixodes scapularis*) disease. *Ixodes scapularis* is also a POWV carrier and can transmit several other pathogens. So, when tests are conducted for Lyme disease, the ability to detect the *Powassan virus* is desirable. *Heartland virus* (HRTV) is in the *Bunyaviridae* family and in the genus *Phlebovirus*. *Phlebovirus* is most commonly observed in animals and can infect humans. According to Centers for Disease Control and Prevention (CDC) in case of humans, vaccines for the prevention or medication to cure *Heartland virus* have not been introduced. Prevention for this virus disease is simply by avoiding ticks and tick bites. Transmission of viruses that belong to *Phlebovirus* are vectored by mosquitos and sandflies. *Heartland virus* is transmitted by *Amblyomma americanum* or the Lone Star tick.

Tick are very common in outdoor areas where there are weeds, woods, tall grasses, and animals. Ticks can easily come in contact with humans both in rural and urban landscapes. There are various opportunistic tick-borne pathogens, which can directly cause illness or even death of human. This study focused on two of the tick-borne viruses, *Powassan virus* (POWV) and *Heartland virus* (HRTV). The objectives of the

research were to find optimal primers (proper thermodynamic, quality and sensitivity) for the detection of two viruses.

Materials and methods

Primer design (ticks, viruses)

Appropriate set of primers was designed. First, numerous sequences were found from NCBI (https://www.ncbi.nlm.nih.gov/) and alignment was performed using Clustal X2 (http://www.clustal.org/). Further aligned sequences are introduced to GeneDoc to get consensus sequences. The consensus sequences were used for primer design using primer3. The parameters of primer design were adjusted to obtain optimal sets of primers. While designing primers, Tm, GC %, "any", "3' complimentarity" values were considered. Once the primers were designed, quality analysis was done using mFOLD software where probable secondary structure and location were identified. In mFOLD, ΔG value (standard $-1 < \Delta G < 1$) were considered. The greater the ΔG value, the greater the chance of forming a primer dimer and secondary structures. Primers were checked for its compatibility using BLASTn software and whether the primers that were designed will attached to other non-target RNA sequences was determined.

Lyophilized primer dilution:

$V_1 \times C_1 = V_2 \times C_2$ Stock solution: 100 µM

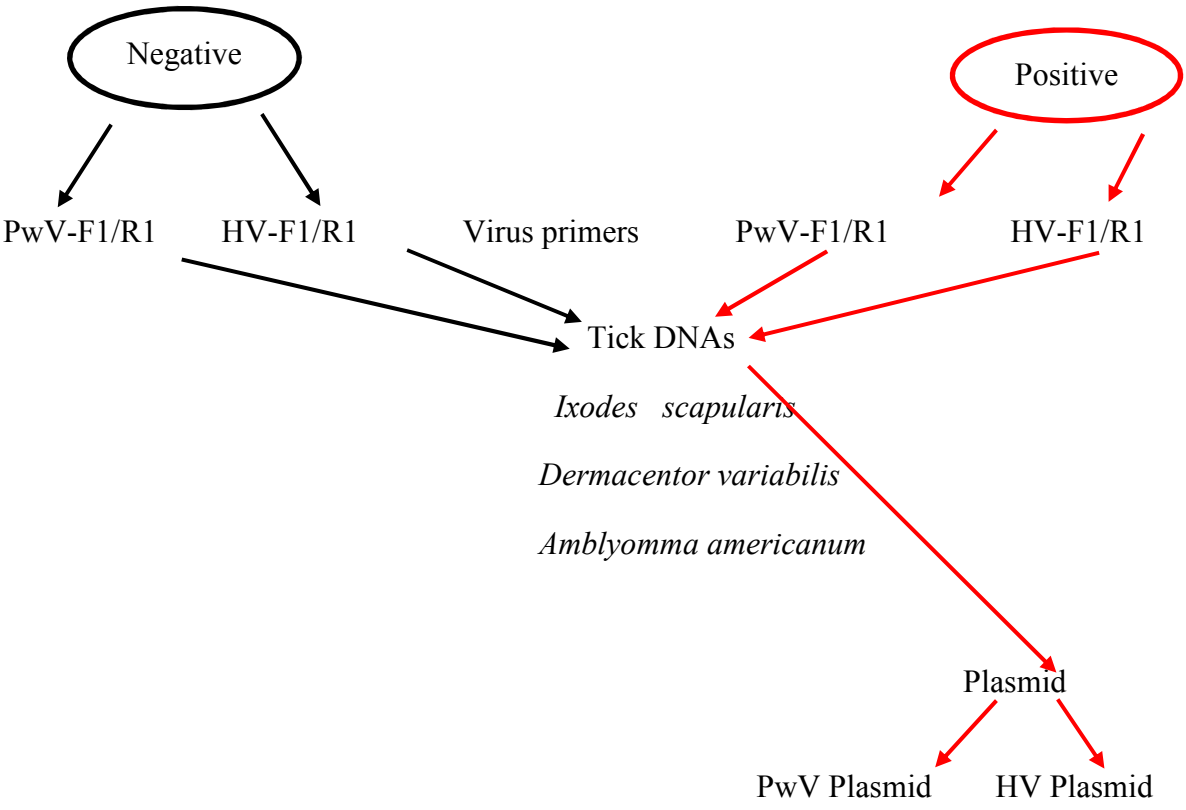
$V_1 = (50\ \mu l \times 5\ \mu M) / 100\ \mu M = 2.5\ \mu l$ Working solution: 5 µM

Gradient PCR of primers (Ticks as well as virus)

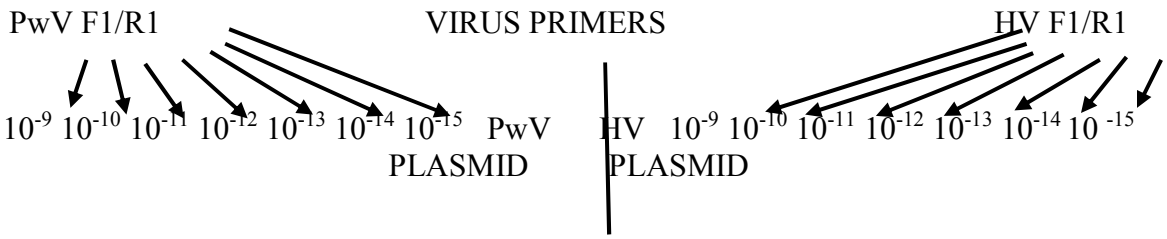
PCR was carried out at initial denaturation of 94□ for 4 min followed by 37 cycles, 94□ for 20 sec (denaturation), 50□-60□ (60, 59.3, 58.1, 56.3, 54, 52.3, 50.9, 50) for 20 sec

(annealing) and 72□ for 20 sec (extension). Final extension was carried out at 72□ for 3 min. Gradient PCR with same regiments was carried out for primers (virus as well as tick) with tick DNA.

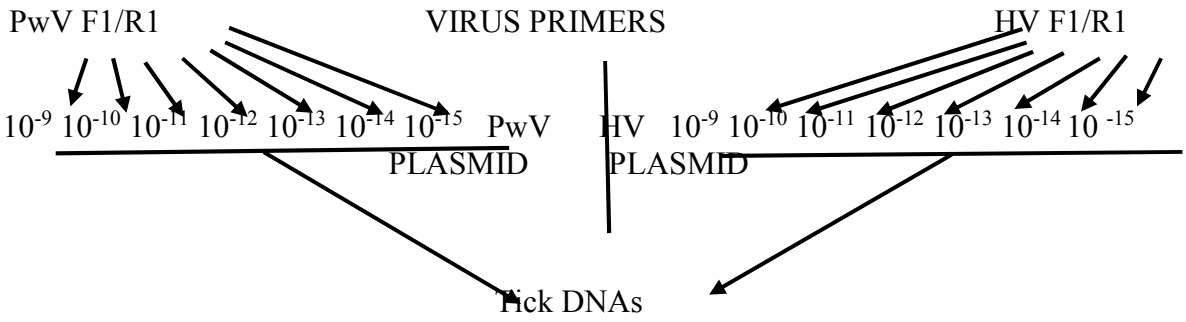
Objective 1



Objective 2



Objective 3



Ixodes scapularis

Dermacentor variabilis

Amblyomma americanum

Results

Primer designing

Many sequences were aligned using clustalX2 and conserved sequence was identified using GeneDoc. Consensus sequence were used for designing primers in primer3 (version 0.4.0)

Powasasn virus

OLIGO	start	len	tm	gc%	any	3'	sequence
LEFT PRIMER	9475	19	60.53	52.63	2.00	0.00	GCCCTCAAIACCATIACAA
RIGHT PRIMER	9735	19	60.47	63.16	2.00	0.00	CTCCCAITCICCCACATCC

PRODUCT SIZE: 261, PAIR ANY COMPL: 2.00, PAIR 3' COMPL: 0.00

```
                                                                  GCCCTC
                                                                  >>>>>>

9481 AAcACCATcACAAACATGAAAGTCCAaCT-ATCCGCATGATGGAAGGaGA-GGAGTCATa
     >>>>>>>>>>>>>

9541 GGACCAGC-GACTCACAGGA-CC-CGtCTgAAAAGGGTaGAA-C-TGG-TGA-GGAgCAc


9601 GGAG-TGAA-G--T-GGGAGGATGCT-GTGAGTGGAGAtGA-TGtGTcGTCAA-CCCATc


9661 GA-GACAGGTT-GGAAAGGC-CTGTACTTCcTGAATGAcATGGC-AAg---CGtAAGGAT
                                                              <<<<

9721 GTGGGaGAgTGGGAG
```

Heartland virus

OLIGO	start	len	tm	gc%	any	3'	sequence
LEFT PRIMER	1951	24	60.03	41.67	3.00	0.00	GGCAATCACTTTCCTTATGAGTCT
RIGHT PRIMER	2153	18	60.01	55.56	3.00	0.00	GAATGGGGATGCAGCAAC

PRODUCT SIZE: 220, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

```
                                        GGCAATCACTTTCCTTATGAGTCTGGAAGA
                                        >>>>>>>>>>>>>>>>>>>>>>>>>>>>>

1981 CAAATCAACAACAGAAGAGCTTGTTACTCTCACGAGGTATTCACAAATGGAGGGATTTGT

2041 GTCTCCACCCCTGCTCCCTAAACCCCAGAAGATGGTGGAAAAGTTAGAAGTTCCtTTGCG

2101 AACAAAGCTTCAAGTGTTTTTGTTTAGGAGGCATCTTGATGCTATTGTTAGAGTTGCTGC
                                                                <<<<<<<<<

2161 ATCcCCATTC
     <<<<<<<<<<
```

Tick primers (internal control)

Ixodes scapularis

OLIGO	start	len	tm	gc%	any	3'	sequences
LEFT PRIMER	18	23	58.99	34.78	4.00	0.00	AAATTGCTGTGGTATTTTGACCT
RIGHT PRIMER	185	23	57.73	39.13	4.00	0.00	AAATTCATAGGGTCTTCTTGTCC

SEQUENCE SIZE: 456
INCLUDED REGION SIZE: 456

PRODUCT SIZE: 168, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

```
    AAATTGCTGTGGTATTTTGACCTATACAAAGGTATTGAAATAA
```

```
  >>>>>>>>>>>>>>>>>>>>>>

 61 GATTTTAAATGAGTGCTAAGAGAATGATTAAACAATTAAAAACTTTCTTTAATTAAATAA


121 TTAAATTTAATTTTTTTGTGCAAAAGCAAAAATAAAATTTAGGGACAAGAAGACCCTATG
                                                  <<<<<<<<<<<<<<<<<<<


181 AATTT
    <<<<<
```

Dermacentor variabilis (16s rRNA)

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	128	26	59.22	34.62	4.00	0.00	AATTAAGGACAAGAAGACCCTAAGAA
RIGHT PRIMER	341	18	59.33	50.00	6.00	0.00	CAACATCGAGGTCGCAAA

SEQUENCE SIZE: 403
INCLUDED REGION SIZE: 403

PRODUCT SIZE: 214, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

```
AATTAAGGACAAGAAGACCCTAAGAATTTTAAAATAATTAATTTCTTATAAGA
>>>>>>>>>>>>>>>>>>>>>>>>>

181 AATTAATTATTTTTAGTTGGGGCAACTAGAAAATATTATAAACTTTTTATTTAATAAAAT


241 GACCCATTATTAATGATTATTTGAGAAAAATACTCTAGGGATAACAGCGTAATAATTTTT


301 GATAGAACCTATAGACAAAATAGTTTGCGACCTCGATGTTG
                             <<<<<<<<<<<<<<<<<<<<
```

Dermacentor variabilis Ferritin (fer) mRNA

OLIGO	len	tm	gc%	any	3'	sequence
Left primer	16	56.02	62.50	4.0	3.0	ATGGCCGCTACTCAGC
Right primer	22	59.24	54.55	5.0	3.0	CTAGTCTGACAGGGTCTCCTTG

product length = 519

```
      ATGGCCGCTACTCAGCCCCGTCAGAACTACCACGTCGAAT
      >>>>>>>>>>>>>>>>
  181 GCGAAGCCCGCATCAACAAGCAGATCAACATGGAACTCTACGCGAGCTACGTCTACACAT
  241 CAATGGCCTACTACTTTGACCGCGATGATGTGGCACTGCCGGGATTCCACAAGTTCTTCA
  301 AGAAAAGCAGCGAGGAGGAGCGTGAACATGCTGAGAAGCTGATGAAGTATCAGAATATGC
  361 GTGGAGGGCGTGTGGTGCTGCAGCCAATCCAGAAGCCAGCACAGGACGAATGGGGTGCAG
  421 GCTTGGACGCCATGCAGGCTTCACTGGAGCTGGAGAAGACTGTTAACCAGTCACTGCTTG
  481 ACCTGCACAAGCTGGCGACTGATCACAATGACGCTCAGCTGTGTGACTTCCTGGAAAGTG
  541 AGTACCTGGCAGAGCAGGTGAAGGCCATCAAGGAGCTGTCCGATTATGTGACCAACTTGA
  601 AGCGTGTTGGCCCTGGCCTTGGAGAGTACATGTTTGACAAGGAGACCCTGTCAGACTAG
                                                  <<<<<<<<<<<<<<<<<<<<
```

Amblyomma americanum

OLIGO	len	tm	gc%	any	3'	sequence
Left primer	20	61.32	65.00	3.00	0.00	GAGGGGAGTCTGGAACGGAG
Right primer	22	61.68	54.55	5.00	0.00	GAAGTCGTCCAGGTCGTTGTTG

product length = 466

```
      gaggggagtctggaac
      >>>>>>>>>>>>>>>>
  841 ggagttaccgatgtgcacaccccactctactcgagatccattgatatcggtccgcagaag
  901 actctgaatgtgaaagacggcctcgctcgcatcgtgagcagcggtgcgccgaagtccaag
  961 gtggtgatgggtatcgctttcttcggccggggcttcacgctcctcgacccggcacagcac
 1021 ggcctccacgccctcgtcaaccgcgacgtgcctccgcgccccggacccctttgtcaggagc
```

```
1081 aacgaggtgttcgcctactacgagatctgtctcaacctgaagggcaactggaagcgcgag
1141 ttcgacgaggagggcaagtgcccgtatgtatactacagggaccagtggatcggctacgat
1201 gatgctgtcagcattcgacacaagatcaacttccttctgcaagagggctaccggggtgtc
1261 tacgtgttcaacaacgacctggacgacttc
             <<<<<<<<<<<<<<<<<<<<
```

Once the primers were designed, melting temperatures for each primer were determined to be close enough to conduct the PCR using all the primers in the same cycles at the same time. “Any” and “3’ complimentarity” value was checked thoroughly. If “any” value is higher (standard usually three but flexible till five) than standard then there was a greater probability of forming secondary structure (such as rattle neck structure) or self-binding of primers (Arif and Ochoa-Corona 2013). Similarly, if “3’ complimentarity” value was greater (standard usually 0 but flexible till 3) than the standard then there is a higher chance of forming primer dimer. A high value of 3’ complimetarity indicates the greater chances of self-binding of primers at 3’ region. By observing the possible secondary structure using mFOLD software the self-binding capacity and location of the primers can be predicted. If primers form a loop by self-binding at 3’ then the primers will not bind with the target sequence with strong affinity. Hence, either high primer dimer could be formed (smear in gel) or primer may not bind at all.

Qualitative analysis of primers

Although, some primer may have “3’ complimentarity” and “3’” value to the threshold, by looking the ΔG value (standard $-1<\Delta G<1$) and possible secondary structure, primers are still usable.

Although there are probabilities of forming secondary structure (such as primer dimer as a result), primer was considered acceptable because the structure formation is in the 5' region (Fig. IV.2.-IV. 6.).

Gradient PCR of primers

The primers were exposed to different annealing temperatures to determine the one where no band would appear (Fig. IV. 9.) Primers PwV-F1/R1, HV-F1/R1, IxSc-F1/R1, DV-F1/R1 and Dv/Fer-F1/R1 did not produce a band in the gel which meant these primers were flexible over a temperature range. However, some bands were observed for the primer set AA-F1/R1 lane with every temperature. Although some primer dimer can be observed in a gel for AA-F1/R1, the band is smaller than 466 bp (amplicon size of AA-F1/R1 primers).

Conclusion and discussion

A PCR program to detect two tick-borne human viruses and an internal control using tick DNA sequence that can be conducted at the same time was established. Because of the increase in POWV and HRTV cases, working primers set to detect both at the same time is needed. The Oklahoma State University tick rearing facility has been supplying ticks for research purposes for many years. This rearing facility must supply pathogen free ticks. Due to the large number of pathogens that ticks can transmit and because these ticks are used for research studies, a detection method to ensure that those ticks are virus free is also desired.

Primer design tools are necessary for the customization of the required primers. Primers to detect *Powassan virus* [TBE-1(5'-3' ACATGGCAGTACTGGGG) and TBE-2 (5'-3' CCCATCATGTTGTACAC)] have been developed before and been used

frequently in many researches (Ebel et al. 1999; Brackney and Armstrong 2016). However, there is no indication of any quality checks using softwares such as mFold. Although, the primers were capable enough to detect the viruses, the sensitivity of the primers were not determined (Ebel et al. 1999; Brackney and Armstrong 2016). Similarly, for *Heartland virus*, primers have been developed before [(HRTV1-forward TGCAGGCTGCTCATTTATTC, HRTV1-reverse CCTGTGGAAGAAACCTCTCC) (HRTV4-forward CCTTTGGTCCACATTGATTG, HRTV4-reverse CACTGATTCCACAGGCAGAT)] but possible secondary structure was not determined (Savage et al. 2013). HRTV1-reverse primer can possibly bind to itself (Fig. IV. 7.) in 3' region. The HRTV4-forward primer can form secondary structure by binding itself at 3' region primer. This self-binding prohibits the primer to bind specifically in target region. Also, the HRTV4-reverse primer can possibly form big loop near 3' region (Fig. IV. 8. b). However, the new set of primers (Table IV. 1.) developed in this research has helped to overcome the limitations of previously used primers. The new primers have been tested for their quality (Table IV. 2.-IV. 3., Fig. IV. 1.-IV. 6.) by checking with NCBI and ensuring no alignment with off target, no self-binding, and no secondary structure formation. In this study, set of primers was designed for the identification of *Powassan virus*, *Heartland virus* and also three primers for tick DNA as an internal control, namely *Ixodes scapularis, Dermacentor variabilis,* and *Amblyomma americanum* (Table IV. 1.). After designing the primers, there were a few flaws with the primers such as low GC content in *Ixodes scapularis*. GC binds each other with a three-hydrogen bond and AT binds with a two-hydrogen bond. Hence, if the GC content was too low for a primer, then the bonding is weak. Therefore, the primer will have a low bonding capacity and so will

be unable to bind properly in target sequence during PCR. However, despite low GC content the ΔG value was within range. Also, the probable secondary structure given by mFOLD software indicated that if there was any secondary structure formation, that is more likely in 5' end. Sequence is elongated from 5' to 3' region, so the 3' region should fully bind to the target sequence. If there was an internal binding of primers at the 3' region, then it is more likely the primers will form a loop-like rattlesnake structure and primers would not bind to the target (Arif and Ochoa-Corona, 2013). After developing the primers, a plasmid containing particular sequence of POWV and HRTV, which respective primers could identify, were developed. Further, serial dilution of the plasmid was carried out to check the sensitivity of the primers. With the data presented in this research, it has been determined that even as small as 1 femtogram of sample is enough to give clear bands at gel electrophoresis for the detection.

When tick primers were subjected to BLASTn individually, the results concluded that both forward and reverse primers can be attached to other sequences than the target. However, both primers do not attach to the same species. Hence, these primers are specific enough to be used as internal control to identify a specific tick species. Also when virus primers were compared using BLASTn, they do not align to any other species than that particular virus itself. Hence, primers are specific for the sequence in target virus and do not bind to any other sequences.

Table IV. 1. List of primers used for tick experiment.

Acronym used	Primer ID	Forward primers	Reverse primers	Expected product size (bp)
Powassan virus	PwV-F1/R1	GCCCTCAAIACCATIACAA	CTCCCAITCICCCACATCC	261
Heartland virus	HV-F1/R1	GGCAATCACTTTCCTTATGAGTCT	GAATGGGGATGCAGCAAC	220
Ixodes scapularis	IxSc-F1/R1	AAATTGCTGTGGTATTTTGACCT	AAATTCATAGGGTCTTCTTGTCC	168
Dermacentor variabilis (16s rRNA)	DV-F1/R1	AATTAAGGACAAGAAGACCCTAAGAA	CAACATCGAGGTCGCAAA	214
Dermacentor variabilis (fer mRNA)	DV/Fer-F1/R1	ATGGCCGCTACTCAGC	CTAGTCTGACAGGGTCTCCTTG	519
Amblyomma americanum	AA-F1/R1	GAGGGGAGTCTGGAACGGAG	GAAGTCGTCCAGGTCGTTGTTG	466

Table IV. 2. ΔG value determination using mFOLD.

Forward primer	ΔG value (kcal/mol)	Reverse primer	ΔG value (kcal/mol)
PwV-F1	0.0	PwV-R1	
HV-F1	0.9	HV-R1	0.8
IxSc-F1	0.0	IxSc-R1	0.9
DV-F1	0.9	DV-R1	0.4
DV/fer-F1	0.8	DV/fer-R1	0.0
AA-F1	0.8	AA-R1	0.9

Table IV. 3. BLASTn to verify the primers compatibility other than its own.

Primers ID	Forward	Reverse
Powassan virus	*Fusarium verticillioides*	
	Oryza sativa indica cv. RP	
	Mus musculus NLR family	*Mus mucus* targeted KO
		Homo sapiens chromosome 4
		Botrytis cinerea B05
Heartland virus	*Brassica napus cullin*	
	Solanum lycopersicum	
	Lupinus angustifolius cv. Tanji	
	Burkholderia multivorans	
	Solanum pennellii	
		Aspergillus bombycis
		Arachis ipaensis
		Sclerotinia sclerotiorum
Ixodes scapularis	*Ixodes persulcatus*	
	Amblyomma dissimile	
	Hemaphysalis qinghaiensis isolate HY	
	Ornithodoros capensis strain KOR	
	Amblyomma mixtum	
	Dermacentor nitens	
		Ixodes kazakstani
		Rhipicephalus haemaphysaloides
		Ixodes inopinatus
Dermacentor variabilis (16s rRNA)	*Amblyomma gervaisi*	
	Haemaphysalis qinghaiensis	
	Dermacentor auretus	
	Dermacentor nitrens	
		Tetraclita rufotincta
		Haemaphysalis japonica
		Phatnoma laciniatum
Dermacentor variabilis (fer)	*Rhipicephalus sanguineus*	
	Hyalomma anatolicum	
	Dermacentor andersoni	
		Dermacentor albipictus
Amblyomma americanum	*Drosophila serrata*	
		Rhipicephalus sanguineus
		Streptomyces pactum

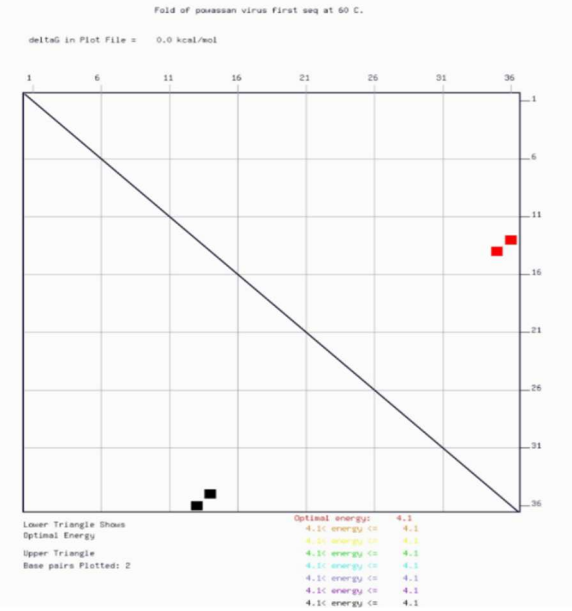


Fig. IV. 1. *Powassan virus* primer mFOLD.

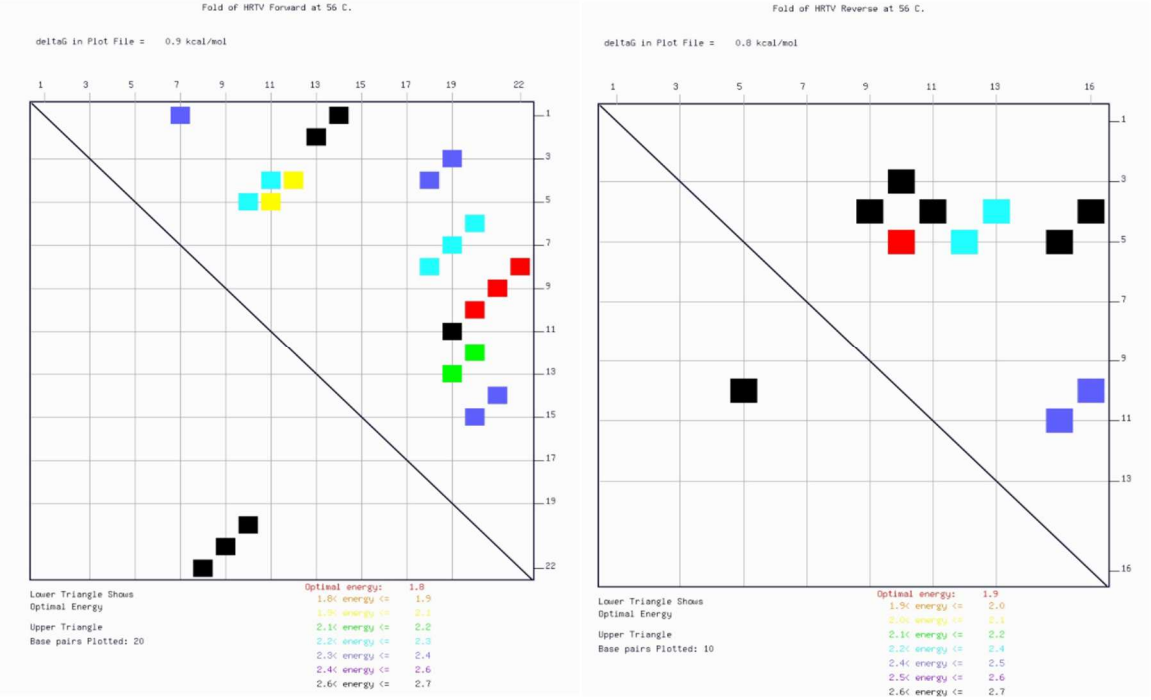
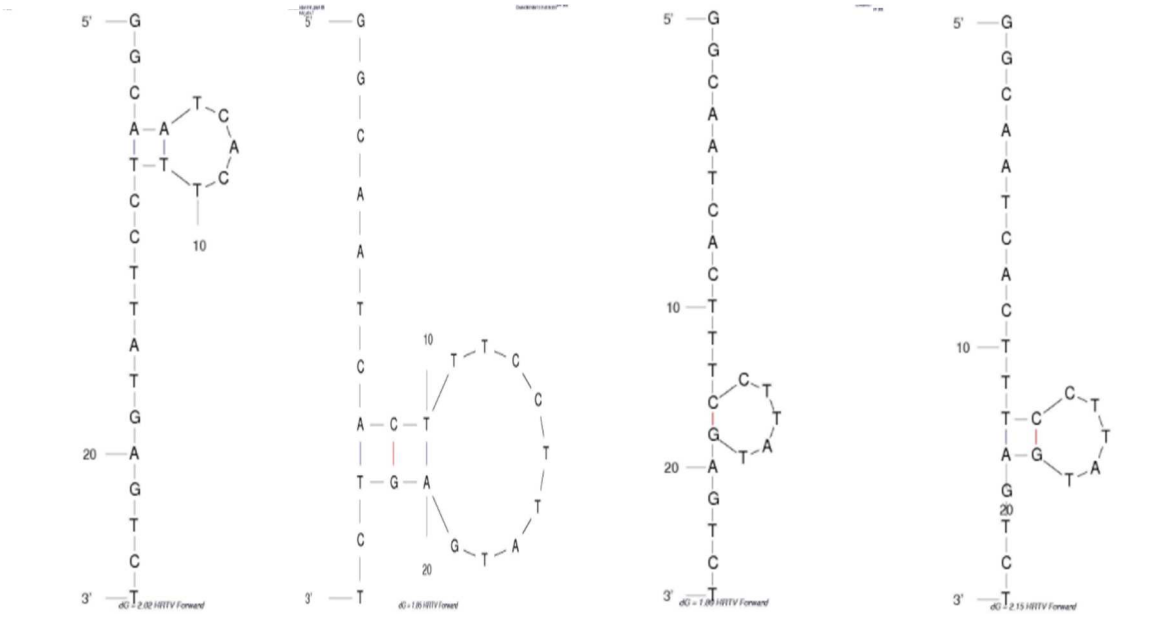
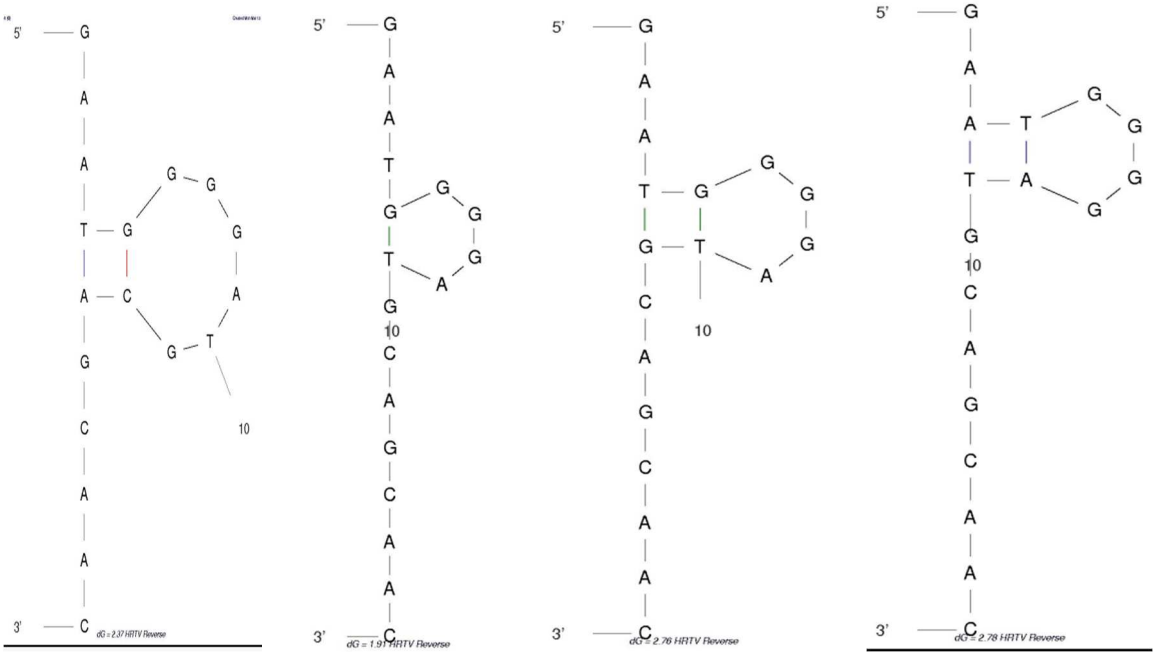


Fig. IV. 2. *Heartland virus* primers mFOLD.

(a)*Heartland virus* Forward. (b)*Heartland virus* Reverse.



(c) Possible secondary structure formation position of *Heartland virus* forward primers.



(d) Possible secondary structure formation of *Heartland virus* reverse primers.

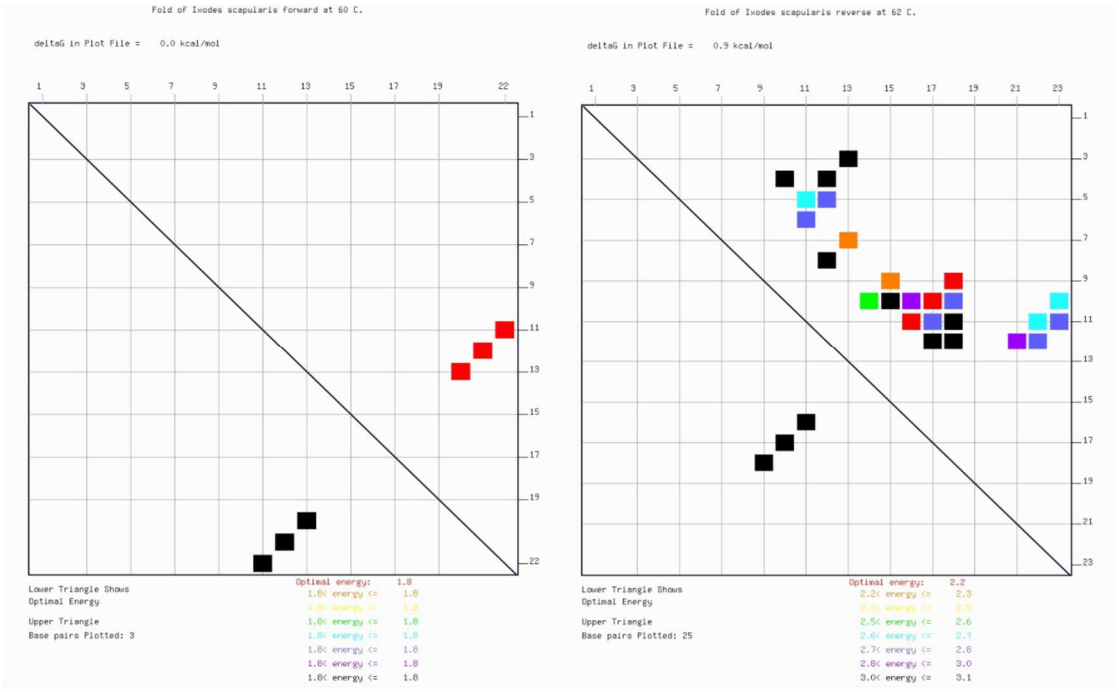
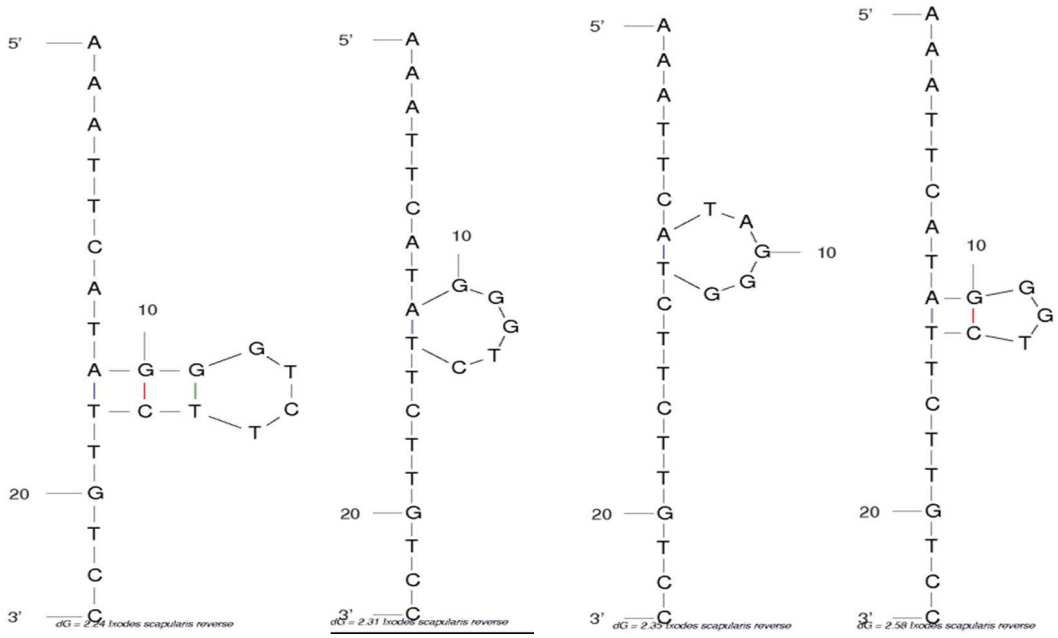


Fig. IV. 3. *Ixodes scapularis* mFOLD.

(a)Ixodes scapularis forward primer. *(b) Ixodes scapularis* reverse primer.



(c) Possible structure formation location for *Ixodes scapularis* reverse primers.

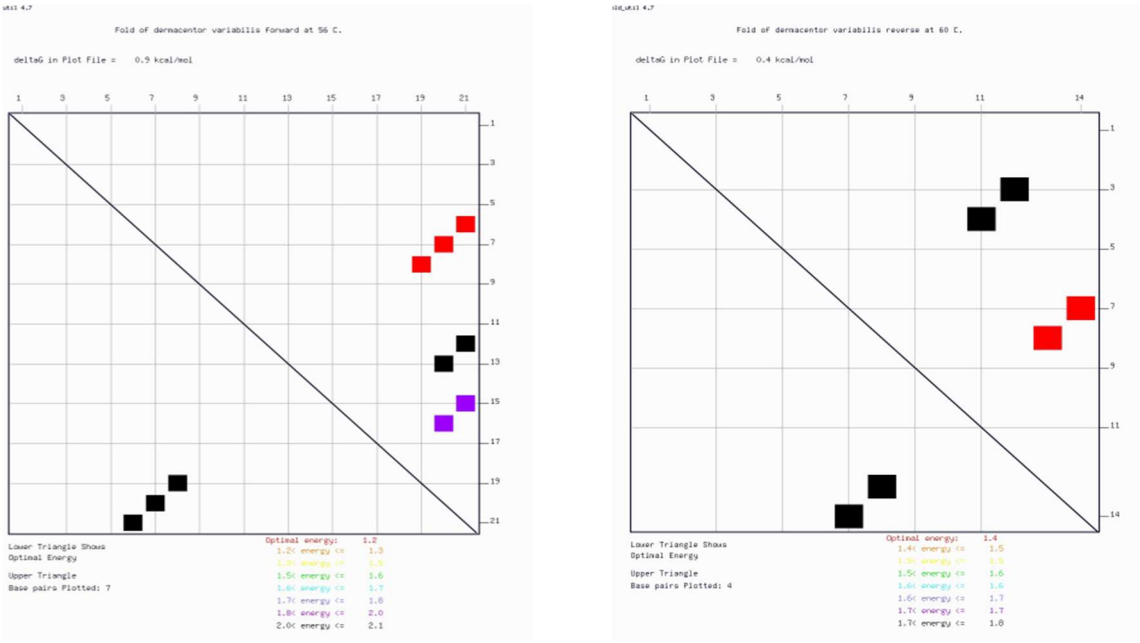
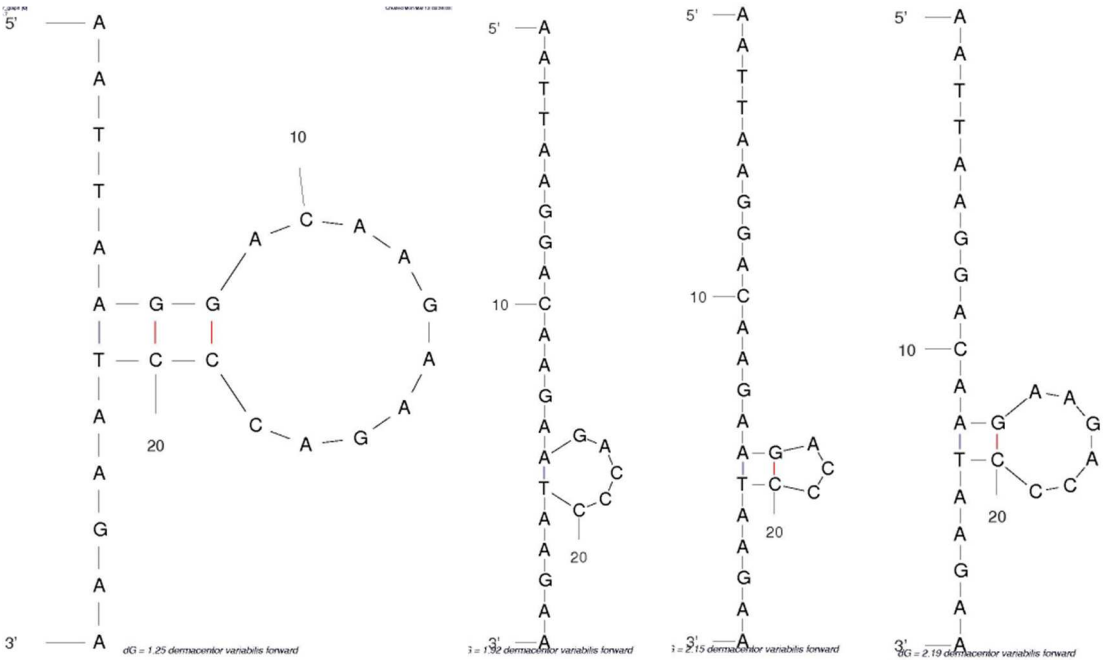
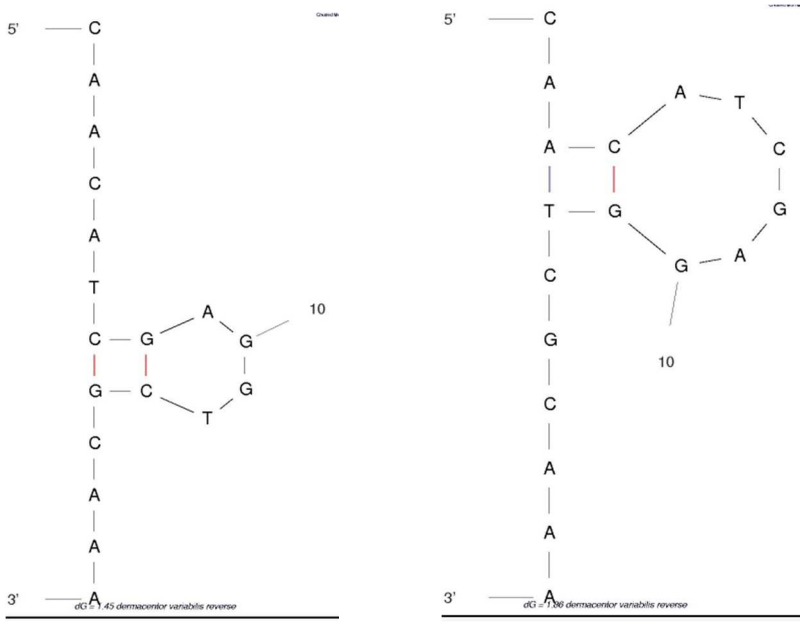


Fig. IV. 4. *Dermacentor variabilis* (16s rRNA) mFOLD.

(a)Dermacentor variabilis forward primer. (b)*Dermacentor variabilis* reverse primer.



(c) Possible structure formation location for *Dermacentor variabilis* (16s rRNA) forward primers.



(d)Possible structure formation location for *Dermacentor variabilis* (16s rRNA) reverse primers.

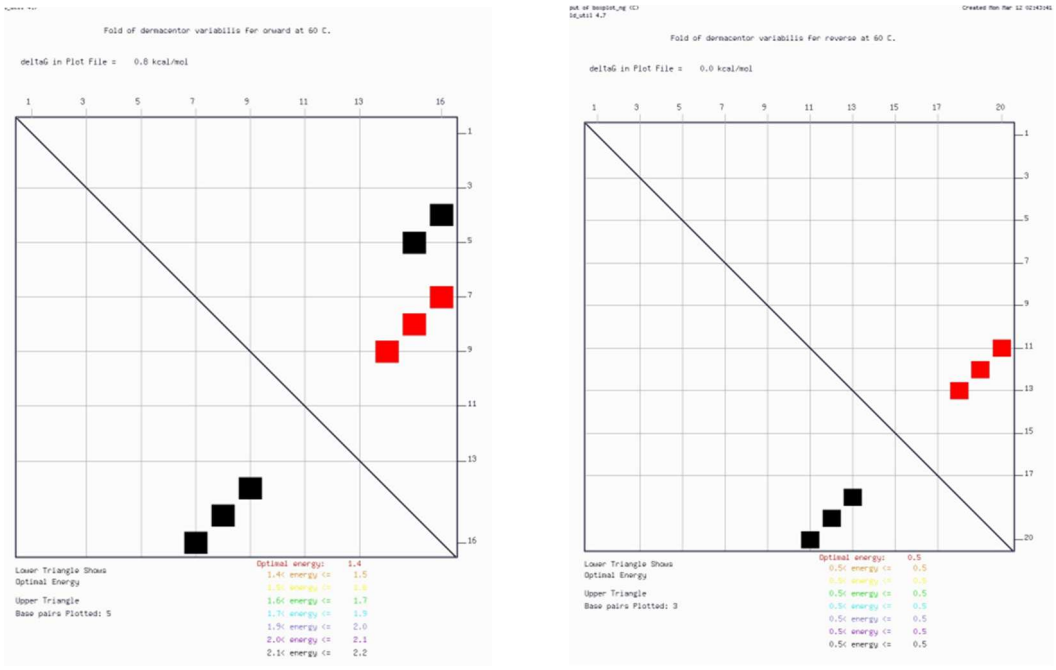


Fig. IV. 5. *Dermacentor variabilis* (fer mRNA) mFOLD.

(a)Dermacentor variabilis(fer) forward primer. (b)*Dermacentor variabilis*(fer) reverse primer.

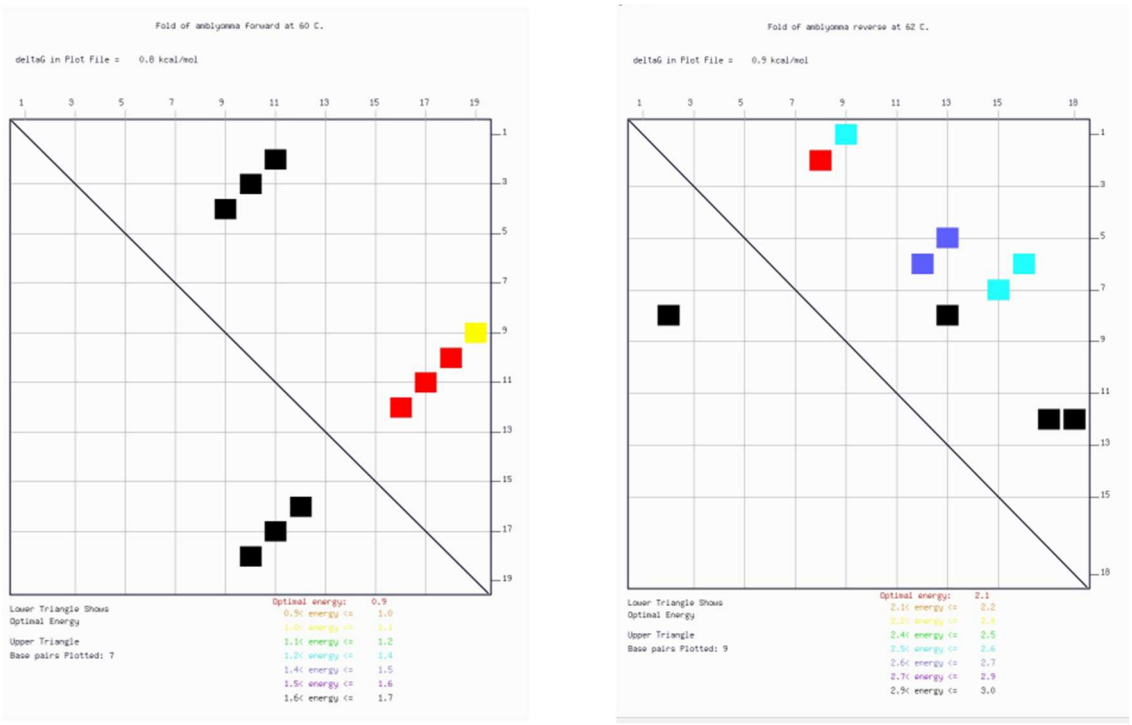
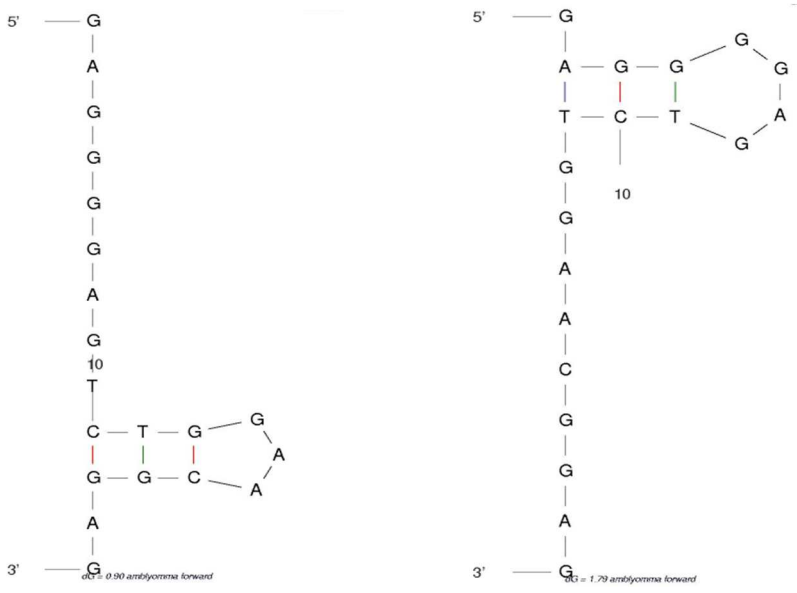
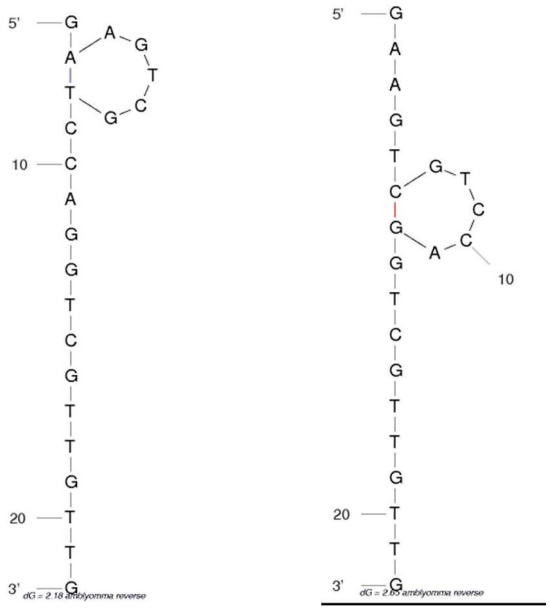


Fig. IV. 6. *Amblyomma americanum* mFOLD.

(a)Amblyomma americanum forward primer.(b) *Amblyomma americanum* reverse primer.



(c)Possible structure formation location for *Amblyomma americanum* forward primers.



(d)Possible structure formation location for *Amblyomma americanum* reverse primers.

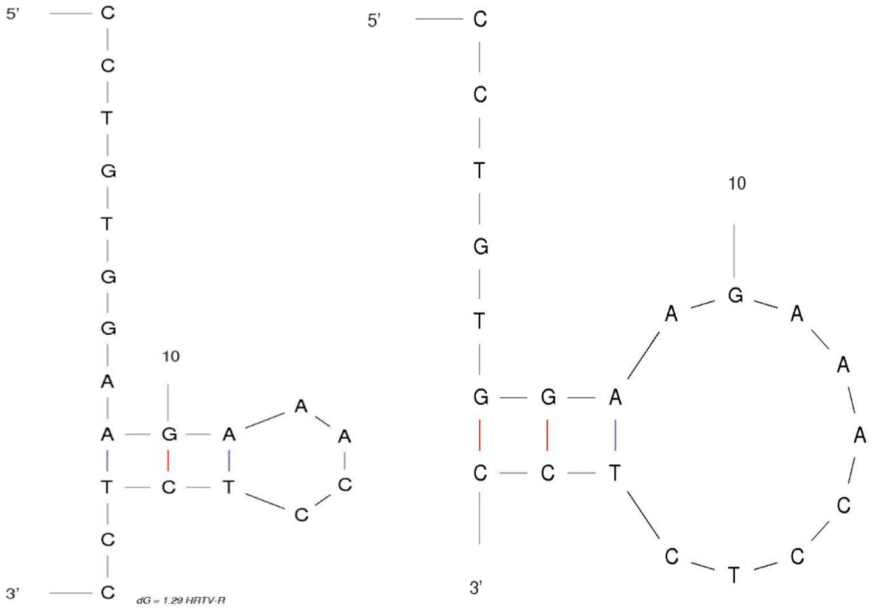


Fig. IV. 7. Possible secondary structure formation of HRTV1-reverse primer.

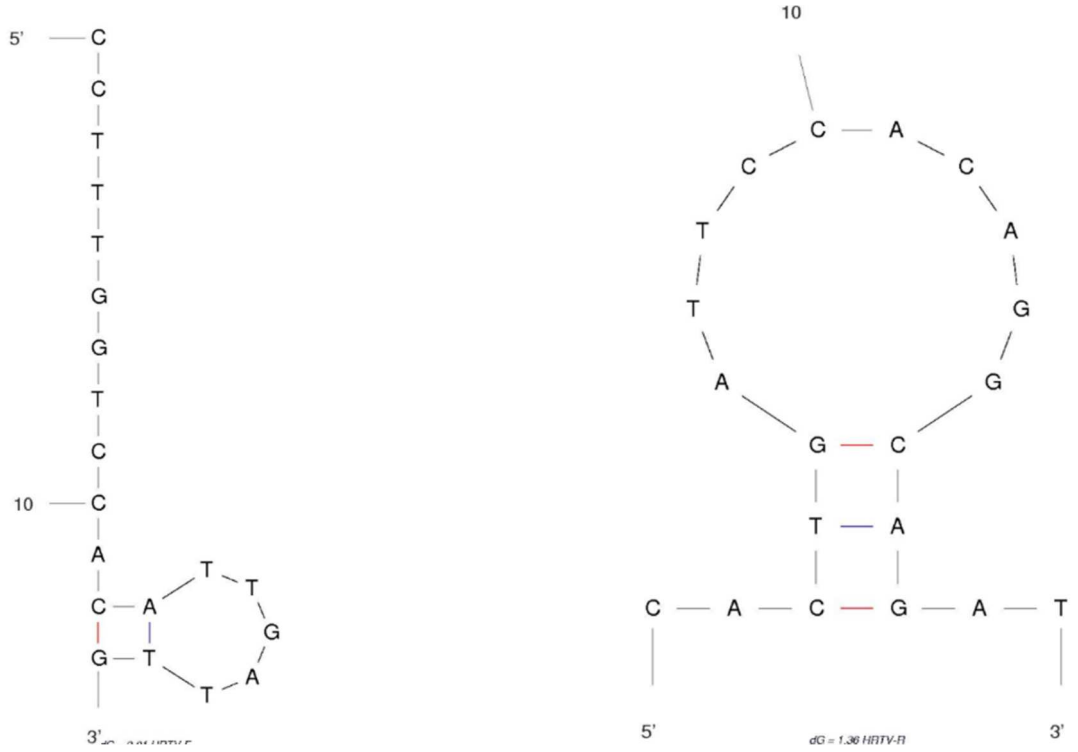
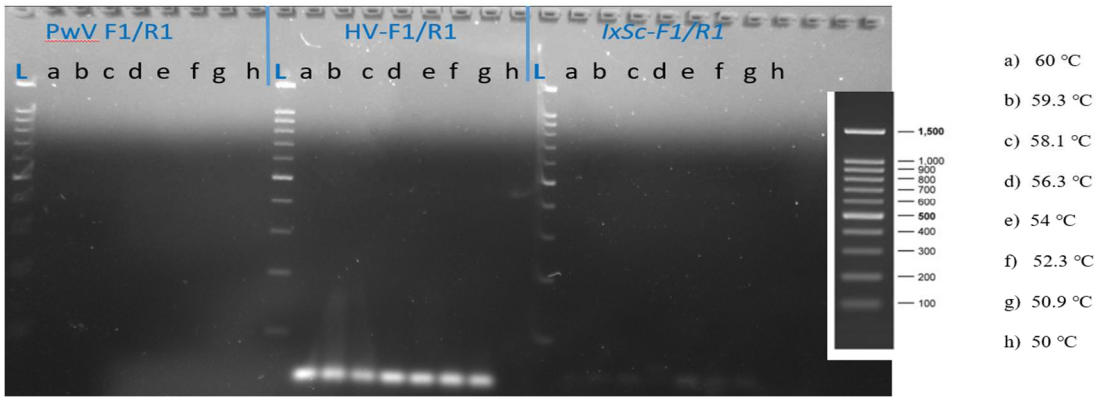


Fig. IV. 8. Possible secondary structure formation of HRTV4 primer.

a) HRTV4 forward.

b) HRTV4 reverse.

(a)



(b)

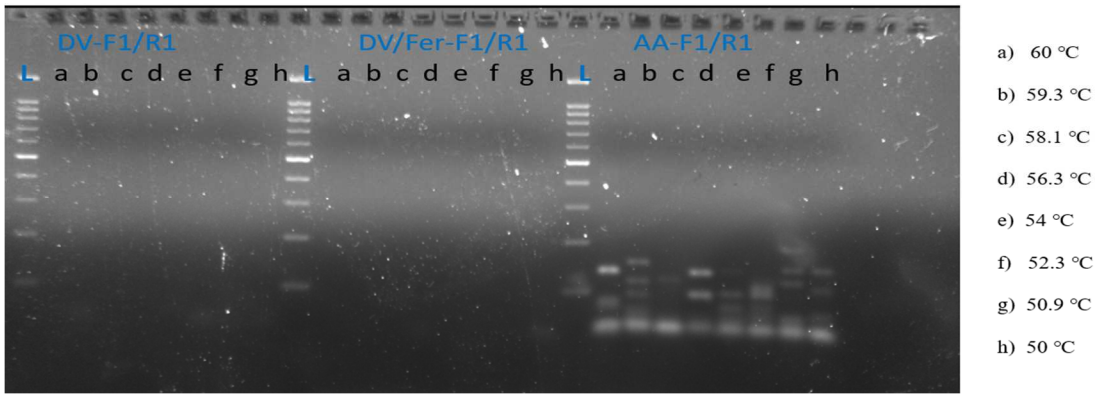


Fig. IV. 9. Gel picture showing gradient PCR of both primers (tick as well as viruses). L=ladder (Diamond DNA Ladder 100-1500bp), 2% agarose gel was used, 90 V, 65 min.

(a) First (a-h) is the temperature gradient for PwV-F1/R1 primers, second (a-h) is for HV-F1/R1 and third (a-h) is for IxSc-F1/R1.

(b) First (a-h) is the temperature gradient for DV-F1/R1 primers, second (a-h) is for DV/Fer-F1/R1 and third (a-h) is for AA-F1/R1. Some bands are visualized where (a-h) AA-F1/R1 primers were used. However, these bands were smaller than 466 bp (amplicon size of AA-F1/R1 primers).

Objective 1

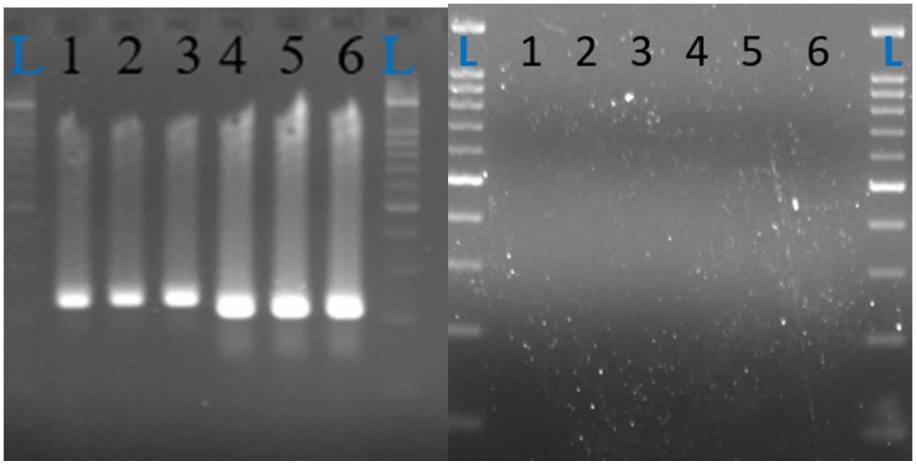
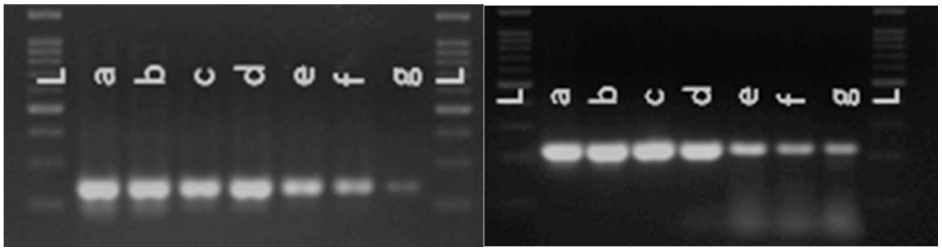


Fig. IV. 10. Gel picture of positive and negative control.

(Left) 1-3, PwV-F1/R1 with tick DNA along with PwV plasmid. 4-6, HV-F1/R1 with tick DNA along with HV plasmid. This is the case of mimicing of infection.

(Right) 1-3, PwV-F1/R1 with tick DNA. 4-6, HV-F1/R1 with tick DNA. It would have been positive only if tick DNA was infected with the viruses.

Objective 2



L= Ladder

a= 10^{-9}

b=10^{-10}

c=10^{-11}

d=10^{-12}

e=10^{-13}

f=10^{-14}

g=10^{-15}

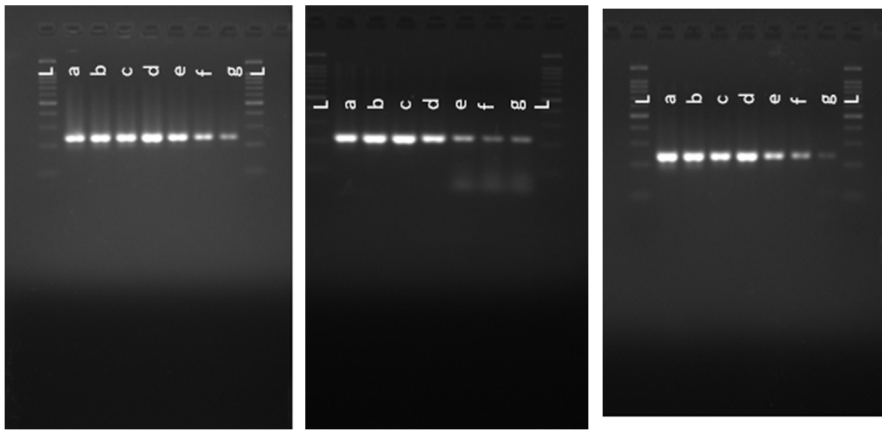
Fig. IV. 11. Gel picture of plasmid with various concentration (a-g).

(Left) *Powassan virus* primers. (Right) *Heartland virus* primers.

Objective 3

To check the sensitivity of the primers when tick DNA present. Both *Powassan virus* and *Heartland virus* primers was very much sensitive. Primers can detect even in as small as quantity as femtogram.

(a)



L= Ladder

a) 10^{-9}

b) 10^{-10}

c) 10^{-11}

d) 10^{-12}

e) 10^{-13}

f) 10^{-14}

g) 10^{-15}

(b)

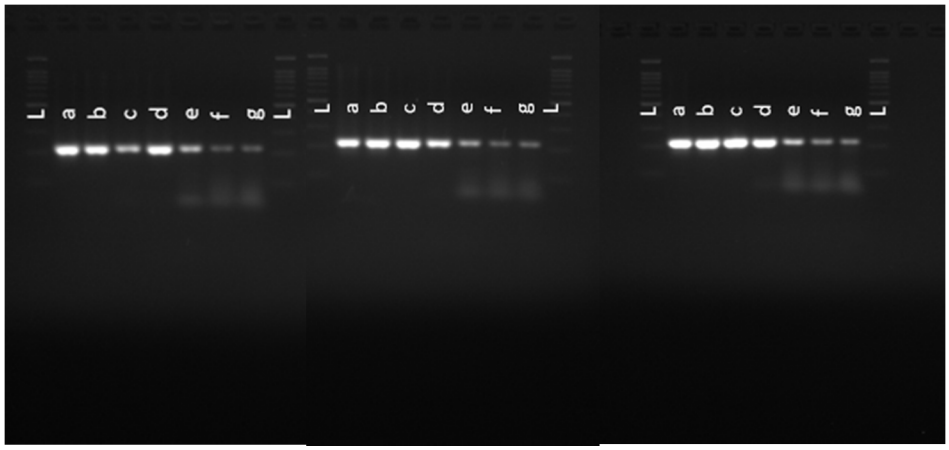


Fig. IV. 12. gel electrophorsis of different concentration of viruses used.

(a) *Powassan viruses* at different concentration (a-g). First (a-g) *Ixodes scapularis* DNA. Second (a-g) *Dermacentor variabilis* DNA. and third (a-g) represents *Amblyomma americanum* DNA.
(b) *Heartland viruses* at different concentration (a-g). First (a-g) *Ixodes scapularis* DNA. Second (a-g) *Dermacentor variabilis* DNA. and third (a-g) represents *Amblyomma americanum* DNA.

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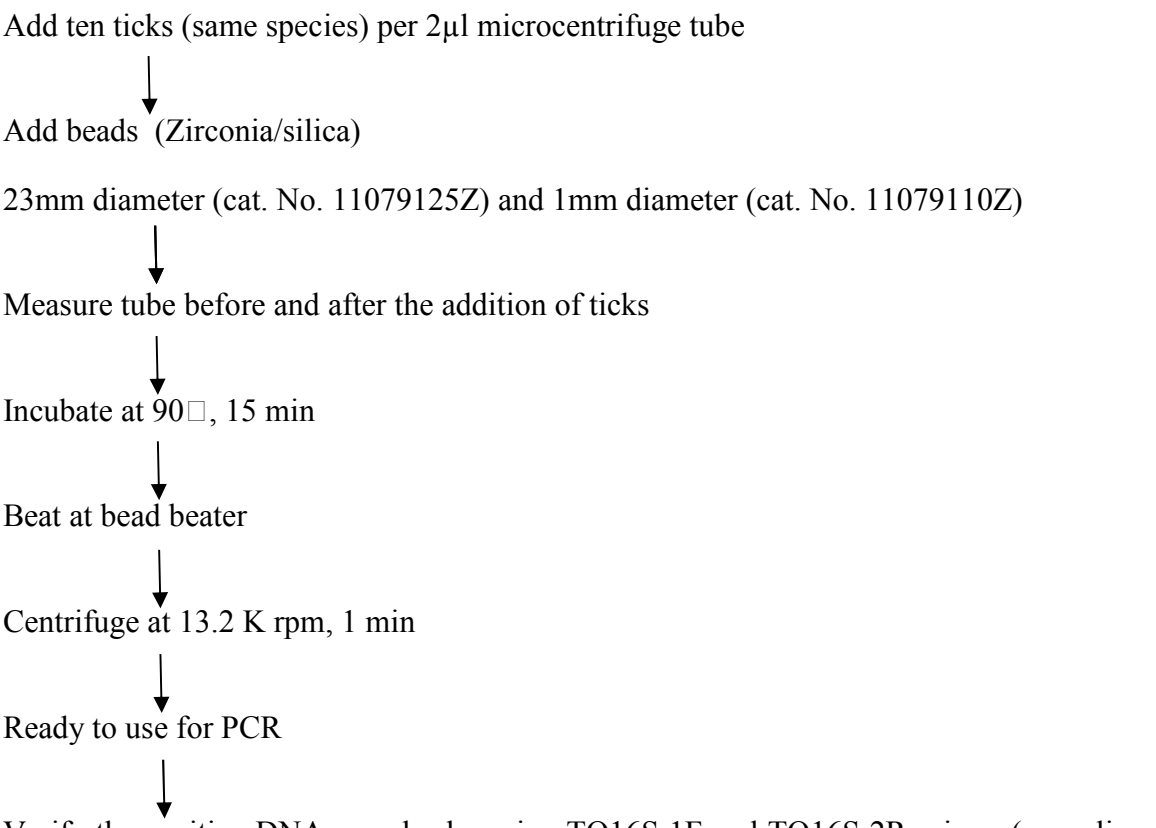
APPENDICES

Fig. A. 1. DNA isolation from tick.



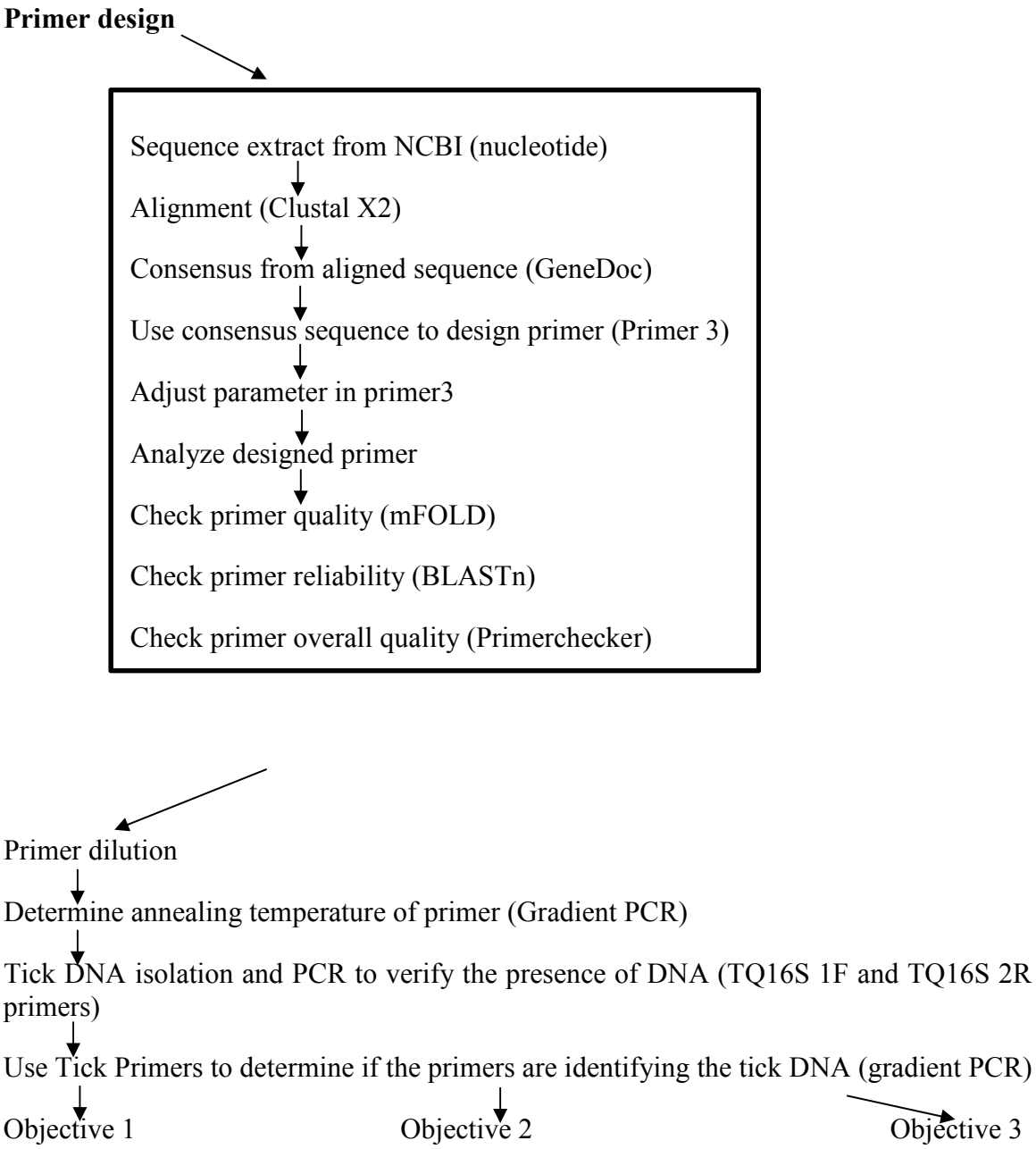


Table A. 1. Plasmid (containing viral sequence) used as control in the study.

Plasmid name	Size (bp)	Virus
pYM0036P1	1296	CaYMV-AP01 (Wijayasekara 2016 et al.)
pBYMV382	382	BYMV (Chauhan et al. 2015)
pYSOK7672	1827	CaYSV (Chauhan et al. 2015)

FASTA format (7385 bp DNA)

>MF074075.1 Canna yellow mottle virus isolate CaYMV-AP01, complete genome

TGGTATCAGAGCTGAGTTGAAGGTATGTCTATGCTGGGGTAAAACCCTAATA
AGGGCCCAAAGGGCTCTGTCTGTATGTTCCAATCTGGTTTATACTATACTGTT
TTGATTTTTCTGGATTCTGCTTAGAAGGAGTTCACTGCGGTGTGTTGTTTTATC
TTTATACTATGCTGCTATTACTAGCAAAGACGACCAGAGTTCTAAGTCTAGAT
CAAGTTTTAACCAGTTGAGGAAAAGATTGTGTTGCAAGCAAAAGACTGCAGG
TCCCGCCGAAGGCGAGGAAGCCGTATAGGGATGAGTCGGGTGAGCTTGCTGC
ACGATTGACCAAAAACTGGAAGAGACTAATCGTGACAAAGAACGAAGGTAG
GATCTGTTAGTAGTGACATACTAGTGTAAAGAAATAATCCACCCGGTTTGAG
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ACTCAGTCTTGTTCCTCTTAGTGCGACTAGACACTAAGATCGCCTCTCTCGAC

GACAAGTTACTGAGACTTGAAGCGAGAGTGAGGAATATTGAAGCGGCCAAA

GCTCCAGCAGGAACAGGAGCCCCCAACTGGAAGGAAGAGCTTGACAAGATC

ACAGGTAAACTCAGTGATCTACGCATCGGCGAACCAAGGCCGAGAGAAGTA

GGAGGTAATCTGAAGGTGATAAGAAACCCCTACAATATCCTGAAAGAAGTCA

AGCAGTGATGGCCAACAGAGGACGAGTCACTGGAAGATCTGGCTCTACCACC

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ACCGTCCCTGCAGAGGTCCTGTACATGACCCGGAGAGACGACATCCACCACC

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TTGTAAGAGTTCCAGTTCTAATAAACTTCGAGTTTCCCTTACATCTTTGTGTTC

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ATTTTC

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