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Follow-up of Tobamovirus infection in cultivated tomato crop using NGS analysis

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Follow-up of Tobamovirus infection in cultivated tomato crop using NGS analysis.

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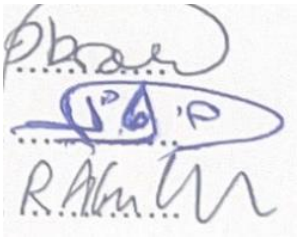

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Dedication

In the name of Allah, the most Gracious, the most Merciful.

Praise be to God, we have not succeeded nor we have not risen nor we have not excelled, except with his satisfaction. Praise be to God, we have not lead any path nor we have not overcome any effort except by his grace. And only Favor, perfection and completion are attributed to him.

In the middle of the most painful events and horrible massacres that took place nearly 8 months ago among our people and beloved Gaza. And in the middle of incursions that extended for days and days, it was not easy for us to continue our journey and reach our goals. Today, we are standing with our heads held high, seeing our great dream and two years journey are nearly ended, crowned with pride and gratitude. The journey was not easy nor was the road fraught with easy, but I did it. Praise be to God, who facilitated the beginnings and we reached the endings with his grace and generosity.
And who ever says, I am for it, he will attain it, and I am for it, despite the circumstances, I achieve my dreams.

I dedicate this success to myself first and foremost, and I am proud of this great achievement.

Then to the one whose name is inseparable from my name, to the one with whom I have always promised success, "my dear father."
To the one under whose feet Paradise was made and whose heart embraced me before her hands, "my beloved mother."

To my brothers and sisters for encouragement and continued support.

To my sister and companion all the times and crises. "Ibtesam"

To my journey companions, and my supporters at all times, "my friends".

To the one whose trusted me and bet on my success and supported me to continue this journey "my unknown soldier".

To the souls of the martyrs of Gaza and all those lost in this war.

I dedicate this work.

Maram Hatem

Declaration:

I certify that this thesis submitted for the degree of master in biochemistry and molecular biology is the result of my own research accept were otherwise cited, and this thesis is not to be submitted for a higher degree to any other University or institution.

Signature: 

Maram Hatem Adel Jaafreh

Date: 18/5/2024

Acknowledgment

First and foremost, praise and thanks be to God Almighty always and forever for his success and for the strength and endurance that he gave me in my life and throughout my educational journey.

I would like to thank my supervisor, Dr. Ibrahim Abbasi. I am grateful to him and his endless endurance, as his office door was always open whenever I faced a problem or had a question about my research or writing. I also thank the professors of the Biochemistry and Molecular Biology program for the knowledge and scientific tools they provided us

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Abstract

The Tomato vegetable plant (*Solanum lycopersicum*), which bears edible fruit, is a common component of the diet, and it is economically importance across the world. Tobamovirus group (family: Virgaviridae) are destructive agricultural diseases that infect plants. The main goal of the current research is to identify the most tomato cultivars that show the best resistance to viral infections. This outcome could help farmers to avoid many financial losses by choosing specific disease tomato resistance varieties. This study is designed to detect the presence of Tobamovirus relied on next generation sequencing (NGS) technology.

The study was performed after collection of leaves, fruit tomato plants and soil samples from 4 different greenhouses located in Jericho district over a period of four months starting from February to May 2023 till the end of the samples collection period, a total of 155 of soil and plant samples were collected. For each collected sample total nucleic acid extraction was done, and processed for genetic analysis. First single stranded cDNA was synthesized from the produced total nucleic acid, followed by amplification via polymerase chain reaction. For each sample two PCR systems were applied for the viral detection and later its DNA sequence analysis was performed by NGS method. All files were uploaded on Galaxy platform program (usegalaxy.org), quality filtered, and analysis were achieved.

Among the 155 PCR-tested samples using PCR system 1 and system 2; there was consistency in the PCR results of the two systems. Samples that were seen to have faint or weak amplification were different among the two used PCR systems. It was clear more positive results were seen among the tested samples using PCR system 1 compared to PCR systems 2. From the total tested samples using PCR system 1, 86 samples revealed to have moderate strong PCR results reflected by showing strong PCR bands on agarose gel electrophoresis of Tobamovirus compared to about 46 samples of the same criteria were obtained by PCR system 2. On the other hand, the samples that showed a faint or weak amplification were calculated to be 45 samples applying PCR system 1 and 86 samples applying PCR system 2. All other samples were revealed to be negative by the two used PCR systems.

Using NGS analysis, the sequences showed 99% similarity to different isolates of Tomato brown rugose fruit virus including the Jordanian tomato Tobamovirus isolate (TBRFV-Jo), (GenBank accession no. KT383474.1).

Key words: Tomato pathogens, Tobamovirus, Next Generation Sequence (NGS), Polymerase Chain Reaction (PCR).

متابعة الإصابة بفيروس التوبامو في محصول الطماطم المزروعة باستخدام تحليل تسلسل الجيل التالي.

إعداد: مرام حاتم عادل جعافره.

إشراف: د. إبراهيم سليمان العباسي.

ملخص

تعتبر نبتة الطماطم (*Solanum lycopersicum*)، التي تحمل ثمارًا صالحة للأكل، مكونًا أساسيًا في النظام الغذائي، ولها أهمية اقتصادية في جميع أنحاء العالم. تعتبر فيروسات التوبامو من الأمراض الزراعيةالخطيرة/المدمرة التي تصيب النباتات. الهدف الرئيسي لهذه الدراسة هو الكشف عن وجود فيروس التوبامو في سلالات الطماطم المختلفة، وتحديد أكثر أنواع الطماطم التي تتمتع بأفضل مقاومة للعدوى الأفضل الاصابة الفيروسية. يمكن أن تساعد هذه النتيجة المزارعين على تجنب العديد من الخسائرالمادية عن طريق اختيار سلالات أخرى من الطماطم المقاومة للأمراض. اعتمدت هذه الدراسة للكشف عن وجود فيروس توبامو على تقنية التسلسل من الجيل التالي.

أجريت الدراسة بعد جمع أوراق ونباتات الطماطم المثمرة وعينات التربة من 4 دفيئات مختلفة تقع في منطقة أريحا على مدى أربعة أشهر بدأت من شباط وحتى أيار 2023 وحتى نهاية فترة جمع العينات، تم جمع 155 عينة من التربة وعينات نباتية. بالنسبة لكل عينة تم جمعها، تم إجراء استخراج إجمالي للحمض النووي، ثم تم تصنيع cDNA من إجمالي الحمض النووي المنتج باستخدام (RevertAid First Strand cDNA Synthesis Kit)، وتبع ذلك التضخيم عبر تفاعل البلمرة المتسلسل. لكل عينة تم تطبيق نظامين من تفاعل البوليميريز المتسلسل للكشف عن الفيروس وبعد ذلك تحليل تسلسل الحمض النووي بطريقة تسلسل الجيل التالي. وتم تحميل جميع الملفات على برنامج منصة Galaxy (usegalaxy.org) وتمت تصفيتها بالجودة وتحليلها.

وقد تبين أن من بين ال 155 عينة تم اختبارها بواسطة تفاعل البوليميراز المتسلسل باستخدام نظام تفاعل البوليميراز المتسلسل 1 والنظام 2؛ كان هناك اتساق في نتائج PCR للنظامين وخاصة تلك العينات التي أظهرت تضخيم قوي لـ Tobamovirus cDNA. كانت العينات التي شوهدت ذات تضخيم خافت أو ضعيف مختلفة بين نظامي PCR المستخدمين. كان من الواضح أنه تم رؤية نتائج أكثر إيجابية بين العينات التي تم اختبارها باستخدام نظام PCR 1 مقارنة بأنظمة PCR 2. ومن إجمالي العينات التي تم اختبارها باستخدام نظام PCR 1، تم الكشف عن أن 86 عينة لها نتائج PCR قوية معتدلة تنعكس من خلال إظهار

نطاقات PCR قوية على هلام الاغاروز. تم الحصول على الترحيل الكهربائي مقارنة بحوالي 46 عينة لنفس المعايير بنظام PCR 2. من ناحية أخرى، تم حساب العينات التي أظهرت تضخيم خافت أو ضعيف لفيروس التوبامو لتكون 45 عينة تطبق نظام PCR 1 و 86 عينة تطبق نظام PCR 2. وتم الكشف عن عينات أخرى سلبية من خلال نظامي PCR المستخدمين. باستخدام تحليل NGS، أظهرت التسلسلات تشابهاً بنسبة 99% مع عزلات مختلفة من فيروس ثمار نبات الطماطم البنية بما في ذلك عزلة فيروس الطماطم الأردنية (TBRFV-Jo)، (رقم دخول بنك الجينات KT383474.1).

الكلمات المفتاحية: مسببات أمراض الطماطم، فيروس توبامو، تسلسل الجيل التالي (NGS)، تفاعل البلمرة المتسلسل (PCR).

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List of Abbreviations

Abbreviation	Full word
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CP	Coat Protein
cDNA	complementary DNA
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
GH	Green House
ICTV	International Committee on Taxonomy of Viruses
kDa	Kilo-Dalton
MP	Movement protein
mRNA	Messenger Ribonucleic acid
NGS	Next Generation Sequence
ORFs	Open Reding Frames
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real time PCR
SNP	Single nucleotide polymorphism
sgRNA	Sub genomic RNA
TMV	Tobamovirus
ToMV	Tomato Mosaic Virus
ToMMV	Tomato Mottle Mosaic Virus
Torf	Tomato Brown Rugose Fruit Virus
TSWV	Tomato spotted wilt virus
TYLCV	Tomato yellow leaf curl virus
UTR	Untranslated Region

+ssRNA	Positive sense single-stranded RNA
+RNA	Positive-sense RNA

Chapter one: Introduction and Literature Review

1.1. Introduction

The Tomato is a vegetable plant (*Solanum lycopersicum*), which bears edible fruit, is a key component of the diet, and it is economic importance across the world (Chanda et al., 2021; Luria et al., 2017). In addition to being consumed directly, tomatoes are vital components of processed meals, drinks, and sauces, which increases their economic value. Tomato plants are widely grown worldwide (a total of 4,762,457 ha) and their fruits are consumed in large quantities in the traditional and daily diet. In Palestine, tomato plants are cultivated in open fields or greenhouses year-round specially in Jordan valley. In 2018, there were 1,543 hectares of agricultural land, and 220,864 tons were produced annually on average (Food and Agriculture Organization of the United Nations, 2020).(Jamous et al., 2022).

1.2. Tomato Plant Pathogens

One of the primary causes of the yield and quality loss in worldwide agriculture output is plant diseases (J. Zhang et al., 2021). A plant disease is a change of the plant's initial condition that impacts with or modifies its important functions. It primarily results from bacteria, fungus, micro-organisms, or viruses and has a significant effect on crop production and farm budgets (Lee et al., 2020). The most frequent cause of plant illnesses worldwide is virus infection, which also poses a significant threat to many agricultural production systems. These diseases lead to losses due to reduced fruit development, production, and deformity (Luria et al., 2017).

Tomatoes, like other cultivated crops, there are many plant pathogens, that can negatively affect the crop productivity of tomato plants (Sabra et al., 2022; S. Zhang et al., 2022). Trellised tomato plants cultivated in protected structures, greenhouses, or net houses are very susceptible to infections by mechanically transmitted viruses or viroid's, especially by the common genera of the *Tobamoviruses, Potexviruses,* and *Pospiviroids* (Alon & , Hagit Hak , Menachem Bornstein , Gur Pines, 2021).

These are the principal disease pathogens that impact tomato crops in the Mediterranean region:

1.2.1. **Bacterial pathogens:** Worldwide, numerous severe plant diseases are caused by plant pathogenic microorganisms. As plant pathogens, bacteria can cause a wide range of diseases that are extremely harmful to the agro production and consequently then the economy, from smelly tuber rots to pustules, spots, or mosaic patterns on leaves and fruits. Some induce a hormone-based deformity of leaves and shoots known as fasciation, also known as crown gall disease, which is a proliferation of plant cells that results in a swelling on roots and at the junction of the stem and soil.

The most significant bacterial plant pathogens are listed below: *Pseudomonas syringae, Ralstonia solanacearum, Pseudomonas corrugate, Clavibacter ichiganensis, Agrobacterium tumefaciens, Xanthomonas oryzae pv. Oryzae, Xanthomonas campestris pathovars, Xanthomonas axonopodis pathovars, Erwinia amylovora, Xylella fastidiosa; Dickeya (dadantii and solani), Pectobacterium carotovorum (and Pectobacterium atrosepticum,* and others (Krueger, 2004; Mansfield et al., 2012).

1.2.2. **Fungal pathogens:** 70% of all plant illnesses that are known to exist are caused by the diverse species of plant pathogenic fungus. Though not all plant parasitic fungus are pathogens, plant pathogenic fungi are parasites. Certain fungi live concealed inside their plant hosts; these are known as endophytes because they only show symptoms when they are inside of asymptomatic plants. Mycorrhizal fungi are another significant category of fungus connected to plants. Mycorrhiza or "Fungus root" is a term used to describe a symbiotic relationship (a kind of mutual benefit) between plant roots and fungi.

These are the most fungal plant pathogens: *Alternaria alternata, Pleosporaherbarum, Phytophtho racapsici, Stemphylium botryosum, Sclerotinias clerotiorum, Magnaporthe oryzae, Botrytis cinerea, Puccinia spp., Fusarium graminearum, Fusarium oxysporum, Blumeria graminis, Mycosphaerella graminicola, Colletotrichum spp., Ustilago maydis, Melampsora lini,* and others (Dean et al., 2012; Fei & Liu, 2023).

1.2.3. **Nematode pathogens:** Nematodes that parasitize plants come in a variety of forms and sizes. The typical nematode shape is a long and slender worm-like animal, but often the adult animals are swollen and no longer even resemble worms. Plant-parasitic nematodes have a length range of 250 um to 12 mm, with an average length of 1 mm, and a breadth of roughly 15-35 um. Nematodes may differ greatly in appearance, yet they all have certain characteristics in common.

Of the most significant nematode plant pathogens are root-knot nematodes *(Meloidogyne spp.),* cyst nematodes *(Heterodera and Globodera spp.),* root lesion nematodes *(Pratylenchus spp.),* the burrowing nematode *Radopholus similis, Ditylenchus dipsaci,* the pine wilt nematode *Bursaphelenchus xylophilus,* the reniform nematode *Rotylenchulus reniformis, Nacobbus aberrans, Aphelenchoides besseyi, Noctuapronuba, Helicoverpazea, Manducaquin, quemaculatasexta, Pleosporaherbarum, Phytophthoracapsici, Belonolaimus longicaudatu,* and others (Bleve-Zacheo & Melillo, 2007; Fei & Liu, 2023; Jones et al., 2013).

1.2.4. **Viral pathogens:** In many crop production systems, viral infections constitute a significant limiting factor (Hanssen et al., 2010), plant viral infections seem to be spreading at an ever-increasing rate. *Tobamoviruses, begomoviruses, tospoviruses, cucumoviruses, potyviruses,* and other significant viruses are some of the principal infections that hinder the development of tomatoes and peppers. These viruses cause significant economic losses by lowering agricultural output and degrading fruit quality and marketability (Scholthof et al., 2011; S. Zhang et al., 2022).

Since viruses are tiny, their existence is only detected when the creature they are infecting exhibits symptoms that may be identified. These symptoms manifest as mosaic patterns, ringspots, flower-break, stunting and leaf deformation, distorted growth, chlorosis or yellowing, and vein clearing in plants. Regretfully, not every virus manifests with severe symptoms. A diagnosis cannot be determined only on the basis of symptoms because virus-like symptoms can occasionally be brought on by other causes, such as environmental conditions, insect damage, or incorrect nutrition (Gullino et al., 2020).

In this study, we focus light on the causes of viral pathogen, especially on tobamovirus, which infects tomatoes and other crops.

1.3. Tobamovirus

Tobamovirus group (family: Virgaviridae) are destructive agricultural diseases that infect plants (Alon & , Hagit Hak , Menachem Bornstein , Gur Pines, 2021). Worldwide, one of the largest hazards to ornamental and vegetable crops is the tobamovirus (Salem et al., 2016), because of the recent appearance of new species, the significance of this genus's species for agricultural productivity is growing (de Andrés-Torán et al., 2023). The Tobamovirus genus contains the greatest number of species among the seven genera in the family Virgaviridae (Jamous et al., 2022). The most significant tobamoviruses that infect tomatoes are the newly identified tomato mild mottle virus (ToMMV), tobacco mosaic virus (TMV), and tomato mosaic virus (ToMV) (Salem et al., 2016), tomato brown rugose fruit virus (ToBRFV) (Jamous et al., 2022).

1.3.1. Biology of the virus:

Tobamoviruses are among the most researched viruses because they are readily available and simple to use in biotechnological applications. A single TMV virion is 300–310 nm length and 18 nm (Figure 1.1) broad that contains a single copy of a monopartite single-strand genomic RNA of ~6,400 nucleotides and ~2,130 copies of a coat protein (CP) of 17.5 kDa. The genomic RNA, or messenger RNA (mRNA), is 7-methylguanosine 5′ triphosphate [m7G (5′) ppp]-capped at the 5′ terminus and acts as a template for the synthesis of proteins. The 5′ untranslated region (UTR) is a region rich in A and C acids, measuring around 70 nucleotides. Around 200 nucleotides long, the 3′ UTR has three successive upstream pseudoknot structures and a structure resembling transfer RNA (de Andrés-Torán et al., 2023; Ishibashi & Ishikawa, 2016).

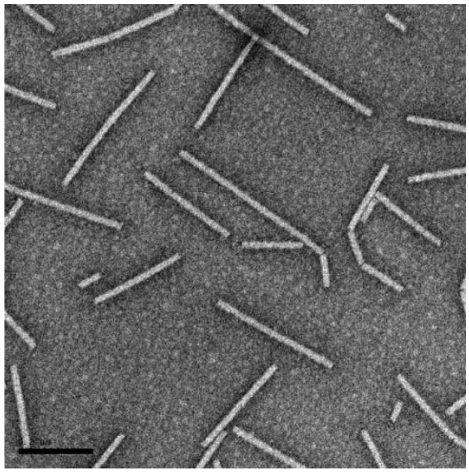


Figure 1.1: Electron micrograph of purified virion of the L strain of tomato mosaic virus. Scale bar 200 nm. Photograph by Yasuhiro Tomitaka (Ishibashi et al., 2023).

Shorter virions measuring 32–34 nm in length is produced by the encapsulation of subgenomic RNA (sgRNA), however they make up a very small portion of the total virion population. The rod-shaped virions have an inner core of 4 nm diameter, which contains a +ssRNA molecule (Figure 2). The external core consists of 2100 coat protein subunits. The subunits are grouped around the RNA molecule in the shape of a right-handed helix. The extraordinary stability of tobamoviruses and their environmental persistence have been attributed to their structural makeup (Ilyas et al., 2022). Tobamoviruses spread horizontally by plant-to-plant contact and by insect pollinators, as well as vertically through seed dispersion (de Andrés-Torán et al., 2023).

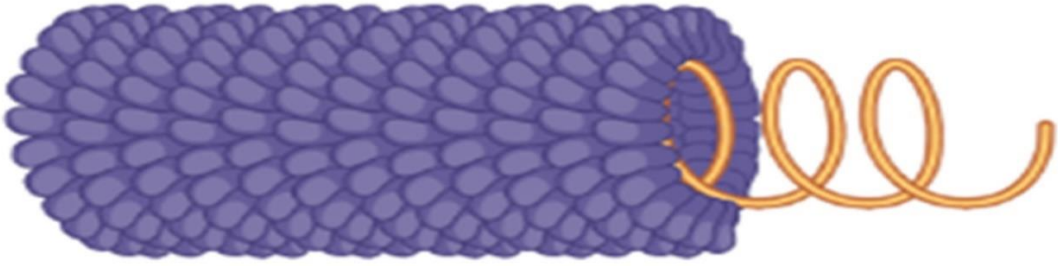


Figure 1.2: Structural diagram of a TMV particle. Protein subunits are shown in purple while the ssRNA is shown in orange. Source: Image created in Biorender.

At least four proteins are encoded by the genome: a 126-kDa protein, its read-through derivative of 183 kDa, a 30-kDa movement protein (MP), and the 17–18 kDa viral coat protein (CP). The open reading frames (ORFs) of CP, 30-kDa, and 183-kDa are arranged in a sequential manner with tiny intergenic noncoding regions or terminal overlaps.

A different ORF, designated ORF6, was discovered in certain Tobamovirus species, but not all. It overlaps the 30-kDa protein and CP ORFs and encodes a tiny protein (4.8 kDa) (Figure 3)(Ishibashi & Ishikawa, 2016).

Figure 1.3: Schematic diagrams of tobamovirus genomic RNA. The thick line and circle denote the TMV genomic RNA and m7G (5′) ppp cap, respectively. Boxes denote open reading frames (ORFs) or proteins.

The 186-kDa and 126-kDa proteins, which are produced by translating the genomic RNA, are involved in the replication of viral RNA. Together, these two proteins are referred to as replication proteins. In the tissues of host plants, the 30-kDa protein takes role in the viral dissemination from infected cells to nearby uninfected cells.

Both the CP and the 30-kDa protein are synthesized from their respective subgenomic mRNAs and are not necessary for the replication of viral RNA. In Nicotiana Benthamian, disruption of ORF6 reduces host responses to TMV, indicating a function for ORF6 in viral pathogenicity. The 30-kDa protein subgenomic RNA is translated in vitro to yield the ORF6 protein. It is well known that the MP is needed for the cell-to-cell movement of Tobamovirus and the CP is critical for symptom development, virion formation, viral replication and the long-distance movement of Tobamovirus (Alon & , Hagit Hak , Menachem Bornstein , Gur Pines, 2021; Ishibashi & Ishikawa, 2016; Luria et al., 2017).

1.3.2. Symptoms of crop infection:

Symptoms of Tobamovirus disease may vary depending on the host plant, viral species and environmental conditions. A mosaic pattern, mottling, or deformity can be seen on the leaves of affected plants. Systemic necrosis and defoliation may occasionally occur in cases of severe infection depending on biological parameters including (plant age, cultivar, virus strain, etc.) and environmental variables (temperature, light intensity, etc.).

The most well researched species are tomato mosaic virus (TMV) and ToMV, which frequently produce chlorosis, mosaic patterns, and leaf deformation on susceptible hosts (Figure.4). Systemic necrosis and defoliation are additional side effects of severe infections (Luria et al., 2017).

Figure 1.4 (A) TMV symptoms on Nicotiana tabacum cv. 'Samsum nn', showing leaf distortion, mosaic pattern and chlorosis; (B–D) ToBRFV symptoms on tomatoes showing mosaic pattern, leaf narrowing and rugose spots on fruits (Luria et al., 2017).

1.3.3. Tobamovirus Classification:

The Tobamovirus genus contains the greatest number of species (37 species) among the seven genera in the family Virgaviridae, according to the 2020 publication of the International Committee on Taxonomy of Viruses (ICTV) (Jamous et al., 2022). The Tobamovirus genus comprises the well-known species the type member Tobacco mosaic virus (TMV) and the Tomato mosaic virus (ToMV), as well as Tobacco mild green mosaic virus (TMGMV) and Pepper mild mottle virus (PMMoV) among the viruses capable of infecting Solanaceae crops (Luria et al., 2017), and other species that infect various agricultural crops.

There are three types of tobamoviruses that infect tomato crops:

1. **Tomato mosaic virus (ToMV):**

   Tomato mosaic virus ToMV is the most prevalent and significant virus often linked to tomato crops. ToMV is a recognized member of the genus Tobamovirus and a member of the Virgaviridae family. It is a widely distributed stable RNA virus that infects many types of plant plants (Ullah et al., 2017).

   ToMV's virion is a rod-shaped particle with a diameter of 18 nm and a length of around 300 nm, similar to other tobamoviruses. ToMV's genome is made up of a single-stranded positive sense RNA (ssRNA), which is approximately 6.4 kb long and encodes at least four proteins: a 126-kDa protein, a 183-kDa protein, a 30-kDa protein, and the coat protein (CP), respectively. The 126- and 183-kDa proteins are expressed from the genomic RNA, with the latter being translated as a read through product of the former. Together, the 186- and 126-kDa proteins are in charge of the virus's reproduction. The 30-kDa protein and the CP are each expressed from a distinct subgenomic RNA. While the CP is the only structural protein of the virus, the 30-kDa protein facilitates cell-to-cell movement. For decades, ToMV has served as a model to comprehend the mechanics of plant-virus interactions due to its

economic significance and suitability for genetic alterations; in certain cases, it has even replaced tobacco mosaic virus (Xu et al., 2021).

Typically, infected tomato plants exhibit mosaic, curling, and distorted leaves, as well as internal browning and uneven fruit ripening. ToMV is a major disease in tomato plants that severely reduces production in cultivars that are susceptible. Significant tomato crop losses are caused by two closely related tobamoviruses called ToMV and TMV. Although tomato is frequently infected with both ToMV and TMV, ToMV predominates in tomato and is thought to be its preferred host (Ullah et al., 2017).

2. **Tomato brown rugose fruit virus (ToBRFV):**

One of the most destructive plant viruses that seriously damages crops and jeopardizes tomato output globally is the tomato brown rugose fruit virus (ToBRFV), which is a member of the genus Tobamovirus and family Virgaviridae. Since 2016, this virus has produced significant issues with tomato and pepper crop production all around the world (Salem et al., 2022).

As of right now, various countries have reported on the prevalence and current distribution of ToBRFV infection: Israel (Luria et al., 2017), Mexico (Cambrón-Crisantos et al., 2018), the USA (Ling et al., 2019), Germany (Menzel et al., 2019), Italy (Panno et al., 2019), Palestine (Alkowni et al., 2019), Turkey (Fidan et al., 2019), and China (Yan et al., 2019).(Oladokun et al., 2019).

Infection with ToBRFV lowers plant vigor, productivity, and fruit quality. It spreads through tomato seeds and eventually has the potential to impact the tomato seed trade by blocking the export of tomato seeds grown in ToBRFV-affected areas to ToBRFV-free areas. The disease's common symptoms, which might change according on the cultivar, infection stage, and meteorological factors, are mostly leaf mosaic, leaf narrowing, and fruit discolouration (N. M. Salem et al., 2022).

ToBRFV mostly spreads mechanically through plant-to-plant contact, long-distance transmission of externally infected seed, and shared cultural behaviors involving workers' hands, clothing, tools, equipment, and circulating water. Regional variations may also exist in the transmission mechanism. For instance, bumblebees (Bombus spp.) are widely employed as pollinators in the UK tomato industry, and it has been established that these insects may spread plant viruses, such as ToBRFV, and viroids, such as tomato apical stunt viroid and tomato chlorotic dwarf viroid, in glasshouses. Furthermore, a large number of weed hosts can act as reservoirs of inoculum for crop infection due to Tobamoviruses' characteristic ability to maintain infectivity in seeds and contaminated soil (Oladokun et al., 2019).

3. **Tomato mottle mosaic virus (ToMMV):**

Tomato mottle mosaic virus (ToMMV) was identified for the first time as a third Tobamovirus infecting tomato crops in Mexico in 2013 utilizing deep sequencing and small RNA assembly (Sui et al., 2017). Since then, reports of ToMMV have come from Australia, China, Brazil, Spain, Israel, and the USA. It is comparable to other Tobamovirus species such as tomato mosaic virus (ToMV), tobacco mosaic virus (TMV), and tomato brown rugose fruit virus (ToBRFV).

ToMMV is distinguished by the presence of a monopartite, positive-sense RNA (+RNA) genome of approximately 6,400-nt, encapsulated in stiff, rod-shaped particles that measure 310 nm in length and 18 nm in width. Currently, ToMMV has become one of the most economically important viruses affecting tomato plants (TETTEY et al., 2022). Tomato plants infected with ToMMV exhibit severe mosaic symptoms, leaf deformation, and stunting (Sui et al., 2017).

ToMMV's genome is made up of four open reading frames (ORFs). The ORF1 and ORF2 encode two replicases with molecular weights of approximately 126 and 183 kDa, respectively. Through a readthrough approach, the larger one is generated. 30 kDa movement proteins (MP) are encoded by ORF3, while 17.5 kDa coat proteins

(CP) are encoded by ORF4 (TETTEY et al., 2022). Interestingly, it has been revealed that the 126 K protein and the 183 K protein are parts of the RNA replication protein complex that play a major role in ToMMV viral RNA replication (Tu et al., 2021).

1.3.4. Transmission:

Tobamoviruses are extremely contagious and spread by mechanical contact with hands, tools, soil, and plant components that have been infected. More important, tobamoviruses are also carried through seed propagation (Luria et al., 2017). While not infecting the embryo, tobamoviruses can transfer from surrounding tissue to the seedling during germination. Tobamoviruses can be discovered in the endosperm and in the seed coat. The process of seed transmission from outside the embryo is slow and dependent on the virus particles' high stability. On the other hand, ToBRFV and other tobamoviruses spread mostly by seed transmission.

In addition to plants cultivated in contaminated soil or grafted with contaminated material, other sources of main inoculum are the juice of infected fruits. It has also been demonstrated that tomato crop pollination by bumblebees, which are known to carry ToBRFV particles, can spread the virus to new facilities through hives from greenhouses (Ilyas et al., 2022).

1.3.5. Economic losses:

Economically, tomato cultivation serves as a vital source of income for farmers worldwide. From small-scale family farms to vast commercial enterprises, the cultivation of tomatoes generates substantial revenue streams, contributing significantly to local and global economies. Whether destined for fresh consumption, processing into sauces and pastes, or as ingredients in countless food products, the demand for tomatoes remains consistently high.

The tomato brown rugose fruit virus (ToBRFV), tomato spotted wilt virus (TSWV), tomato yellow leaf curl virus (TYLCV), and others are among the viruses that can have an impact on tomato production from year to year. (Ilyas et al., 2022).

In agricultural settings, these viruses result in decreased crop yields and quality, which leads to severe economic losses. For multiple reasons, the economic consequences of Tobamovirus infections can be significant.

- Reduced Yield: Infected plants typically exhibit stunted growth, reduced fruit size, and lower yields compared to healthy plants. Lower profitability for farmers who rely on these crops for a living will result from this.
- Quality Issues: Infected fruits may show symptoms such as mottling, yellowing, or distortion, rendering them unmarketable or less desirable to consumers. This may result in decreased prices or buyer rejection, which would further impair the crop's capacity to make money.
- Long-term impact: Because of their extended persistence in soil and plant debris, tobamoviruses continually threaten crops in the future. Consequently, until efficient control measures are put in place, impacted farmers might continue to suffer financial losses across several growing seasons (Ilyas et al., 2022; Rao & Reddy, 2020).

1.4. Detection of Tobamovirus

The recent decades have seen the development of several techniques for nucleic acid hybridization (including microarray), polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA), which together offer quick and affordable diagnostics for known viruses and viroids. These techniques are extensively employed in both agriculture and medicine. When diagnosing and identifying viruses and viroids, plant pathologists can utilize the total nucleic acid content of a biological sample obtained by NGS technology as a valuable tool (Wu et al., 2015).

- **Polymerase chain reaction (PCR):** Plant virus identification can be achieved with sensitive and specific nucleic acid-based techniques like PCR and RT-PCR (Sabra et al.,

2022). The virus can be detected more effectively and economically by applying a generic PCR with universal primers (Li et al., 2018).

There are many previous studies that examined the development of these other DNA-based methods (Li et al., 2018; Tiberini et al., 2022), as well as those that used these methods to detect tobamovirus in tomato crops (Jamous et al., 2022; Rizzo et al., 2021; Sui et al., 2017; Yoon et al., 2008).

In a previous study in 2022, Jamous and his colleagues studied the biological and molecular characterization of tomato brown fruit virus (ToBRFV) on tomato plants in the State of Palestine. They collected samples from *Solanum lycopersicum* L. (tomato) and *Nicotiana tabacum* L. (cultivated tobacco) plants, tested them for tobavirus infection by reverse transcription polymerase chain reaction, and performed a sequence analysis of the ToBRFV-PAL genome. As a result: Tomato leaf samples collected from Tulkarm and Qalqilya are infected with ToBRFV-PAL with an infection rate of 76% and 72.5%, respectively. Leaf samples collected from Jenin and Nablus were found to be mixed infected with ToBRFV-PAL and Tobacco mosaic virus (TMV) (100%) (Jamous et al., 2022).

- **Enzyme-linked immunosorbent assay (ELISA):** ELISA is regarded as a valuable instrument for detecting viruses and is a simple test to handle a large number of samples. The powerful method known as ELISA makes it possible to identify the viral capsid protein subunits of tobamoviruses.

  In a previous study, Ahmed Sabra and colleagues used this technique to screen the most common tomato viruses using kits that were available in their laboratory, and the ELISA results clearly showed that ToBRFV was present in commercial tomato crops collected from the Riyadh region, Saudi Arabia (Sabra et al., 2022).

- **Next Generation Sequencing (NGS):** NGS, deep sequencing or massively parallel and short read sequencing are related terms which can facilitate sequencing of a large amount of genomic data in a single reaction by different methodology (Chelsie, 2016).

Next generation DNA sequence analysis (NGS) also called high throughput sequencing method is one of the best used methods for the discovery of genetic differences, wide genome sequencing, and for multi-sequence analysis of different amplified DNA segments. This method can sequence several DNA segments at once in high quality, sensitivity and accuracy and it can effectively provide a huge information on short sequence repeats or single nucleotide polymorphism (SNP) (Kesanakurti et al., 2016).

NGS analysis is superior over other DNA based methodologies in that it a sensitive and specific method for pathogen detection without a previous knowledge of the pathogen. RNA genomes are present in the majority of plant viruses. Next-generation sequencing (NGS) may identify viral RNA genomes in infected plant material by extracting and sequencing total RNA, total RNA free of ribosomal RNA, tiny RNA interference (RNAi) molecules, or double-stranded RNA (dsRNA) (Kesanakurti et al., 2016).

Sequencing by NGS based on either sequencing by ligation (SBL) or sequencing by synthesis (SBS). The first method sequencing used in NGS was Roche/454 launched in 2005 which is an SBS method, then another method was developed including Illumina/Solexa in 2006, Ion torrent sequencing and in 2007 the ABI/SOLiD. The principle of Roche/454 sequencing based on detection of pyrophosphate which are released after incorporation of each nucleotide in new synthetic DNA (Kchouk et al., 2017).

During preparation, the DNA sample must be fragmented randomly and each fragment attached to the bead through modified primer on its surface that has complementary oligonucleotides to DNA fragment, after binding, amplification of each bead using PCR emulsion leads to the production of ~ one-million copy of each DNA fragment on the surface of bead, then pyrosequencing technique using picotiter wells is utilized which permit hundreds of thousands of reactions to be done in parallel, after activation of series of downstream reaction and producing of light at each nucleotide incorporation, the light emission is detected after each incorporation and the DNA sequence will be derive. Although, the identification of DNA size based on the detection of intensity of light, the

low intensity of light may lead to under-or over-estimation of number of nucleotides and cause errors (Kchouk et al., 2017; Slatko et al., 2018).

Ion torrent sequencing is similar to Roche/454 sequencing but it is not based in using light intensity for identification of DNA size, it is based on detection of hydrogen ions that are released during sequencing after each incorporation of each nucleotide, leading to change in pH. This change is converted to voltage which is proportional to number of nucleotides incorporated (Kchouk et al., 2017).

Illumina/Solexa sequencing is the most commonly used method in next generation DNA sequencing, it is based on PCR amplification bridge on the solid plate. During the preparation, the DNA sample must be fragmented, each fragment must be ligated with unique adaptors that are complementary to adaptors on surface of the solid plate, each attached sequence will amplify using PCR bridge amplification which leads to create many identical copies of the same sequence, a collection of the same sequence from the same origin is called cluster. Each type of nucleotide is labelled with a fluorescent, the nucleotide that has inactive 3'-hydroxyl group permit for only one nucleotide to be incorporated. Using of laser for detection the light signal from exiting clusters which is specific for each nucleotide then a program by couple- charged device is used for transforming these light signals into nucleotide sequence (Kchouk et al., 2017).

The Illumina/Solexa technique improves sequencing through its ability to produce paired-end sequence reads in which the sequence at both ends of clusters is listed. The total error of this method is about 1% (Kchouk et al., 2017). Illumine offers a series of many platforms which include (iSeq, MiSeq, MiniSeq, NextSeq, HiSeq, and NovaSeq) (Gu et al., 2019).

Metagenomics NGS (mNGS) which is refer to application of NGS for detection of pathogen or microbe in clinical sample. mNGS is unbiased sampling, its capable of identification of unknown, unexpected pathogen even new organism, mNGS can help to provide genetic information of microorganisms including strain identification and prediction of drug resistance, and can provide quantitative and semi quantitative

information according to concentration of microorganism in samples. Also, mNGS can identify the species-level, it can use primers from conserved 16S rDNA for bacterial detection and primers from internal transcribed spacer sequences for fungal detection (Bragg & Tyson, 2014; Gu et al., 2019).

Nowadays, Life Technologies Ion Torrent Personal Genome Machine (PGM) and Illumina MiSeq are the two most widely utilized systems in research and clinical labs. Because to the development of these and other NGS platforms, more labs can now do sequencing, which has led to a sharp rise in the use of nucleic acid sequencing in clinical diagnoses and research.

The Ion Torrent PGM and the Illumina MiSeq have a same underlying approach that consists of template preparation, sequencing and imaging, and data processing, even if each NGS platform has its own unique way for accomplishing sequencing. Every generic phase has certain characteristics specific to each platform. Figure 1.5 provides an overview of the sequencing approaches (Grada & Weinbrecht, 2013).

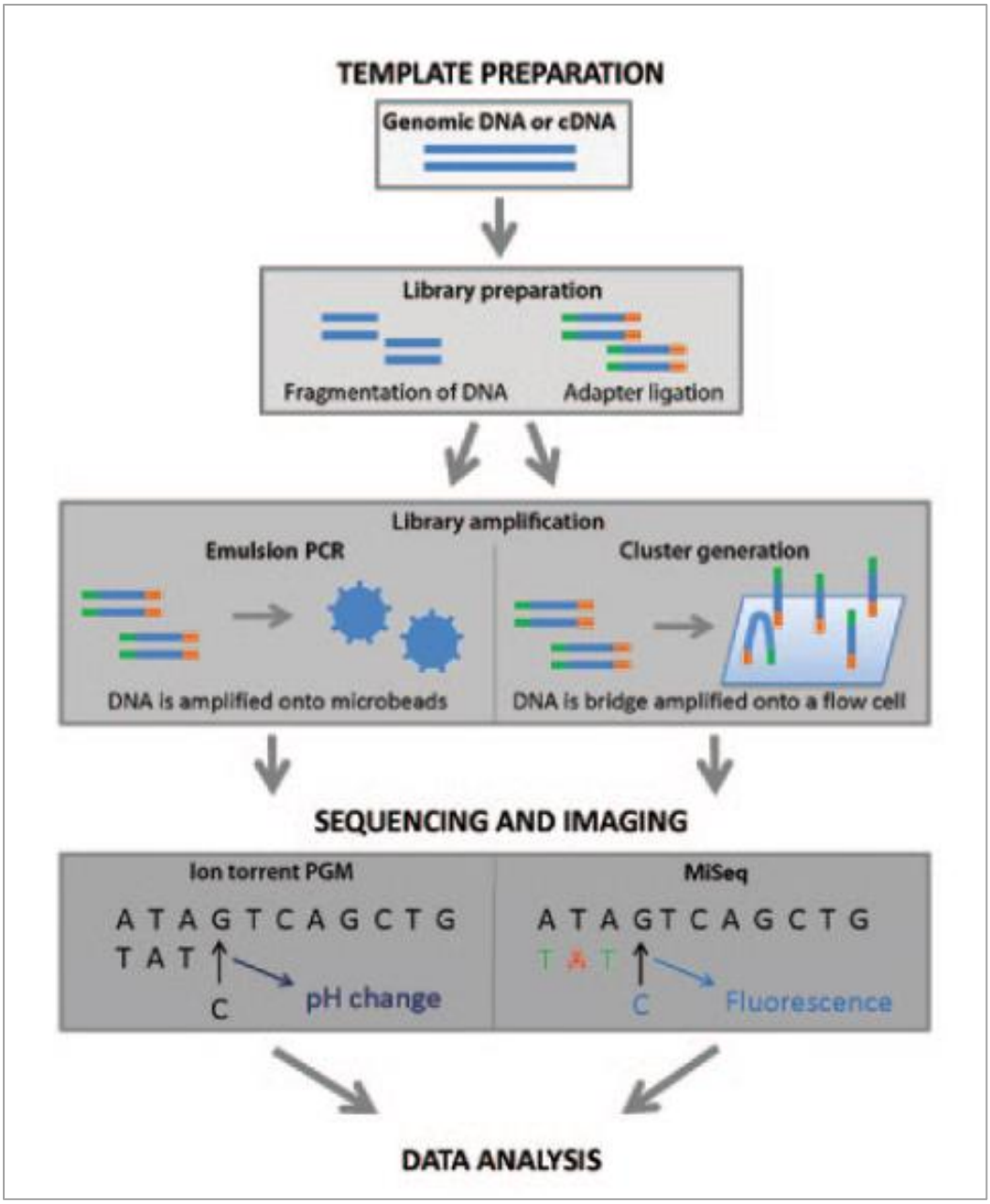


Figure 1.5: Next-generation sequencing methodology (Grada & Weinbrecht, 2013).

Next-generation sequencing (NGS) is a massively parallel sequencing technology that offers ultra-high throughput, scalability, and speed. The technology is used to determine the order of nucleotides in entire genomes or targeted regions of DNA or RNA. By The combination of NGS technology with advanced bioinformatics have been made possible, recent advancements in the study of plant virology, notably in the areas of genome

sequencing, ecology, discovery, epidemiology, transcriptomics, replication, detection, and identification (Barba et al., 2013).

With the advancement of NGS technology and its comparatively low cost, NGS has expanded in knowledge and potential to diagnose viral pathogens without prior knowledge of the invasive pathogen. This allows for the timely and accurate detection of these viral pathogens in plants for efficient disease management and control (Akinyemi et al., 2016).

In this study, we used next-generation sequencing technology to detect tobamovirus in tomatoes.

Research objectives:

The main goal of the current research is to identify the most tomato cultivars that have best resistance to viral infections. This outcome could help farmers to avoid many financial losses by choosing other disease tomato susceptible varieties.

The specific objectives are to:

1. Follow up of Tobamovirus infection in different cultivated tomatoes green houses.
2. Comparison of tomato cultivars according to their resistance or infection with this virus.
3. To quantify the viral infection and confirm its sequence by NGS DNA sequence analysis.

Study significance:

Tomatoes are one of the most economically important vegetable crops around the world. However, viral diseases are considered one of the most dangerous factors that affect the productivity of this crop, and cause total losses of production in some cases. Among these viral diseases that affect tomatoes, which has recently begun to spread both in Palestine and other countries, is Tobamovirus.

Next generation sequencing (NGS) is a technology that allows comprehensive sequencing of genetic material, and enables the production of a wide range of genomic information from many organisms. In NGS sequencing, it is possible to perform millions of sequencing reactions of a portion of whole genes or reactions involving different amplified PCR segments.

In the current study, we use next-generation sequencing technology to detect tobamovirus affecting tomato crop from different greenhouses and during their growth period.

Chapter two: Materials and Methods

2.1 Sample collection:

In this study samples of tomato plants and soil were collected from 4 different green-houses located in Jericho city. Sample collection was started in the middle of the cultivation period of tomato plant culturing; starting from February to May 2023. Over the collection period a total of 5 visits were performed from the 4 green-houses (Table 2.1).

In each collection visit; three plant samples and three soil samples were collected from each growing green-house. Samples were collected using sterile collection equipment (scissors, forceps, 100 ml plastic collection cup, gloves). Caution was highly given not to introduce any contamination to the green-house and not to do cross contamination between the collected samples. Regarding soil samples, the two samples were collected randomly from different locations of the green-house.

Plant samples were collected randomly from different corners of the green-house and each time few leaves were collected from different tomato plantlets. At the end of the samples collection period a total of 155 soil and plant samples were collected and stored in a refrigerator at 4^{o}C.

Table 2.1: Sampling, visiting date, and type of crop.

Visit	Date	Number of collected samples	Tomato cultivar
Visit 1	23.2.2023	25	GH1: Senator F1, Master GH2: Senator F1, Master GH3: Nikran, GH4: Senator F1
Visit 2	7.3.2023	40	
Visit 3	27.3.2023	40	
Visit 4	10.4.2023	40	
Visit 5	16.5.2023	10	

2.2 Total nucleic acid extraction:

Total RNA and DNA that were present in the leaves tomato plants, soil, and fruits samples were precipitated by simple isopropanol precipitation and ethanol washing method, as follows: (Gambino et al., 2008)

1. 0.5 ml (about 0.2 grams) of each sample was placed in a tube containing 500 µl of lysis Buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.1% triton X-100), the tubes then were incubated at 60°C for one hour.
2. After this incubation, samples were centrifuged at maximum speed (14,000 rpm) for 5 minutes, followed by the addition of 15µl of 5M NaCl, and then 700µl of cold isopropanol.
3. The tubes were left at -20°C for overnight and in the second day they were centrifuged at maximum speed (14,000 rpm) for 10 minutes.
4. The supernatant was decanted and to the precipitate 500µl of 70% alcohol was added and centrifuged another time for 5 minutes to wash the excess remained salts by centrifugation at 14,000 rpm for 5 minutes followed by alcohol removal.
5. The precipitate was air dried for about 10 minutes and then suspended in 50µl double distilled.
6. Samples were stored at -20°C until further analysis.

2.3 cDNA synthesis:

First strand cDNA was synthesized from the produced total nucleic acid using (RevertAid First Strand cDNA Synthesis Kit) from (Fermentas, USA) using oligo dT primers and according to supplier's protocol. In brief the following protocol that was used to synthesis the first-strand cDNA: (Periasamy et al., 2006)

1. To 10µl of the total nuclic acids 2µl of 100 pmoles of Oligo$(dt)_{18}$ primer were added.
2. The tube was incubated at 65 °C for 5 minutes then chilled at 4°C for 5 minutes.
3. To this mixture the following reagents were added (4µl 5X RT buffer, 2µl RiboLock RNAase inhibitors, 1µl RevertAid RT, 1µl dNTPs), then incubated for 5 min at 25°C followed by 60 min at 42°C and 5 minutes at 70°C.

4. The produced product at this stage conatin first starnd cDNA from both plants and viral sources and they are ready for further downstream applications.
5. The produced cDNA was kept at -20°C for later uses.

2.4 Viral DNA amplification by polymerase chain reaction (PCR):

PCR amplification was done using ready mix tubes of Taq DNA polymerase obtained from (Synthezza company, Jerusalem). Each PCR reaction has a total volume of 25µl. In each PCR reaction 20 pmoles of each direct and reverse primers were added, and 5µl of the produced cDNA material was added. Double-distilled water was used to increase the reaction volume to a maximum of 25µl before starting the PCR process.

PCR amplification was performed in MJ Mini™ personal thermal cycler (Bio-Rad, USA), the thermo-cycler uses a thermal profile (the PCR conditions) that starts with initial denaturation at 95°C for 5 min, then 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C Tm, extension for 1 min at 72°C, and a final elongation step for 10 min at 72°C.

For each sample two PCR systems were applied for the viral detection and later its DNA sequence analysis by NGS method. The used primers are newly designed by us and they enable the detection of 1100 bp from the viral ORF3 gene. The specific tomato virus primers are indicated in the below table, and they are adapted to be used with the new sequencing technology of NGS.

Table 2.2: Newly designed specific tomato virus primers suitable with the new sequencing technology of NGS.

PCR systme	Primer name	Sequence	Expected Band size
System 1	D239	GAGGAACAGACGCTTATTGC	341 bp
	R580	GTAYTGTGACAGACAACTTC	
System 2	D3666	ATGGTACGAACGGCGGCAG	315 bp
	R3981	CATATGCCTGTACTGATCAAC	

*All used primers synthesized using the below forward and reverse overhang adaptor.

- **Forward adaptor :** TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
- **Reverse adaptor:** GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC

2.5 Agarose Gel Electrophoreses:

The PCR products were run on a 1.5% agarose gel (1.5g agarose, 100ml 1X TAE and 10µl Ethidium bromide). The 50X TAE electrophoresis running buffer (242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0)). The Gene Ruler 100bp DNA ladder (Thermo Scientific™, #SM0371) was used as size marker

2.6 Next generation sequencing using Illumina MiSeq platform

This approach was adapted from the Nextera microbiome MiSeq DNA sequence to be used for detecting the presence of tobamovirus cDNA in the tested samples. For this purpose, two different PCR systems were applied on each sample as indicated above (Table 2.2) by using distinctive primers which were adapted to be used with Illumina MiSeq next generation sequencing system. Each primer consisted from two parts:

First 5'-part: the direct or reverse primers that were specifically designed to target selected DNA sequences.

Second part: the universal 5' tailed oligonucleotides (complementary region for R1 connected to the direct primer or R2 connected to reverse primer) known as Read 1 and Read 2, later these sites will be used for DNA sequencing from both sides.

To be able to identify each sample sequence of different DNA samples (in this case different pooled samples), dual barcode sequences were added at each end of all amplicons which were related to the specific plant sample, these specific barcode sequences called indices (N7XX, S5XX), so each sample was labeled with unique dual indices which were bound to illumine sequencing adapter by second PCR (see below: Index addition by PCR). As explained in (Figure 2.1) which is adapted from (Microsynth: The Swiss DNA sequencing company); MiSeq analysis requires two PCR systems; the first PCR required for specifically amplifying the target gene and the second PCR for addition of unique dual indices to each amplicon to be labeled.

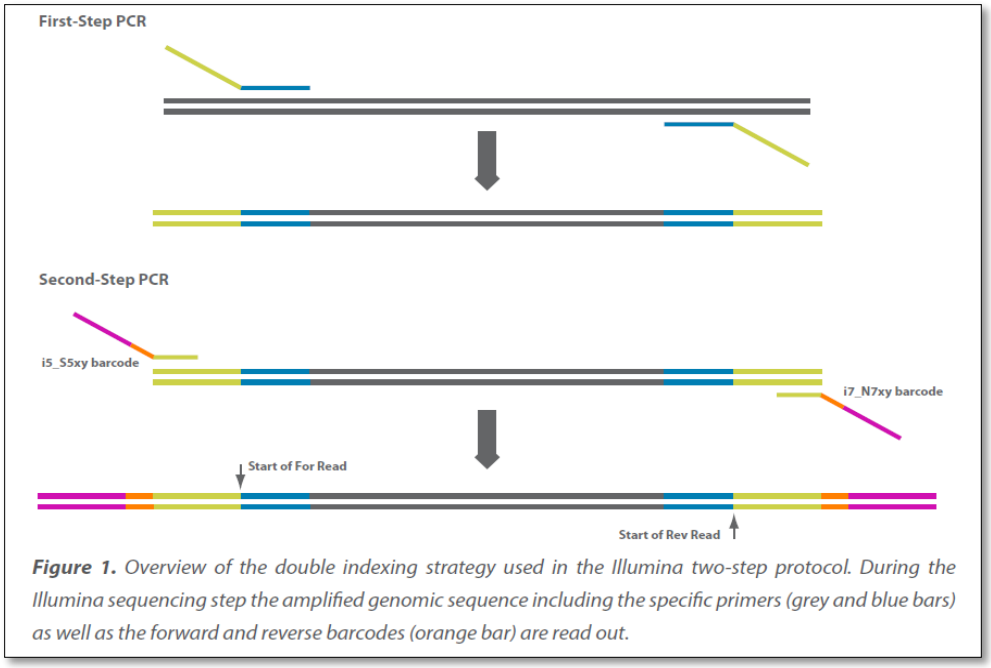


Figure 1. *Overview of the double indexing strategy used in the Illumina two-step protocol. During the Illumina sequencing step the amplified genomic sequence including the specific primers (grey and blue bars) as well as the forward and reverse barcodes (orange bar) are read out.*

Figure 2.1: Strategy of first and second NGS PCR reactions adapted from (Microsynth: The Swiss DNA sequencing company). (Illumina, 2013)

2.7 PCR products purification using AMPure XP beads (magnetic beads):

For achieving pure amplicons without any impurities that can result from the conventional PCR such as un-incorporated nucleotides, primers and salts; highly efficient purification protocol with magnetic beads (AMPure XP beads kit / Beckman coulter, USA) were used according to following purification protocol:

1. A total of 15µl of each PCR products were transferred into fresh 200µl tube, each tube was containing 20 µl of magnetic beads, tubes were kept at room temperature for 5 min, then they were transferred into 96 well magnetic plate stand and kept for 5 min until the beads attached to the side, after that the solution which containing PCR buffer and salt were removed without disturbing the attached beads, washing with 200 µl of 80% ethanol for 1 min.
2. Then tubes were returned to the magnetic plate for 1 min allowing magnetic beads to attach to the sides before removing ethanol, washing with ethanol was repeated another time and at the end of second washing step; tubes containing beads without any ethanol were left for 5 min on the magnetic plate to dry. Then tubes were transferred into PCR tube rack and 30 µl of DDW was added to elute bound DNA from magnetic beads, finally 20 µl of eluted DNA was transferred into a fresh tube.

2.8 Index addition by PCR

This is the second stage PCR that was used to bind the dual barcodes indices (i5 and i7) attached to illumine sequencing adapters. For this PCR system, the below indicated N7XX and S5XX indices. The index addition was performed using ready PCR tubes contained ready dehydrated mix with total volume of each reaction 25µl.

The second PCR was performed using the following program: (Illumina, 2013)

1. 5 min at 95^{o}C.
2. 12 cycles: each composed of
    - 30 seconds at 95^{o}C.
    - 30 seconds at 55^{o}C.
    - 30 seconds at 72^{o}C.
3. A final elongation step at 72^{o}C for 5 min.

Representative samples were analysed by agarose gel electrophoresis in order to verify the addition of the dual index.

2.9 Final preparation of MiSeq pooled and barcodes sequencing library:

After addition of dual index by a second PCR, each five tubes were pooled into a fresh new tube (about 125 in each tube). All the PCR products then were purified using magnetic beads. After this final purification all the PCRs were pooled in one tube that was identified as the DNA library. At this stage the library is prepared to be sent for next generation DNA sequencing (NGS) service company, (sequencing was done on MiSeq machine using 500 cycle kit from Illumina Co.).

2.10 NGS sequence analysis:

The produced sequences were analyzed using specialized software that enables the handle of NGS obtained data files of type (FASTq). Professional free software is available from Galaxy bioinformatics that enables sequence classification and arrangements according to length and sequence (*Galaxy*, n.d.).

2.11 Bioinformatics and data analysis:

Normally the recovered NGS data using the MiSeq machine, give the FASTq R1 and R2 data of all NGS samples.

In this study we used a summary Excel classification file that was provided for each sample and showed all samples potentially infected with the virus.

For virus identification the Illumina machine is using BLAST bioinformatics analysis tools, which is identifies homologous sequences by locating short matches between the two sequences 2being compared. (Donkor, 2014).

Chapter three: Results

3.1 Testing the new designed tobamovirus primers.

At the beginning the primers were tested for their utility to amplify tobamovirus genetic material and their sensitivity of PCR amplification. For this purpose, we used already isolated viral DNA (obtained from the National Agricultural Research Center/ Palestine). Both designed PCR primers were used to amplify different concentration of viral cDNA (1ng, 0.1ng, 0.01ng, 1pg, and 0.1 pg). Both PCR systems were succeeded to clearly amplify cDNA down to 1pg, amplification of 0.1pg was more obvious in PCR systems 1 compared to PCR system 2 (Figure 3.1). Both PCR systems amplified DNA fragment of the expected size (PCR 1=341 bp and PCR 2= 315 bp) as shown in the (Figure 3.1).

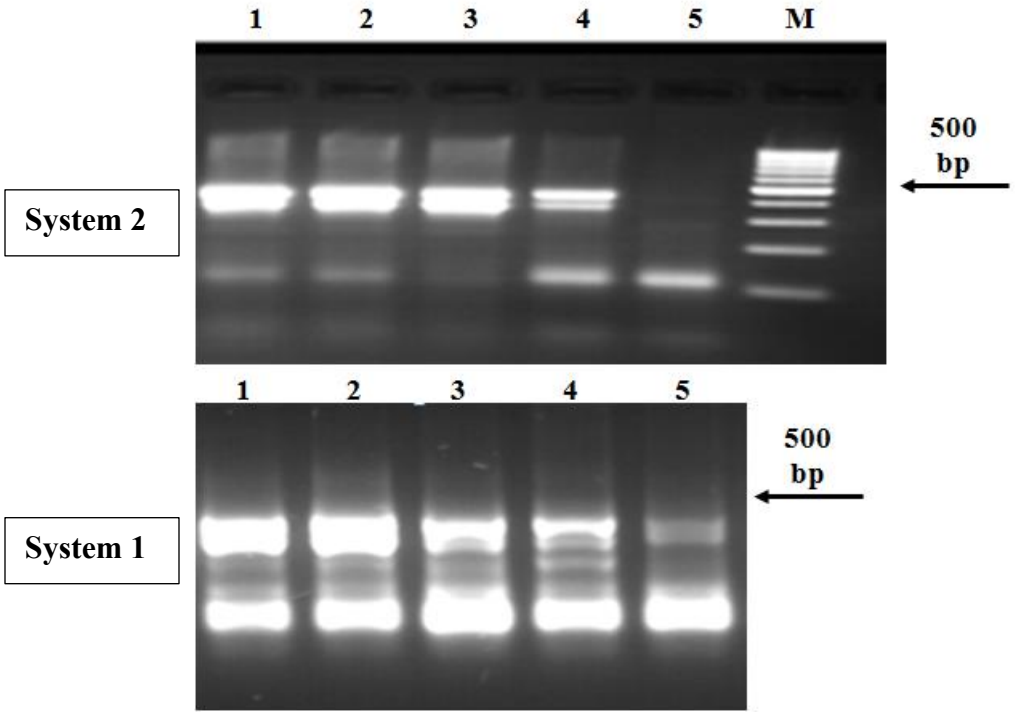


Figure 3.1: Agarose gel electrophoresis depicting sensitivity examination of the two used PCR systems. Different concentrations of viral DNA were used (1=1ng, 2=0.1ng, 3=0.01ng, 4=1pg, 5= 0.1pg).

3.2 PCR amplifications of viral cDNA from different analyzed samples:

Successful PCR amplification was obtained using the two PCR systems targeting the prepared cDNA material from different plant, whether this was plants' leaves or plants' fruits. The PCR reactions of each used PCR system was performed separately although it was possible to do multiplex reactions, this was done to show the efficacy of each PCR reaction in detecting the viral cDNA and to ensure the consistency of the obtained results by the two different PCR systems.

Figure 3.2 to Figure 3.7 depicting agarose gel electrophoresis of the obtained PCR results for all the tested samples (total 155 samples including negative controls). In general, there was consistency in the PCR results of the two systems especially those samples that showed strong amplification of Tobamovirus cDNA. Samples that were seen to have faint or weak amplification were different among the two used PCR systems. It was clear more positive results were seen among the tested samples using PCR system 1 compared to PCR systems 2.

From the total tested samples using PCR system 1, 86 samples revealed to have moderate strong PCR results reflected by showing strong PCR bands on agarose gel electrophoresis compared to about 46 samples of the same criteria were obtained by PCR system 2. On the other hand, the samples that showed a faint or weak amplification were calculated to be 45 samples applying PCR system 1 and 86 samples applying PCR system 2. All other samples were revealed to be negative by the two used PCR systems (Table 3.1).

Table 3.1: PCR system 1 compared to PCR systems 2.

	PCR system 1	**PCR system 2**
Strong to medium positive band	86/155	46/155
Weak band	45/155	86/155
Only by one PCR system +ve	8/155	9/155
All negative	24/155	23/155

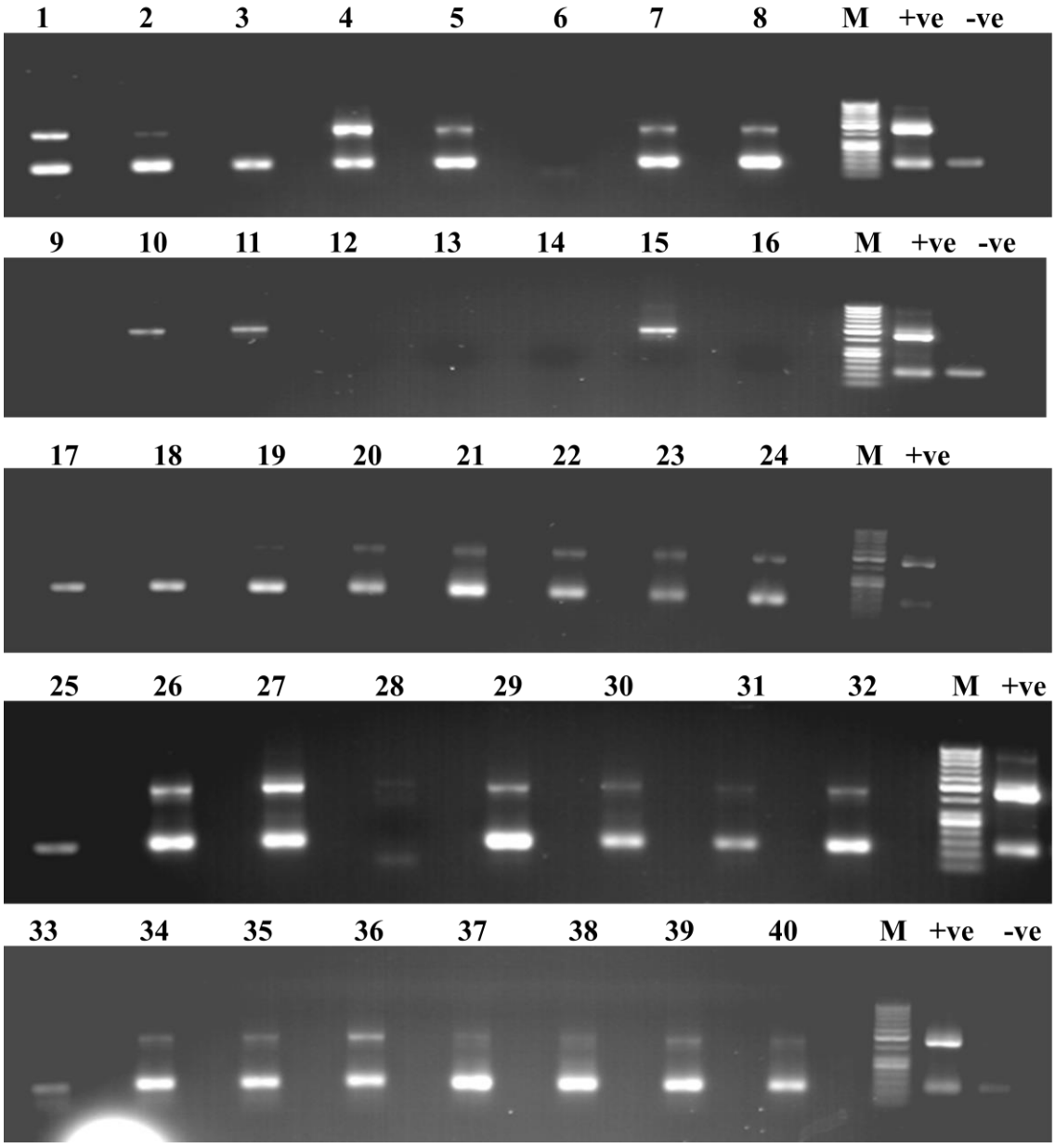


Figure 3.2: Agarose gel electrophoresis of the PCR products using PPCR system 1 targeting cDNA material that was prepared from different plants' samples. (samples from 1 to 40). The rest of the results are in Appendix 1.

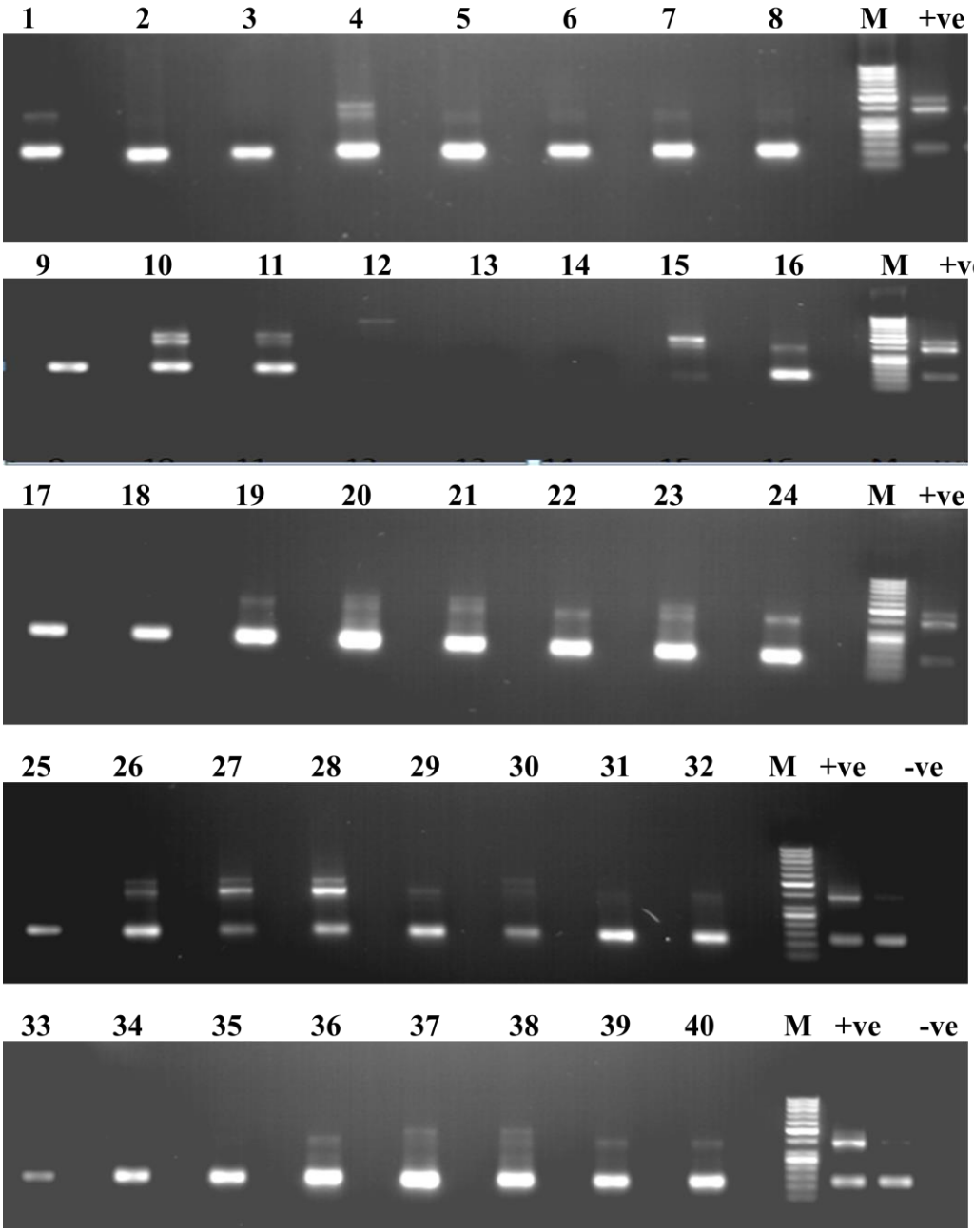


Figure 3.5: Agarose gel electrophoresis of the PCR products using PPCR system 2 targeting cDNA material that was prepared from different plants' samples. (samples from 1 to 40). The rest of the results are in Appendix 2.

3.3 MiSeq DNA library analysis and index addition quality control check:

After the second PCR that involves the addition of two indices (S5xx and N5xx), a quality control analysis of the obtained pooled and purified DNA amplicons was performed. Indices addition introduces about 130 bp on each PCR amplicon. So, after the second PCR (indices addition) random samples were chosen to ensure that the index addition step was successful. As seen in (Figure 3.8), for each type of PCR system there was a 70 bp advanced shift in amplicon size as results of indices addition. Later and after the purification of all indexed labeled amplicons and their pooling in one tube to be used in MiSeq Illumina sequences analysis, a Tapestation electrophoresis analysis (Agilent Technologies, CA, USA) was performed using SYPBR green I fluorescence detection system and this in order to analyze the produced library at the quantitative and qualitative level. (Figure 3.9), shows the Tapestation results

3.4 Next generation DNA sequence analysis:

The received results included the direct and the reverse sequences of each of the tested samples (155), a total of 310 FASTQ files were received from the service company that performed the NGS run. The 310 FASTQ files were considered the raw Illumina sequencing data and they consisted of two different files: Read 1 and Read 2 for each tested sample. All files were uploaded to Galaxy platform program (usegalaxy.eu) as indicated in material and methods. A workflow for sequence analysis that based on joining each direct and reverse sequence reads (taken from read1 and read2) (**Figure 3.10**).

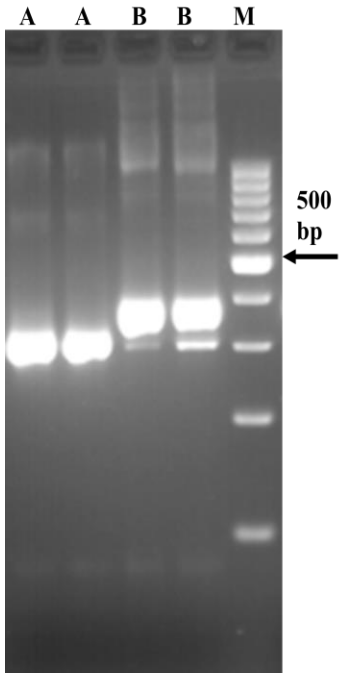
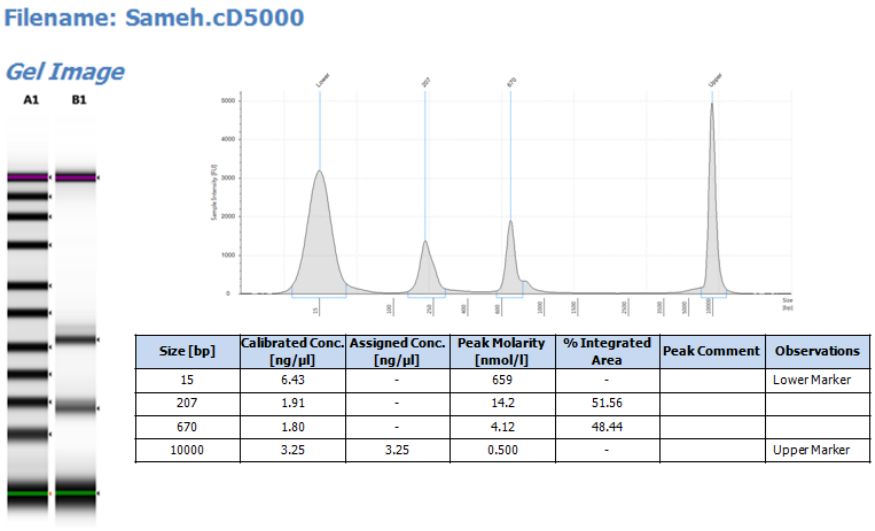


Figure 3.8: Band size comparative analysis for some selected amplicons used in MiSeq library preparation, before and after indices addition. About 70 bp shift in amplicon size seen in (b: after index addition) and (a: without index).



Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	% Integrated Area	Peak Comment	Observations
15	6.43	-	659	-		Lower Marker
207	1.91	-	14.2	51.56		
670	1.80	-	4.12	48.44		
10000	3.25	3.25	0.500	-		Upper Marker

Figure 3.9: MiSeq library quality control analysis using a sensitive fluorescent DNA analysis performed on Tapestation electrophoresis machine. Band size of about 400 bp is clearly seen that is related to PCR system1 system 2.

This was followed by transforming the sequences from FASTQ files into FASTA files and then sequence length selection and then selection of sequences that represents the amplified tobamovirus gene segments. The last step in the workflow was to compile all sequences in one file for easier follow up and consequent BLAST analysis using BLAST tool from NCBI/NIH.

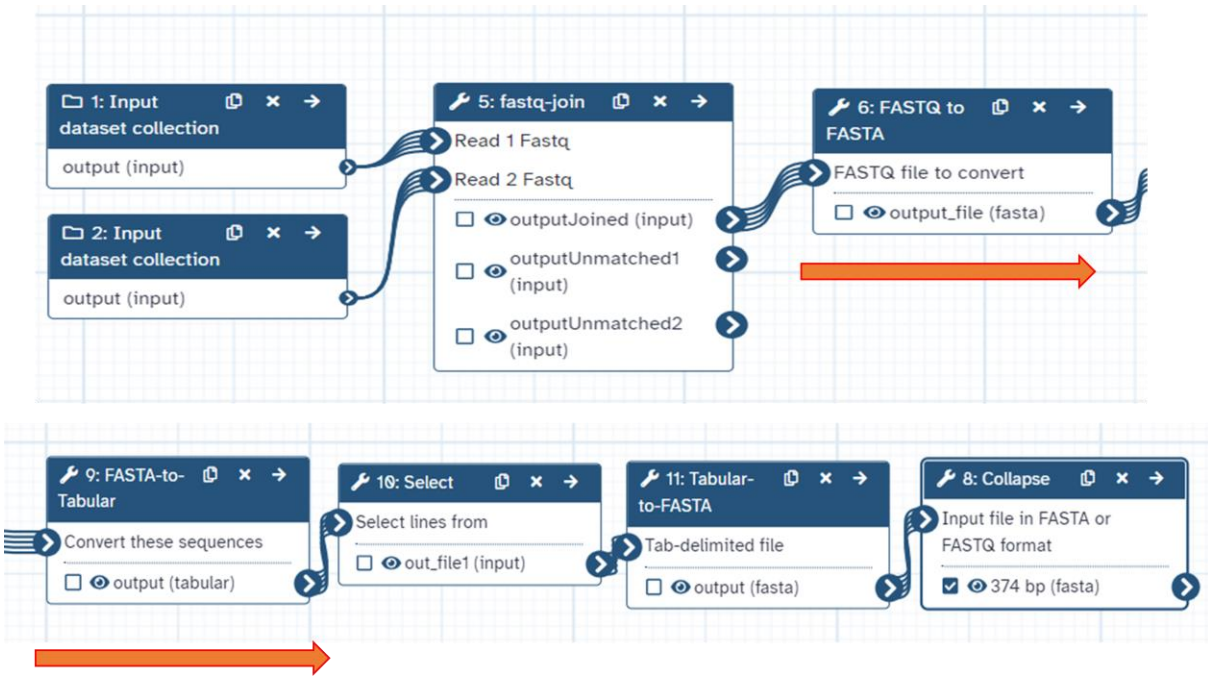


Figure 3.10: Workflow used for NGS sequence data analysis. The workflow starts with input data reads for each sequence and at the end their selection by sequence length and according to the specific used primers.

3.5 NGS sequence data analysis:

The amplified PCR products from both system 1 and systems 2 were all specifically labeled and grouped together as indicated in materials and methods and then they were used in NGS sequence analysis. The obtained data of NGS was analyzed as it is indicated in the previous section that enabled a quantitative measurement of viral load in each analyzed sample. Using the NGS analysis it was possible to identify two targets DNA segments of the virus that were amplified by PCR system 1 and PCR system 2. These DNA amplicons were identified using the above indicated workflow and using selection criteria based on the sequences of the direct primers for both PCR system 1 and PCR system 2. (Figure 3.11) shows the obtained DNA sequencing of the two obtained amplicons that were compared by (BLAST) analysis tools against nucleotide sequence data in NCBI GenBank. The sequences showed 99% similarity to different isolates of **Tomato brown rugose fruit virus including** the Jordanian tomato Tobamovirus isolate **(TBRFV-Jo), (GenBank accession no. KT383474.1).**

From the quantitative viral data analysis that was obtained from calculating number of amplicons reads (Number of fasta reads) of each utilized PCR system; it was possible to produce a relationship between the abundance of viral infections load in different studied green houses along the collection period. Based on this analysis it is clearly seen that greenhouses 3 and 4 showed much less viral load compared to greenhouse 1 and 2. (Figure 3.12).

>PCR system 1 (380 bp)(Tomato brown rugose fruit virus). (different isolates), 100% coverage and 98.9% identity

GAGGAACAGACGCTTATTGCTACTAGGGCATATCCAGAATTCCAGATAACCTTCTATAATACGCAGA
ACGCCGTGCATTCGCTTGCCGGTGGACTACGATCCTTAGAACTGGAATATCTAATGATGCAGATCCC
GTACGGATCACTCACATATGATATAGGTGGGAATTTTGCATCTCATCTGTTCAAAGGACGGGCATAT
GTTCACTGCTGTATGCCCAATCTTGATGTCCGCGACATAATGCGGCACGAAGGCCAGAAAGACAGTA
TAGAATTATACCTTTCCAGGATTGAGCGGGGCAACAAAGTTGTCCCAAATTTCCAAAAGGAAGCCTT
TGACAGATACGCTGAACCGCCAGACGAAGTTGTCTGTCACAATAC

>PCR system 2 (312bp)(Tomato brown rugose fruit virus). (different isolates), 100% coverage and 99.3% identity

ATGGTACGAACGGCGGCAGAAATGCCACGCCAGACTGGACTATTGGAAAATTTGGTGGCGATGATCA
AAAGAAACTTTAATTCACCGGAGTTATCAGGAATAATCGACATTGAGAATACTGCATCTTTAGTAGT
AGATAAATTTTTTGGTAGTTACTTGCTTAAAGAAAAAAGAAAACCAAATAAAAATGTCTCTTTATTT
TGTAGAGAGTCTCTCAATAGATGGTTAGAGAAGCAGGAGCAAGTGACCATTGGTCAGCTTGCAGATT
TTGATTTTGTGGATCTTCCTGCCGTTGATCAGTACAGGCATATG

Figure 3.11: Amplicons DNA sequences of two amplified Tobamovirus gene segments utilizing PCR system1 and PCR system.

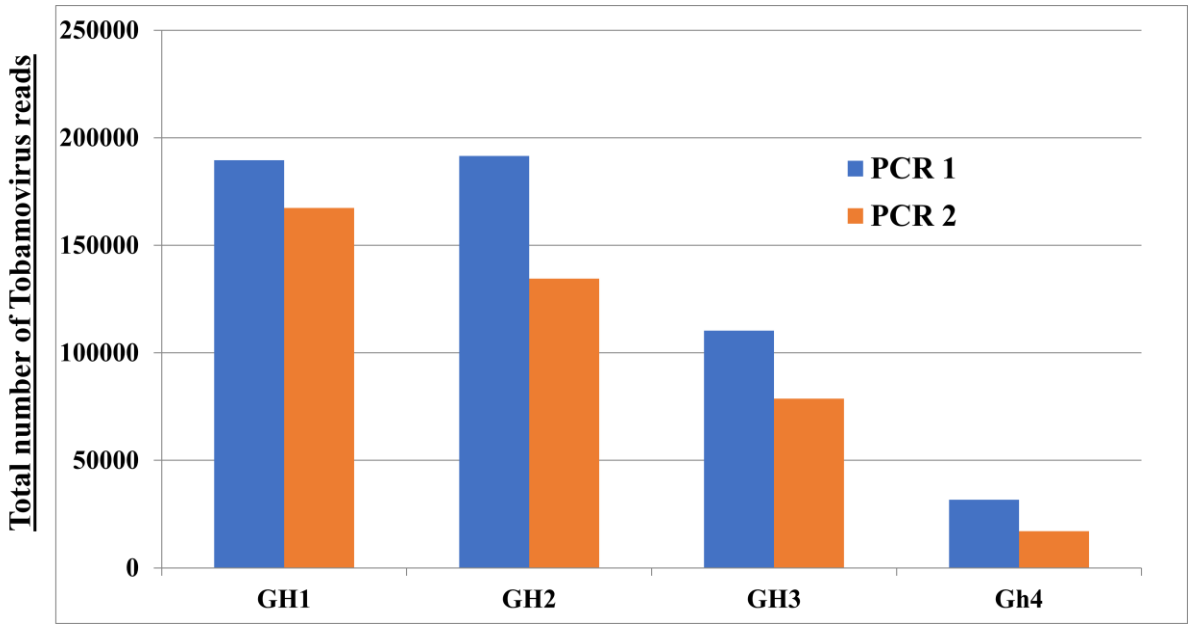


Figure 3.12: Total viral load represented by number of viral reads using PCR system 1 and 2 as it is seen among the four studied green houses.

The calculated number of reads as they obtained from the NGS data analysis were also used to follow up the viral load in each greenhouse along the study period (Table 3.2). In this analysis greenhouse number 1 and 2 were compared together because the same tomato variety (Senator F1, Master) was cultivated in these greenhouses (table 1 in materials and methods). Also, the viral profile was depicted independently for both greenhouse 3 and greenhouse 4, in which Nikran and Senator F1 varieties were used respectively. (Figures 3.13 and 3.14) show a comparison of viral load profile in the four studied greenhouses for both PCR system 1 and 2, it clearly seen that there is an overall consistency in the obtained results of both PCR systems. This indicates that either of the two systems could be used in the future in order to save reagents and time work. Greenhouse 1 and 2, they started as clean environment from tomato viral infections and a month later the viral load increased dramatically. This situation was urgently managed by the farmers and the viral load gradually decreased over the next collection dates. In general greenhouses 3 and 4 showed less viral load over the study period, and this is consistent with a previous study that was done by Al-Quds university team in Jenine and it was concluded that Nikran and Senator F1 varieties were more resistant to Tobamovirus infections.

Table 3.2: Viral load as they calculated based on number of total reads for each greenhouse along the study period.

		PCR system 1				PCR system 2			
Collection date	**No. of analyzed samples**	**GH1**	**GH2**	**GH2**	**GH4**	**GH1**	**GH2**	**GH2**	**GH4**
23.2.2023	**11**	3140	280	103663	861	1711	360	73408	354
07.3.2023	**10**	1558	37	ND	ND	1032	27	ND	ND
27.3.2023	**10**	114661	127218	3500	659	102136	86550	1471	693
10.4.2023	**10**	69924	59369	3152	30195	61970	47021	3817	15978
16.5.2023	**5**	306	4604	ND	ND	480	550	ND	ND

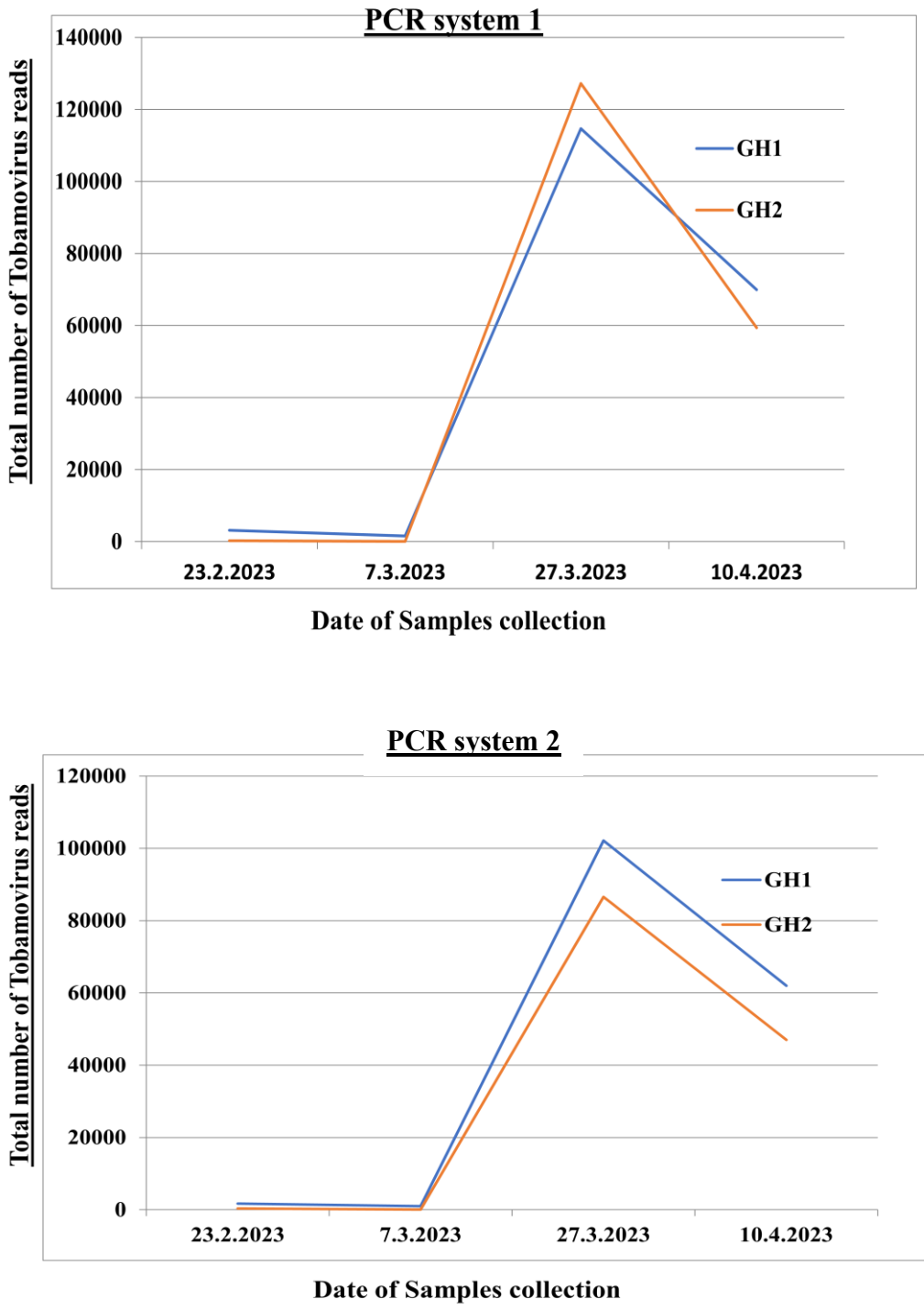


Figure 3.13: Viral load profile based on NGS analysis of PCR system 1 and system 2 over the collection dates for both greenhouse 1 and greenhouse 2.

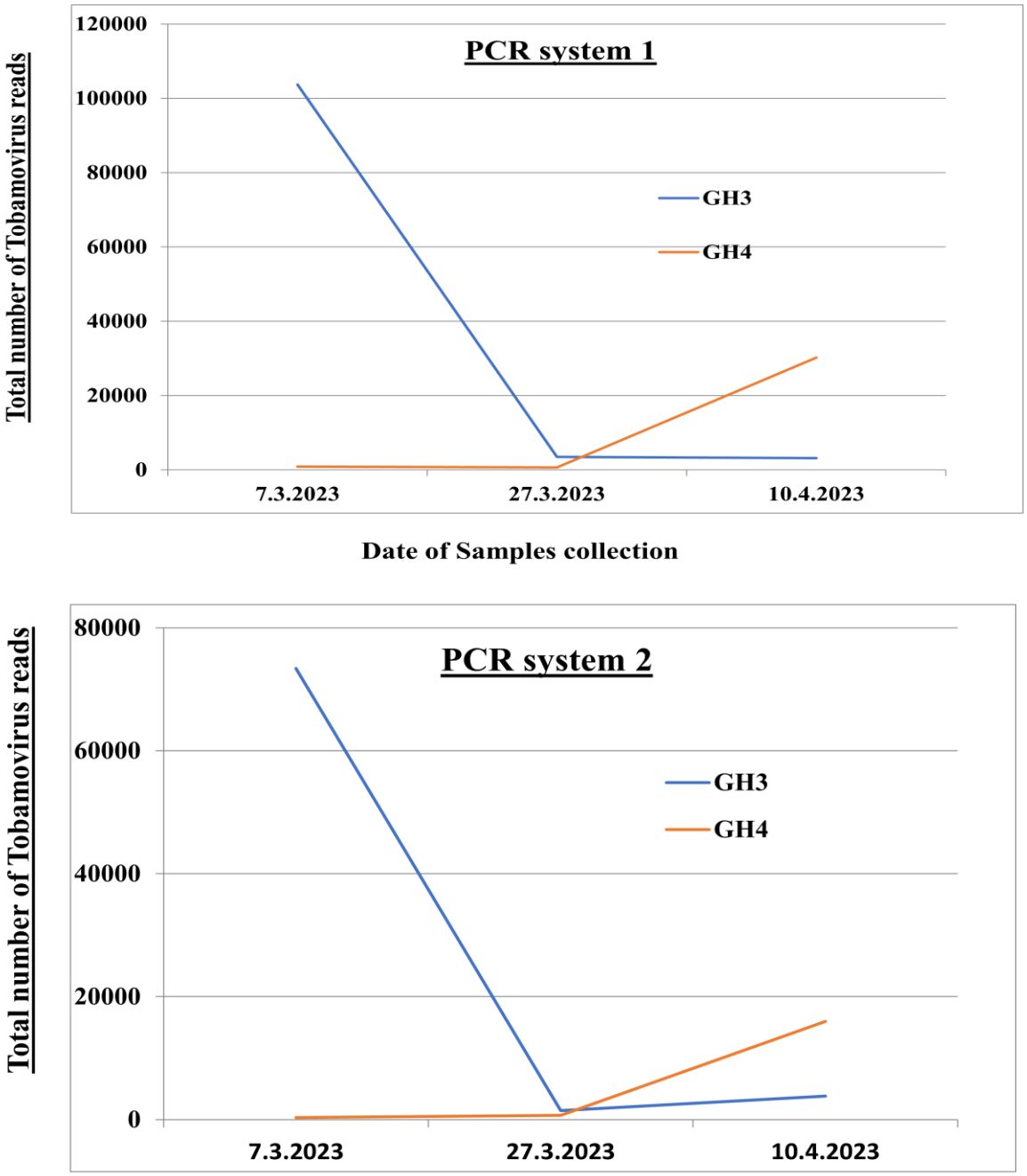


Figure 3.14: Viral load profile based on NGS analysis of PCR system 1 and system 2 over the collection dates for both greenhouse 3 and greenhouse 4.

Chapter four: Discussion

4.1 Review PCR amplification Results

The First part of this research, the obtained PCR amplification was successful using two PCR systems targeting cDNA prepared from different plants, both leaves and fruits. where PCR reactions were performed for each PCR system used separately. Although it was possible to perform multiple reactions, this was done to demonstrate the effectiveness of each PCR reaction in detecting viral cDNA and to ensure consistency of the results obtained by the two different PCR systems. According to our results, there was consistency in the PCR results for the two systems, especially those samples that showed strong amplification of Tobamovirus cDNA.

The findings show that the newly created PCR primers were successful in amplifying the genetic material of the tobamovirus. Both PCR systems demonstrated sensitivity in amplifying viral cDNA down to 1pg, with PCR system 1 showing more obvious amplification at 0.1pg compared to PCR system 2. Additionally, both PCR systems amplified DNA fragments of the expected size, confirming the specificity of the primers.

The study successfully amplified viral cDNA from various plant samples, including leaves and fruits, using the designed PCR systems, as for soil samples, they were used as a negative control. Even though multiplex reactions were feasible, individual PCR reactions were carried out to evaluate each PCR system's effectiveness separately. Agarose gel electrophoresis of the PCR results revealed consistency in the amplification patterns of the two systems, particularly in samples showing strong amplification of Tobamovirus cDNA. However, PCR system 1 consistently yielded more positive results compared to PCR system 2. Quantitative analysis showed that PCR system 1 detected more samples with moderate to strong PCR bands, while PCR system 2 detected more samples with faint or weak amplification.

Overall, the results demonstrate the effectiveness of the newly designed PCR primers in amplifying tobamovirus genetic material and the reliability of the PCR systems in detecting viral cDNA in various plant samples.

4.2 Detection of ToBRFV by NGS analysis in the plant samples

The tomato brown rugose fruit virus (ToBRFV), which was discovered in Jordan in 2016, is one of the Tobamovirus species that has raised serious concerns recently. It is known that ToBRFV mostly infect tomato and pepper (*Capsicum annuum*) plants. It can cause a variety of symptoms, including mild to severe mosaicism, dark green bulges on leaves, deformities, and constriction of the leaves. (Esmaeilzadeh et al., 2023).

In this current study, using NGS analysis, shows the obtained DNA sequencing of the two obtained amplicons that were compared by (BLAST) analysis tools against nucleotide sequence data in NCBI GenBank. Where its sequence showed 99% similarity to different isolates of Tomato brown rugose fruit virus including the Jordanian tomato Tobamovirus isolate (TBRFV-Jo), (GenBank accession no. KT383474.1).

As we also mentioned, this is consistent with a previous study conducted by the Al-Quds University team in Jenin, as this study follow-up a previous study for different regions. It is the same type that is consistent with a previous study when an outbreak of a new disease affecting tomatoes occurred in October and November 2014 in the village of Ohad in southern Israel. After studying it, the complete genome sequence of the new Israeli tobamovirus showed a high sequence identity to the Jordanian isolate of the tomato brown rugose fruit virus (Luria et al., 2017).

Quantitative viral data analysis, based on the number of amplicons reads obtained from NGS data, allowed for the establishment of a relationship between the abundance of viral infections load in different studied greenhouses over the collection period. Notably, greenhouses 1 and 2 exhibited significantly lower viral load compared to greenhouses 3 and 4. This difference was particularly evident in the comparison of viral load profiles between greenhouses 1 and 2 versus greenhouses 3 and 4, where the same tomato cultivars

were cultivated. The viral load of the Senator F1 and Master cultivars grown in greenhouses 1 and 2 was much higher than that of the Senator F1 and Nikran varieties grown in greenhouses 3 and 4. This also indicates resistance of the Senator F1 and Nikran cultivars to Tobamovirus.

As for the comparison between tomato cultivars resistant to the virus, we concluded through this study and the results we obtained, as previously described, that the Nikran and Senator F1 cultivars were more resistant to Tobamovirus infection than the Master cultivar, this is because it showed resistance to the virus and there were not many infections among the samples collected and tested throughout the study period. which are the types on which our current study was based.

Based on the results we obtained in this study; we note that there is no specific period for infection with the virus. The crop can be infected with the virus at any stage of plant growth, whether at the beginning of planting or in the middle of season. According to the results we obtained, infection with the virus in each greenhouse varied in severity and time of infection. Unlike others, and as mentioned previously, the virus can be transmitted to the crop through contact with workers' hands, water, or through soil contamination. It is also possible to transmit it by bees present in greenhouses and other factors i.e. insects that help in infecting the crop with tobamovirus.

The NGS sequencing strategy showed the ability to identify specific Tobamovirus cDNA in biological samples from different plant, whether this was plants' leaves or plants' fruits. Using high-resolution DNA sequence data this is based on the use of PCR primers that allow detection of 1100 bp of the viral ORF3 gene. Tomato virus specific primers are also indicated in the (table 2.2) in Materials and Method and have been adapted for use with the new NGS sequencing technology.

Next-generation sequencing (NGS) has enabled in-depth investigations and sequence analysis of any DNA amplicon, this method was largely used in identifying viral communities found in different biological samples. Also, it was widely used in sequences

to find a single nucleotide polymorphism among the same DNA amplicons, that is difficult to identify by the conventional DNA sequence analysis. The major added value of NGS analysis over its sensitivity is being a quantitative method.

4.3 Conclusion and limitation

In conclusion, according to previous results, we have detected the presence of the tomato brown rugose fruit virus. Also, according to the types of tomatoes that were studied, we found that the tomato varieties (Nikran and Senator F1) were more resistant to tobamovirus infection than the tomato variety (Master). The NGS sequencing strategy showed the ability to identify specific Tobamovirus cDNA in biological samples from plants.

The limitations of the study are the small sample size that was collected for many reasons; this does not enable us to generalize the results to the region as a whole. Likewise, the tomato cultivars used in comparison are limited, few, and mixed, as we discussed previously.

4.4 Recommendation

Based on our results, we recommend conducting a more extensive study in other regions for the rest of the Palestinian regions that have not been studied previously and for other varieties of tomatoes and using larger samples, so that it gives us the opportunity to generalize the results to the regions and varieties studied.

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Appendix 1

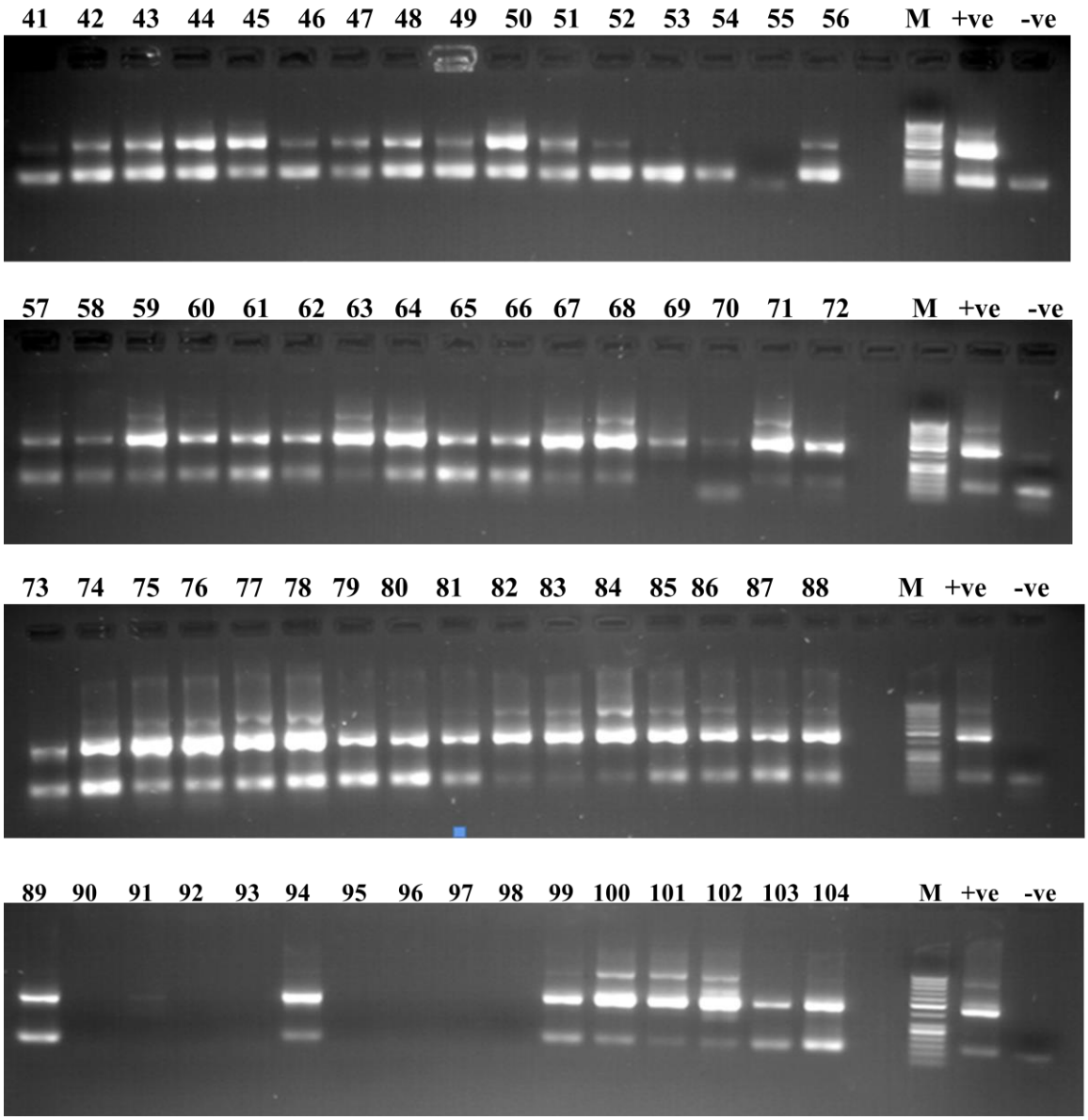

Figure 3.3: Agarose gel electrophoresis of the PCR products using PPCR system 1 targeting cDNA material that was prepared from different plants' samples. (samples from 41 to 104).

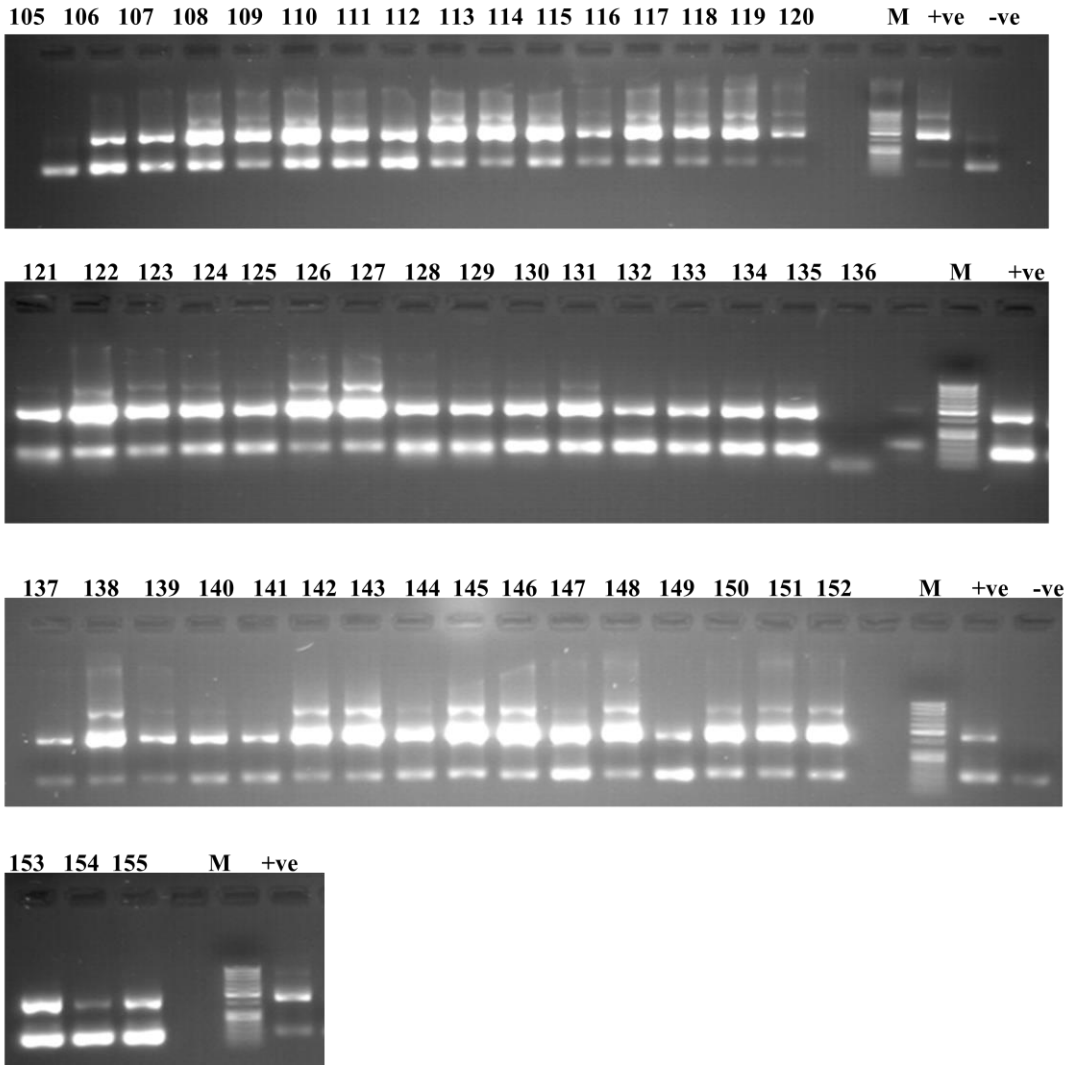

Figure 3.4: Agarose gel electrophoresis of the PCR products using PPCR system 1 targeting cDNA material that was prepared from different plants' samples. (samples from 105 to 155).

Appendix 2

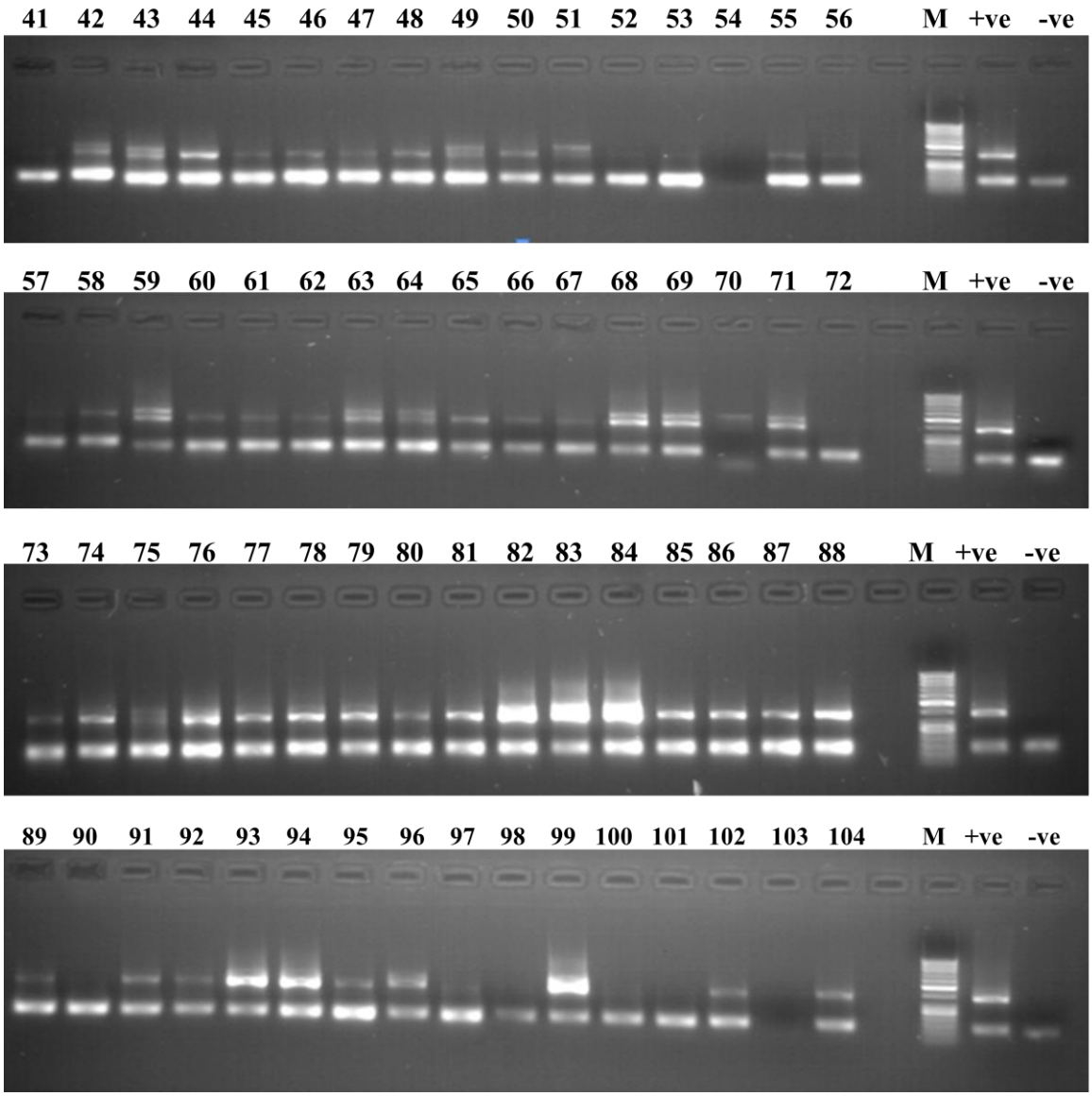

Figure 3.6: Agarose gel electrophoresis of the PCR products using PPCR system 2 targeting cDNA material that was prepared from different plants' samples. (samples from 41 to 104).

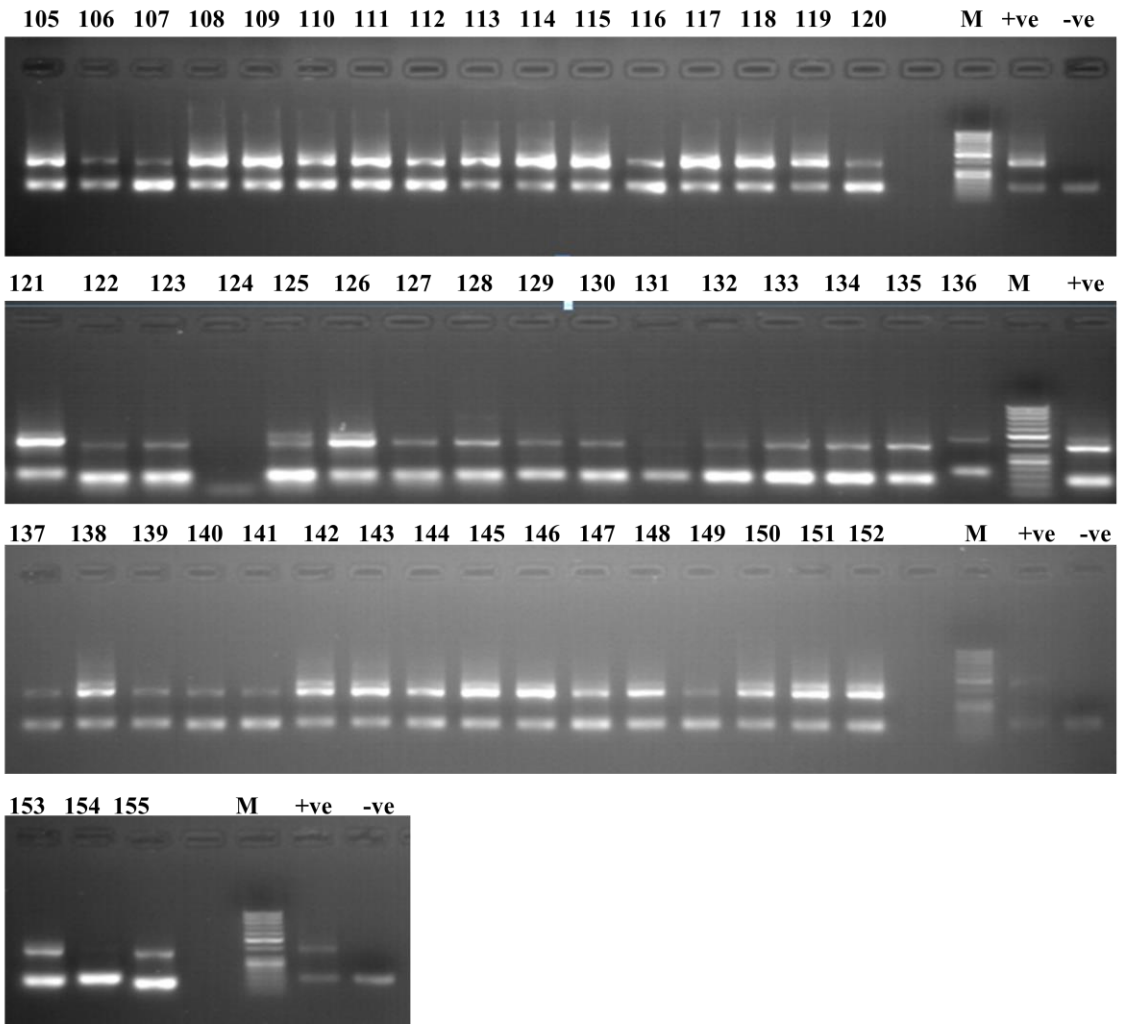

Figure 3.7: Agarose gel electrophoresis of the PCR products using PPCR system 2 targeting cDNA material that was prepared from different plants' samples. (samples from 105 to 155).

Appendix 3

Sample number	Collection date	Greenhouse number	Tomato type	System result 1	System result 2
1	23.2.2023	GH1	Senator F1	positive 2	positive 0
2	23.2.2023	GH1	Senator F1	positive 0	negative
3	23.2.2023	GH1	Senator F1	negative	negative
4	23.2.2023	GH1	Senator F1	positive 3	positive 0
5	23.2.2023	GH1	Senator F1	positive 1	positive 0
6	23.2.2023	GH1	Master	negative	positive 0
7	23.2.2023	GH1	Master	positive 1	positive 0
8	23.2.2023	GH1	Master	positive 1	positive 0
9	23.2.2023	GH1	Master	negative	negative
10	23.2.2023	GH1	Master	positive 1	positive 1
11	23.2.2023	GH1	Master	positive 1	positive 1
12	23.2.2023	GH2	Nikran	negative	positive 0
13	23.2.2023	GH2	Nikran	negative	negative
14	23.2.2023	GH2	Nikran	negative	negative
15	23.2.2023	GH2	Nikran	positive 1	positive 1
16	23.2.2023	GH2	Nikran	negative	positive 0
17	23.2.2023	GH2	Nikran	negative	negative
18	23.2.2023	GH2	Nikran	negative	negative
19	23.2.2023	GH2	Nikran	positive 0	positive 0
20	23.2.2023	GH2	Nikran	positive 1	positive 0
21	23.2.2023	GH2	Nikran	positive 1	positive 1
22	7.3.2023	GH1	Senator F1	positive 1	positive 1
23	7.3.2023	GH1	Senator F1	positive 1	positive 1
24	7.3.2023	GH1	Senator F1	positive 1	positive 1
25	7.3.2023	GH1	Senator F1	negative	negative
26	7.3.2023	GH1	Senator F1	positive 1	positive 0
27	7.3.2023	GH1	Senator F1	positive 2	positive 1
28	7.3.2023	GH1	Senator F1	positive 0	positive 2
29	7.3.2023	GH1	Senator F1	positive 1	positive 0
30	7.3.2023	GH1	Senator F1	positive 0	positive 0
31	7.3.2023	GH1	Senator F1	positive 0	positive 0
32	7.3.2023	GH2	Master	positive 1	positive 0
33	7.3.2023	GH2	Master	negative	negative
34	7.3.2023	GH2	Master	positive 0	negative
35	7.3.2023	GH2	Master	positive 0	negative
36	7.3.2023	GH2	Master	positive 1	positive 0
37	7.3.2023	GH2	Master	positive 0	positive 0
38	7.3.2023	GH2	Master	positive 0	positive 0

39	7.3.2023	GH2	Master	positive 0	positive 0
40	7.3.2023	GH2	Master	positive 0	positive 0
41	7.3.2023	GH2	Master	positive 0	negative
42	7.3.2023	GH3	Nikran	positive 1	positive 1
43	7.3.2023	GH3	Nikran	positive 2	positive 1
44	7.3.2023	GH3	Nikran	positive 3	positive 2
45	7.3.2023	GH3	Nikran	positive 3	positive 0
46	7.3.2023	GH3	Nikran	positive 0	positive 0
47	7.3.2023	GH3	Nikran	positive 1	positive 0
48	7.3.2023	GH3	Nikran	positive 2	positive 0
49	7.3.2023	GH3	Nikran	positive 1	positive 1
50	7.3.2023	GH3	Nikran	positive 3	positive 1
51	7.3.2023	GH3	Nikran	positive 1	positive 1
52	7.3.2023	GH4	Senator F1	positive 0	negative
53	7.3.2023	GH4	Senator F1	negative	negative
54	7.3.2023	GH4	Senator F1	negative	negative
55	7.3.2023	GH4	Senator F1	negative	positive 0
56	7.3.2023	GH4	Senator F1	positive 0	positive 0
57	7.3.2023	GH4	Senator F1	positive 0	positive 0
58	7.3.2023	GH4	Senator F1	positive 0	positive 0
59	7.3.2023	GH4	Senator F1	positive 3	positive 1
60	7.3.2023	GH4	Senator F1	positive 2	positive 0
61	7.3.2023	GH4	Senator F1	positive 2	positive 0
62	27.3.2023	GH1	Senator F1	positive 2	positive 0
63	27.3.2023	GH1	Senator F1	positive 3	positive 1
64	27.3.2023	GH1	Senator F1	positive 3	positive 1
65	27.3.2023	GH1	Senator F1	positive 2	positive 1
66	27.3.2023	GH1	Senator F1	positive 2	positive 0
67	27.3.2023	GH1	Senator F1	positive 3	positive 0
68	27.3.2023	GH1	Senator F1	positive 3	positive 2
69	27.3.2023	GH1	Senator F1	positive 1	positive 2
70	27.3.2023	GH1	Senator F1	positive 0	positive 0
71	27.3.2023	GH1	Senator F1	positive 3	positive 2
72	27.3.2023	GH2	Master	positive 2	negative
73	27.3.2023	GH2	Master	positive 1	positive 1
74	27.3.2023	GH2	Master	positive 4	positive 2
75	27.3.2023	GH2	Master	positive 5	positive 1
76	27.3.2023	GH2	Master	positive 5	positive 3
77	27.3.2023	GH2	Master	positive 4	positive 2
78	27.3.2023	GH2	Master	positive 5	positive 2
79	27.3.2023	GH2	Master	positive 2	positive 2
80	27.3.2023	GH2	Master	positive 2	positive 1
81	27.3.2023	GH2	Master	positive 2	positive 2

82	27.3.2023	GH3	Nikran	positive 3	positive 4
83	27.3.2023	GH3	Nikran	positive 3	positive 5
84	27.3.2023	GH3	Nikran	positive 3	positive 5
85	27.3.2023	GH3	Nikran	positive 3	positive 2
86	27.3.2023	GH3	Nikran	positive 3	positive 2
87	27.3.2023	GH3	Nikran	positive 2	positive 2
88	27.3.2023	GH3	Nikran	positive 2	positive 3
89	27.3.2023	GH3	Nikran	positive 2	positive 0
90	27.3.2023	GH3	Nikran	negative	negative
91	27.3.2023	GH3	Nikran	negative	positive 5
92	27.3.2023	GH4	Senator F1	negative	negative
93	27.3.2023	GH4	Senator F1	negative	negative
94	27.3.2023	GH4	Senator F1	positive 2	positive 1
95	27.3.2023	GH4	Senator F1	negative	negative
96	27.3.2023	GH4	Senator F1	negative	positive 1
97	27.3.2023	GH4	Senator F1	negative	positive 0
98	27.3.2023	GH4	Senator F1	negative	negative
99	27.3.2023	GH4	Senator F1	positive 2	positive 1
100	27.3.2023	GH4	Senator F1	positive 3	positive 0
101	27.3.2023	GH4	Senator F1	positive 2	positive 3
102	10.4.2023	GH1	Senator F1	positive 3	positive 3
103	10.4.2023	GH1	Senator F1	positive 1	positive 0
104	10.4.2023	GH1	Senator F1	positive 2	positive 1
105	10.4.2023	GH1	Senator F1	negative	positive 2
106	10.4.2023	GH1	Senator F1	positive 2	positive 0
107	10.4.2023	GH1	Senator F1	positive 2	positive 0
108	10.4.2023	GH1	Senator F1	positive 4	positive 3
109	10.4.2023	GH1	Senator F1	positive 3	positive 3
110	10.4.2023	GH1	Senator F1	positive 5	positive 2
111	10.4.2023	GH1	Senator F1	positive 3	positive 3
112	10.4.2023	GH2	Master	positive 3	positive 2
113	10.4.2023	GH2	Master	positive 4	positive 2
114	10.4.2023	GH2	Master	positive 4	positive 4
115	10.4.2023	GH2	Master	positive 4	positive 4
116	10.4.2023	GH2	Master	positive 2	positive 1
117	10.4.2023	GH2	Master	positive 4	positive 4
118	10.4.2023	GH2	Master	positive 3	positive 4
119	10.4.2023	GH2	Master	positive 3	positive 3
120	10.4.2023	GH2	Master	positive 2	positive 1
121	10.4.2023	GH2	Master	positive 3	positive 3
122	10.4.2023	GH3	Nikran	positive 5	positive 0
123	10.4.2023	GH3	Nikran	positive 4	positive 0
124	10.4.2023	GH3	Nikran	positive 4	negative

125	10.4.2023	GH3	Nikran	positive 3	positive 1
126	10.4.2023	GH3	Nikran	positive 5	positive 3
127	10.4.2023	GH3	Nikran	positive 5	positive 1
128	10.4.2023	GH3	Nikran	positive 3	positive 1
129	10.4.2023	GH3	Nikran	positive 3	positive 1
130	10.4.2023	GH3	Nikran	positive 3	positive 1
131	10.4.2023	GH3	Nikran	positive 4	positive 0
132	10.4.2023	GH4	Senator F1	positive 2	positive 0
133	10.4.2023	GH4	Senator F1	positive 2	positive 1
134	10.4.2023	GH4	Senator F1	positive 3	positive 1
135	10.4.2023	GH4	Senator F1	positive 3	positive 1
136	10.4.2023	GH4	Senator F1	negative	positive 1
137	10.4.2023	GH4	Senator F1	positive 1	positive 1
138	10.4.2023	GH4	Senator F1	positive 4	positive 2
139	10.4.2023	GH4	Senator F1	positive 1	positive 1
140	10.4.2023	GH4	Senator F1	positive 2	positive 1
141	10.4.2023	GH4	Senator F1	positive 1	positive 1
142	16.5.2023	GH1	Senator F1	positive 5	positive 2
143	16.5.2023	GH1	Senator F1	positive 5	positive 2
144	16.5.2023	GH1	Senator F1	positive 3	positive 2
145	16.5.2023	GH1	Senator F1	positive 5	positive 3
146	16.5.2023	GH1	Senator F1	positive 5	positive 3
147	16.5.2023	GH2	Master	positive 4	positive 1
148	16.5.2023	GH2	Master	positive 5	positive 2
149	16.5.2023	GH2	Master	positive 1	positive 0
150	16.5.2023	GH2	Master	positive 4	positive 2
151	16.5.2023	GH2	Master	positive 4	positive 2
153	23.3.2023	GH1	Fruit	positive 3	positive 2
155	23.3.2023	GH2	Fruit	positive 2	positive 1
Strong band= 4,5					
Medium band = 2,3					
Weak Band= 0,1					