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Kerimbek N.M., Master of Technical Sciences, Ph.D. student, **the main author** https://orcid.org/0000-0001-6955-7454
Institute of Plant Biology and Biotechnology, Almaty, st. Timiryazev 45, 050040, Kazakhstan, nazym_kerimbek@mail.ru
Kapytina A.I., Master of Technical Sciences, https://orcid.org/0000-0001-5029-1107
Institute of Plant Biology and Biotechnology, Almaty, st. Timiryazev 45, 050040, Kazakhstan, anastasiya.kapytina@mail.ru
Khusnitdinova M. A., Ph.D., https://orcid.org/0000-0003-0378-9337
Institute of Plant Biology and Biotechnology, Almaty, st. Timiryazev 45, 050040, Kazakhstan, germironame@gmail.com
Taskuzhina A.K., Master of Natural Sciences, PhD student, https://orcid.org/0000-0001-6819-7426
Institute of Plant Biology and Biotechnology, Almaty, st. Timiryazev 45, 050040, Kazakhstan, ataskuzina@gmail.com
Gritsenko D.A., Ph.D., https://orcid.org/0000-0001-6377-3711
Institute of Plant Biology and Biotechnology, Almaty, st. Timiryazev 45, 050040, Kazakhstan, d.kopytina@gmail.com

ASSESSMENT OF OCCURRENCE AND DIVERSITY OF APPLE VIRUSES IN SOUTH KAZAKHSTAN

ANNOTATION

Apples (*Malus* spp.) are one of the popular fruits in the world. Apple cultivars face significant threats from various viruses and virus-like diseases. These include well-known ones such as *Apple mosaic virus* (ApMV), *Apple necrotic mosaic virus* (ApNMV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), and *Apple chlorotic leaf spot virus* (ACLSV). These viruses are known to infect a wide range of apple cultivars, leading to various symptoms such as stem grooving, chlorotic leaf spots, stem pitting, and mosaic patterns on the fruits and leaves. The objective of this study was to assess the incidence of the five viruses in apple-growing regions of southern Kazakhstan. The average positive rates of ACLSV, ASPV and ASGV were 42.1%, 57% and 12.6% respectively. ApMV and ApNMV were not detected. The study revealed a high incidence of mixed infections of the three apple viruses in Kazakhstan, with 60.6% of the infected apple plants harboring more than one species of virus. The most frequent virus combination was ACLSV+ASPV, with an incidence of 31.7%, followed by ACLSV+ASGV (10.9%), ASPV+ASGV (10%) and ACLSV+ASPV+ASGV (8.2%). The high prevalence of single and mixed infections in surveyed regions underscores the need for comprehensive and integrated approaches to disease management such as multiplex RT-PCR. The assay allows for the simultaneous detection of multiple apple viruses in a single reaction, providing a rapid and efficient means of screening for multiple pathogens in apple plants.

Key words: *apple viruses, ApMV, ApNMV, ACLSV, ASPV, ASGV, RT-PCR.*

Introduction. Apples (*Malus* spp.) are one of the popular fruits in the world consumed in various forms such as fresh, frozen, canned, or cooked. World apple production has been increased by 61.7% since 2000 and reached 95.8 million tons in 2022. According to Food and Agriculture Organization (FAO) in Kazakhstan the harvested area reached about 35.8 ha and 268 k tons of apple produced in 2022. The largest quantity of apple produced by Asia (61.5%), Europe (21.7%) and Americas (12.4%) [1]. The main exporters are mainland China (US$1 billion), Italy ($913.4 million), the United States ($886 million) and apple imported to Kazakhstan mainly from Europe countries [2].

Apple cultivars worldwide, including in Kazakhstan, face significant threats from various viruses and virus-like diseases. These include well-known ones such as *Apple mosaic virus* (ApMV), *Apple necrotic mosaic virus* (ApNMV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), and *Apple chlorotic leaf spot virus* (ACLSV). ApMV and ApNMV are members of the genus *Ilarvirus* (family *Bromoviridae*) and can cause apple mosaic disease (AMD) [3, 4]. Subsequent

studies revealed that other viruses, such as prunus necrotic ring spot virus (PNRSV) and cucumber mosaic virus (CMV) [5, 6], are also associated with AMD. The disease stands as a significant economic threat, with global prevalence in apple cultivation, posing a severe risk to the industry. Initially documented in Europe in 1930 [7], this disease adversely impacts apple trees, leading to a substantial reduction in the net photosynthetic rate of infected leaves, ranging from 2.93% to 45.83% [8]. Furthermore, it can result in a notable decline in fruit yield, reaching up to 30% to 50% [9].

The genome of ApMV is tripartite, consisting of single-stranded positive-sense RNA (RNA1, RNA2, and RNA3) and an encapsidated subgenomic RNA4 [3]. The transmission of ApMV primarily occurs through grafting and mechanical inoculation to several herbaceous plants [10]. The novel ilarvirus ApNMV is close to *Prunus necrotic ringspot virus* (PNRSV) and ApMV [11] that discovered by using deep sequencing assay without prior knowledge. ASPV is a member of the genus *Foveavirus* in the family *Alphaflexiviridae* with a flexuous filamentous particle, 800 nm in length and a diameter of about 12-15 nm [12]. The genome of ASPV is a single-stranded, positive-sense RNA molecule of approximately 9.3 kb in size [13]. ASGV belongs to the genus *Capillovirus* within the family *Betaflexiviridae* and characterized by flexuous filamentous particles measuring approximately 750 nm in length and 12 nm in diameter. Genome of ASGV consists of a single-stranded, positive-sense RNA molecule, approximately 6.5 kb. ACLSV is a member of the genus *Trichovirus* in the family *Betaflexiviridae* with filamentous flexuous particles in a diameter of 12-72 nm. Its genome consists of a single-stranded positive-sense RNA molecule approximately 7555 bp in length, with a polyA in the 3' terminal. It is considered one of the most detrimental viruses affecting pome fruits and has been reported to cause significant yield losses, especially in mixed infections.

ASPV, ASGV and ACLSV is primarily transmitted through vegetative propagation methods such as grafting and budding, and no natural vector for the viruses has been identified. Their genomes encode a polyprotein that undergoes processing to yield various functional proteins, including a replicase, a movement protein, a coat protein, and a putative protease. Additionally, the genomes contain non-coding regions crucial for viral replication and gene expression [14].

These viruses are known to infect a wide range of apple cultivars, leading to various symptoms such as stem grooving, chlorotic leaf spots, stem pitting, and mosaic patterns on the fruits and leaves. In addition, some of these viruses can cause graft-transmissible disorders, phloem necrosis, and decline of commercial cultivars, ultimately affecting the quality and yield of the fruits [15]. Moreover, latent or dormant viruses can be unintentionally transmitted during propagation because infected plants may not exhibit visible viral symptoms. Using virus-free plant material is essential to minimize production costs and prevent the spread of viral infection in crops.

Methods for detection these viruses encompass biological, serological, and molecular assays. Biological assays are less popular due to time-consumption, labor-intensity, sensitivity to environmental conditions, and usually obtained results are difficult to interpret or inconclusive. Additionally, biological assays may not be suitable for detecting viruses that do not induce clear symptoms in the indicator plants or for viruses that have a long latent period. Serological assays have some limitations, including low sensitivity and specificity, cross-reactivity with related viruses, and the requirement for good quality antibodies. Therefore, serological assays are often used in combination with other diagnostic methods, such as molecular assays, to increase the accuracy and reliability of virus detection. RT-PCR is highly sensitive and specific, allowing for the detection of low levels of viral RNA in plant samples. The assay demonstrated high sensitivity and specificity in detecting these viruses, making it a valuable tool for virus diagnosis and research [16].

Up to date there is no available data regarding the prevalence of viruses in apple orchards. The objective of this study was to assess the incidence of the five primary viruses, namely ApMV, ApNMV, ASPV, ASGV, and ACLSV, in apple-growing regions of southern Kazakhstan.

Materials and methods. The study involved collecting symptomatic and asymptomatic apple samples in the southern region of Kazakhstan, specifically in five sites, including Chundja, Ketpentau, Sumbe, Chilik and Taraz with a total number of 221 leaf and branch samples. The collected samples were stored at -80℃ until nucleic acid extraction.

Total RNA was extracted from leaf and branch tissue using the CTAB method as described by Rahmani and Amraee with modifications [17]. In details, 250 mg of tissue was homogenized by liquid nitrogen with following 1.2 ml of extraction buffer adding (0.1M Tris-HCl, 25 mM EDTA, 2M NaCl, 2% CTAB, 2% PVP). Homogenate was incubated at 65℃ for 30 min and then centrifuged at 16 000 g

for 10 min. The supernatant was transferred to a new tube, to which equal volume of chloroform:isoamyl alcohol (24:1) was added. Then it centrifuged at 16 000 g for 15 min and supernatant was transferred to a new tube. Total RNA was precipitated by using 8M LiCl. After overnight precipitation, dried pellets were dissolved by 50 µl of water. The quality of the isolated RNA was verified by a 2% TAE agarose gel electrophoresis.

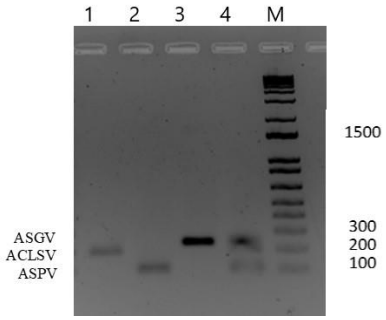
Sets of primers have been designed for multiplex RT-PCR assay by using NCBI (Primer-BLAST module) and UGENE program. Specific primers for virus detection were designed based on conservative regions of genomes such as RNA-dependent RNA polymerase and capsid protein genes. The primer sequences and the expected size of the amplified fragments for each template are provided in Table 1.

Table 1 – Primer sets developed for specific apple viruses

Primer name	Forward 5'-3'	Reverse 5'-3'	Amplicon size (bp)
ASPV	ACGCAAACAAACTCTGAACAAG	TGCGATTACTTCCTCTGCGG	98
ACLSV	AGGTGAGAGGCTCTATTCACA	TCCCCTTCTGCGATCTTCAA	190
ASGV	ACGTGCTTCAACAAGCGAGA	TCCAACAGCGGGAAACTGG	230
ApMV	TCCGAATCCGATGGACCGAA	ACAAATCCCTCATAAAGACTTTCGC	167
ApNMV	GGTGTGCAATCGCTGTCATC	GTCCTCCAACATTCCCACCC	388

Subsequently, reverse transcription was performed using RevertAid Reverse Transcriptase from Thermo Scientific, USA. This involved incubating a mixture of 200 ng RNA, 0.5 µM Oligo-dT, and 0.5 µM random hexamer primers in a final volume of 15 µl at 72 ℃ for 10 minutes, followed by cooling on ice for 3 min. Then, 5x RT reaction buffer, 0.5 mM dNTPs, and 200 U reverse transcriptase were added, and the mixture was incubated for 1 hour at 45 ℃. The PCR reaction mixture, with a total volume of 25 µl, included 0.2 mM of specific forward and reverse primers for each virus and 2 µl of cDNA used as a template. The PCR cycling conditions consisted of an initial denaturation at 96℃ for 3 minutes, followed by 30 cycles of 30 seconds at 96℃, 20 seconds at 55℃, and 60 seconds at 72 ℃. Subsequently, a final extension step of 5 minutes at 72℃ was performed. To verify the presence of the viruses, 10 µl of each PCR reaction was loaded and separated on a 2.0% (w/v) TBE agarose gel.

Results and their discussion. To investigate the presence and distribution of five apple viruses in south Kazakhstan, 221 samples were randomly collected across five sites in the apple-growing regions. All collected leaf and branch apple samples were tested by multiplex RT-PCR assay (figure 1). The obtained results showed that all of tested viruses except ApMV and ApNMV detected in five surveyed sites (table 2). The average positive rates of ACLSV, ASPV and ASGV were 42.1%, 57% and 12.6% respectively.



1 – sample infected by ACLSV; 2- sample infected by ASPV; 3- sample infected by ASGV; 4- sample infected by ASGV+ASPV; M-1kb Plus DNA Ladder, Invitrogen

Figure 1 – Analysis of single and mixed virus infections by multiplex RT-PCR

Table 2 – Multiplex RT-PCR assay of 221 samples from five sites in south Kazakhstan

Collection area	Quantity of samples	Positive rate		
		ACLSV	ASPV	ASGV
Ketpentau	50	23	30	0
Sumbe	50	28	28	0
Chundja	4	0	2	0
Chilik	14	0	5	1
Taraz	103	42	61	27
All samples	221	93	126	28
Positive rate (%)		42.1	57	12.6

The study revealed a high incidence of mixed infections of the three apple viruses in Kazakhstan, with 60.6% of the infected apple plants harboring more than one species of virus (table 3).

Table 3 – Mixed infections of three viruses in apple trees of south Kazakhstan

Mixed infection	Quantity of positive samples	Positive rate (%)
ACLSV+ASPV	70	31.7
ACLSV+ASGV	24	10.9
ASPV+ASGV	22	10
ACLSV+ASPV+ASGV	18	8.2
Total	134	60.6

The most frequent virus combination was ACLSV+ASPV, with an incidence of 31.7%, followed by ACLSV+ASGV (10.9%), ASPV+ASGV (10%) and ACLSV+ASPV+ASGV (8.2%) (figure 2). This indicates that mixed infections are common in apple growing regions of country, which could have significant implications for disease management and the development of resistant cultivars. Various researches on apple cultivars conducted in the Czech Republic, Romania, Albania, and Bosnia and Herzegovina have consistently reported increased infection rates by ACLSV and ASPV viruses [18, 19, 20]. In a recent study by Wright and colleagues [21], they employed High Throughput Sequencing (HTS) to characterize instances of multiple co-infections in apple trees suffering from diseases. HTS technology has revolutionized virus detection, eliminating the need for antibodies or prior viral sequence data. It enables the identification of both known and unknown viruses, offering enhanced sensitivity and the capacity to detect multiple viruses simultaneously in a single test [22, 23]. The findings hinted at a potential synergistic effect among known and unidentified viruses, which could be contributing to the overall disease condition in the affected apple trees. Mix infections involving at least two viruses were frequently observed, with the most prevalent combination being ACLSV+ASPV [14] as same for our findings.

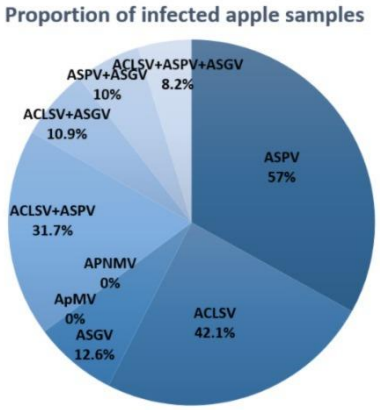


Figure 2 – The incidence of single and mixed infections in apple samples in south Kazakhstan

Mixed infections can lead to increased disease severity, as well as the potential for recombination and synergistic interactions between viruses, which may exacerbate the impact on apple production. Additionally, the presence of multiple viruses in the same plant can complicate disease diagnosis and management strategies, posing challenges for effective control measures.

The high prevalence of mixed infections with 60.6% incidence in surveyed regions underscores the need for comprehensive and integrated approaches to disease management such as multiplex RT-PCR. The assay allows for the simultaneous detection of multiple apple viruses (ACLSV, ASGV, ASPV, ApMV and ApNMV) in a single reaction, providing a rapid and efficient means of screening for multiple pathogens in apple plants.

Since crucial apple viruses lack a known natural vector, the primary transmission way continues to be through vegetatively propagated saplings, either as scion or rootstock, originating from infected parent stock [24]. Consequently, the principal control approach involves the production of propagation material that is free from viruses [25, 26]. The high occurrence of single and mixed infections emphasizes the necessity for comprehensive and integrated approaches to disease management. This includes the imperative development of robust diagnostic tools and the implementation of effective control measures. These measures should be tailored to address the specific combinations of viruses prevalent in distinct apple growing regions in Kazakhstan. Continuous research on the prevalence of viruses in both cultivated and wild apple populations, along with monitoring the dynamics of virus transmission, helps in developing effective preventive strategies.

Conclusion. Understanding the dynamics of single and mixed virus infections and their impact on apple production is crucial for the development of sustainable disease management strategies and the breeding of resistant apple cultivars in Kazakhstan. This highlights the importance of continued research into the epidemiology and ecology of single and mixed virus infections in apple growing regions. Molecular methods such as multiplex RT-PCR assay presents a valuable tool for the efficient and reliable detection of multiple apple viruses, offering practical benefits for disease management and surveillance in apple producing places. Obtained results showed that 42.1% of apple samples were infected by ACLSV, 57% and 12.6% by ASPV and ASGV, respectively. ApMV and ApNMV were not found during survey. In addition, study revealed a 60.6% incidence of coinfection with the most frequent virus combination ACLSV+ASPV (31.7%). To our knowledge, this is the first systematic analysis of simultaneous detection of ACLSV, ASPV, ASGV, ApMV and ApNMV in apple-growing regions of Kazakhstan.

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ТҮЙІН

Алма ағашы (*Malus* spp.) - әлемдегі ең көп таралған жеміс дақылдарының бірі. Әртүрлі вирустар мен вирус тәрізді аурулар алма ағашының әртүрлі сорттарына қатты қауіп төндіреді.

Оларға алма мозаикасының вирусы (ApMV), алма некротикалық мозаикасының вирусы (ApNMV), алма сабағының жарасы вирусы (ASPV), алма сабағының ойық жарасы вирусы (ASGV) және алма жапырағының хлоротикалық дақ вирусы (ACLSV) сияқты белгілі вирустар жатады. Бұл вирустар алма сорттарының кең ауқымына әсер ететіні белгілі, бұл сабақтың бороздылығы, жапырақтардағы хлоротикалық дақтар, сабақтың жарасы және жемістер мен жапырақтардағы мозаикалық үлгілер сияқты әртүрлі белгілерге әкеледі. Бұл зерттеудің мақсаты Қазақстанның оңтүстігіндегі алма өсіретін аймақтарда бес вирустың таралуын бағалау болды. ACLSV, ASPV және ASGV оң нәтижелерінің орташа көрсеткіштері сәйкесінше 42,1%, 57% және 12,6% құрады. ApMV және ApNMV табылған жоқ. Зерттеу Қазақстанда үш алма вирусымен аралас инфекциялардың жоғары жиілігін анықтады, жұқтырған алма өсімдіктерінің 60,6% вирустың бірнеше түрі бар. Вирустардың ең көп таралған тіркесімі 31,7% жиіліктегі ACLSV+ASPV, содан кейін ACLSV+ASGV (10,9%), ASPV+ASGV (10%) және ACLSV+ASPV+ASGV (8,2%) болды. Зерттелген аймақтарда жалғыз және аралас инфекциялардың жоғары таралуы мультиплексті КТ-ПТР сияқты ауруларды емдеудің кешенді тәсілдерінің қажеттілігін көрсетеді. Талдау бір реакция кезінде бір уақытта бірнеше алма вирусын анықтауға мүмкіндік береді, бұл алма өсімдіктерінде көптеген патогендердің болуын жылдам және тиімді скринингті қамтамасыз етеді.

РЕЗЮМЕ

Яблоня (*Malus* spp.) - одна из наиболее распространенных плодовых культур в мире. Различные сорта яблони подвергаются серьезной угрозе со стороны различных вирусов и вирусоподобных заболеваний. К ним относятся хорошо известные вирусы как вирус мозаики яблони (ApMV), вирус некротической мозаики яблони (ApNMV), вирус изъязвления стеблей яблони (ASPV), вирус образования бороздок на стеблях яблони (ASGV) и вирус хлоротической пятнистости листьев яблони (ACLSV). Известно, что эти вирусы поражают широкий спектр сортов яблони, приводя к различным симптомам, таким как бороздчатость стебля, хлоротичные пятна на листьях, изъязвление стебля и мозаичные узоры на плодах и листьях. Целью данного исследования было оценить распространенность пяти вирусов в регионах выращивания яблони на юге Казахстана. Средние показатели положительных результатов ACLSV, ASPV и ASGV составили 42,1%, 57% и 12,6% соответственно. ApMV и ApNMV обнаружены не были. Исследование выявило высокую частоту смешанных инфекций тремя вирусами яблони в Казахстане, при этом 60,6% зараженных растений яблони содержат более одного вида вируса. Наиболее частой комбинацией вирусов была ACLSV+ASPV с частотой 31,7%, далее ACLSV+ASGV (10,9%), ASPV+ASGV (10%) и ACLSV+ASPV+ASGV (8,2%). Высокая распространенность одиночных и смешанных инфекций в обследованных регионах подчеркивает необходимость комплексных подходов к лечению заболеваний, таких как мультиплексная ОТ-ПЦР. Анализ позволяет одновременно обнаруживать несколько вирусов яблони в ходе одной реакции, обеспечивая быстрое и эффективное средство скрининга на наличие множества патогенов в растениях яблони

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Бекишова Г. К., магистр агрономии, **основной автор,** https://orcid.org/0000-0003-4387-5483
НАО «Кокшетауский университет им. Ш. Уалиханова», г. Кокшетау, улица Абая 76, 020000, Казахстан, gulden-kaz@mail.ru
Маханова С.К., PhD, ассоциированный профессор, https://orcid.org/0000-0001-9084-348
НАО «Кокшетауский университет им. Ш. Уалиханова», г. Кокшетау, улица Абая 76, 020000, Казахстан, saulemach@mail.ru
Нурмуханбетова Н.Н., кандидат химических наук, ассоциированный профессор, https://orcid.org/0000-0002-0595-3535
НАО «Кокшетауский университет им. Ш. Уалиханова», г. Кокшетау, улица Абая 76, 020000, Казахстан, nn_nurgul@mail.ru