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Distribution of potato (*Solanum tuberosum*) viruses in Kazakhstan

Abstract: Potato is one of the most popular crops, cultivated around the world. The decrease in potato yield is caused by the degeneration of the seed material associated with the defeat of various diseases. Viruses pose a great threat to potato farming all over the world. Viral infections are dangerous not only by a significant decrease in the potato yield, but also by the absence of a phenotypic manifestation of diseases. The use of modern mpRT-PCR and ELISA methods for the diagnostics allows detecting viral infection quickly and reliably. The purpose of our research was to compare the efficiency of the molecular diagnostic methods of ELISA and mpRT-PCR and on their basis to study the degree of potato damage by the PLRV, PVM, PVS, PVX and PVY viruses in the southeast and north of Kazakhstan. There were analyzed 119 samples from Almaty and 138 samples from Kostanay regions. Most of tested potato samples were infected by PVM and PVS. PLRV was absent in the southeast and north of Kazakhstan. Complex viral infections were dominance over the monoviral infection. The reliability of ELISA and mpRT-PCR methods for the diagnostics of PVX, PVY, PVM, PVS and PLRV was assessed. Viruses PVS, PVX and PVY were more effectively detected by the mpRT-PCR than ELISA. ELISA was more effective for PVM. The reliability and efficiency of analytical methods highly depended on the variability of the nucleotide and amino acid sequences of virus isolates. We found two isolates of the PVS and three PVM isolates in the course of optimization of the mpRT-PCR method.
Key words: potato viruses, PVX, PVY, PVM, PVS, PLRV, mpRT-PCR, ELISA.

Introduction

Originally grown in the Andes, introduced to Europe in the 16th century, potato is one of the main agricultural crops cultivated all over the world, which production is growing every year [1]. It is the second most popular crop stable food after wheat, which is cultivated in all areas of Kazakhstan except Mangystau region. The potato crop area was 183.4 thousand hectares in 2017 and the largest areas were in Almaty and Kostanay (39 and 27.8 thousand hectares, respectively) [2]. In our country, this culture of foreign and domestic selection are grown [3]. However, potato production in the southeast part of Kazakhstan does not provide the needs of the local population due to its low yields. The productively of potato was 1852 kg per hectare in Almaty and 1957 kg per hectare in Kostanay in 2017. The decrease in potato yield is caused by the degeneration of the seed material associated with the nature defeat of various diseases (viral, bacterial, and fungal).

One of the most important problems of potato growing is viral infections, leading to annual significant crop losses [3]. To date, about 40 potato viruses have been identified [4]. The most common and harmful potato viruses are Potato leaf roll virus (PLRV), Potato virus M (PVM), Potato virus S (PVS), Potato virus X (PVX) and Potato virus Y (PVY). Propagation of viruses occurs in nature by insect vectors (various types of aphids, leafhoppers, whitefly), nematodes, and also by mechanical contact between infected and healthy plants. There are cases of transfer of viruses through pollen and seeds [5].

There are primary and secondary infections of plants. In the case of a primary infection, the infection of the plant occurs during the growing season and is characterized by the spread phase of the viral infection from the diseased stalk to the healthy, and

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then the infection accumulates in the tubers, while a part of the tubers may remain uninfected. In secondary infection, the virus spreads systematically from the mother tuber throughout the plant and is transmitted to all daughter tubers [6].

The symptoms of viral infection are different types of mosaic: wrinkled, banded, ordinary as well as necrosis, leaf curling, broom tops, etc. [3]. However, often the phenotypic manifestation of viral infections is absent or weakly expressed. It is not possible to determine the presence of a particular virus by external manifestations reliably in this case.

At present, the methods of enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) are widely used to diagnose viral infections of potato plants [7-9]. These methods are fairly simple to use and have a high degree of reliability of the results of the analysis.

Diagnosis of potato for the presence of viral infections is a necessary stage of the evaluation of seed material, potato recovery technologies, as well as a general assessment of the viral background in a separate area.

This paper describes the compare of efficiency of molecular diagnostic methods, including double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and multiplex RT-PCR (mpRT-PCR) and gives the characteristics of the prevalence level of potato viruses on the territory of Almaty and Kostanay regions in 2013-2017.

Materials and methods

In vitro potato plant samples obtained by the method described earlier [10] from tubers growing in Almaty region and potatoes leaves and tubers from fields of Kostanay region were used for the analysis. The potato tubers were germinated before testing in individual pots in laboratory conditions.

There were analyzed 119 plant samples from Almaty (from field collection of Kazakh Research Institute of Potato and Vegetables Growing) and 138 samples from Kostanay (from field collection North-Kazakhstan Research Institute of Agriculture and Kostanay Research Institute of Agriculture) regions in total. Leaves and tubers were collected in 2013-2017.

Potato leaves were tested for PVX, PVY, PVM, PVS and PLRV infection by DAS-ELISA using the kits "BIOREBA" (Switzerland). The 405 nm values were recorded using a plate reader (model Stat Fax-2100, "Awareness Technology", USA). Samples were considered positive if the absorbance values were high than the value of corresponding negative control and higher than 0.1 after incubation for 45 min at room temperature. Assay for each sample was carried out at least three times.

Total RNAs were extracted from fresh or freeze leaves (0.5-1.0 g) using TRIzol reagent (Sigma-Aldrich, USA) according to the manufacture instruction. The pellets were dissolved in 50 µl RNase-free water.

To synthesized first-strand complementary DNA (cDNA) we used 2 µg of total RNA, an oligo$(dT)_{18}$ as reverse primer at 10 pmol final concentration and Maxima (Moloney murine leukemia virus) Reverse Transcriptase (Thermo Fisher Scientific, Lithuania).

Reaction was carried out in 30 µl according the manufacture's instruction. Synthesized cDNA was amplified using *Taq* DNA polymerase (Thermo Fisher Scientific, Lithuania).

The primers were designed to conservative regions of coat protein-coding sequences of PVX, PVS, PVY and PVM or a large capsid protein P3 of PLRV (Table 1). PCR was carried out in volume of 25 µl using 2.5 µl cDNA and five primer pairs at final concentration of 2 pmol.

The PCR program consisted of 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 57°C and 45 s at 72°C followed by the final extension for 5 min at 72°C. PCR was conducted on Gene Amp PCR System 9700 (Applied Biosystems, USA). PCR products were analyzed in 2% agarose (TopVision Agarose, Thermo Fisher Scientific, Lithuania) gel.

Results and discussion

Potato viruses in Kazakhstan. We have studied the potato samples from Almaty and Kostanay regions. Some viruses were detected PVM, PVS, PVY, while PVX and PLRV could not be diagnosed in tested plant samples, although it was believed that these viruses were spread throughout the potato growing regions of Kazakhstan [2; 3]. This indicates that there is currently no PLRV in the southeast and north of Kazakhstan. Perhaps this is due to the peculiarities of interaction between the virus and the insect vector, however this issue is still insufficiently studied.

PVM was the most common potato virus in Kazakhstan (Figure 1, A). As can be seen from the Figure 1 (A), the total number of samples infected with the virus, was as follows: for the Almaty region – 84.03%, for the Kostanay region – 80.84%. PVS was more common in the north of the Republic (46.11% vs. 36.97% in southeast), and PVY, on the contrary, was more typical for southeast Kazakhstan (24.37% vs. 5.99% in north). The incidence of PVX was almost equal for Almaty and Kostanay areas and was 2.52% and 2.99%, respectively.

Table 1 – Nucleotide sequences of primers used for mpRT-PCR

Primer names	Genome position*	Full nucleotide sequences	Fragment size (bp)	References
#PVX-Forward	5664-5683	5' – tagcacaacacaggccacag – 3'	562	8
#PVX-Reverse	6205-6225	5' – ggcagcattcatttcagcttc – 3'		8
#PVY-Forward	8723-8742	5' – acgtccaaaatgagaatgcc – 3'	480	8; 17
#PVY-Reverse	9183-9202	5' – tggtgttcgtgatgtgacct – 3'		8; 17
#PVM-Forward	7242-7264	5' – gaaagctgaaactgccaaagatg – 3'	521	19
#PVM-Reverse	7737-7762	5' – catctgcagttatagcacctcttgg – 3'		19
#PLRV-Forward	3653-3672	5' – cgcgctaacagagttcagcc – 3'	336	8; 18
#PLRV-Reverse	3969-3988	5' – gcaatgggggtccaactcat – 3'		8; 18
#PVS – Forward	7543-7561	5' – tggcgaacaccgagcaaatg – 3'	187	16
#PVS – Reverse	7707-7728	5' – atgatcgagtccaagggcactg – 3'		16
#PVS-new-Forward	7457-7477	5' – atgaaatgcggaggaatccgg – 3'	280	This paper
#PVS-new-Reverse	7690-7715	5' – actgctccagttgggaactcaacagt – 3'		This paper

*Primer positions of gRNA were chosen according following NCBI GenBank acc. numbers: EU571480.1 (PVX), NC001616.1 (PVY), NC001361.2 (PVM), AF453394.1 (PLRV), KC430335.1 (PVS)

Variants of viruses and maximal values for each infection are indicated on the abscissa. The percent of infected plant samples on ordinate. A – The general level of distribution of viruses in Almaty and Kostanay regions; B – Levels of individual and complex viral infections in Almaty and Kostanay regions

According to literature data, in 1994-1996 the degree of virus infections was as follows in the territory of the Republic of Kazakhstan: PLRV – up to 85.7%, PVM – up to 85.7%, PVS – up to 100%, PVX – up to 96%, PVY – up to 85.7% [6; 11-12]. Consequently, PVS and PVX were the most common, although the level of infection with other viruses was also very high.

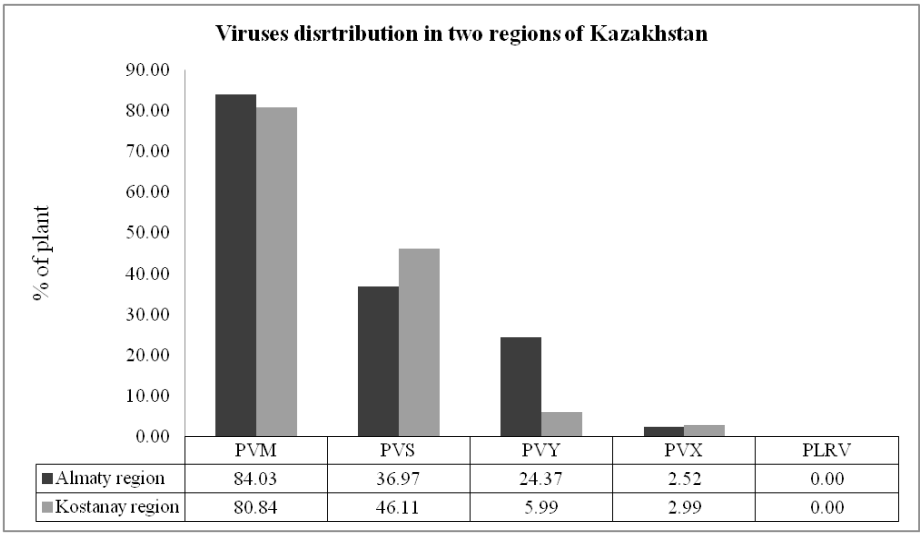
In the Southern region of Kazakhstan in 2014, rate of potato infestation was: PLRV – 5%, PVM – 8.5%, PVS – 31.7%, PVX – 41.9%, PVY – 14.5% [13].This fact also indicates the prevalence of PVX and PVS, although the overall level of virus infection of potato plants was lower by 2-3 times compared to the mid-1990s.

The study of the prevalence of viral infections in East Kazakhstan indicated the prevalence of PVM (10 varieties of 15 were infected), the incidence of PVS and PVY was the same (4 out of 15) [14]. The lowest level of spread was observed for PVX – 3 of 15 varieties were infected. Unfortunately, there was no information about the level of PLRV infection.

Based on the results obtained, at present the level of potato infection in Kazakhstan for PVX and PVY had decreased, but for PVM and PVS had increased. These results not only testify to the significant spread of viruses in the territory of Kazakhstan, but also that the degree of prevalence of a particular virus is not constant and changes over time.

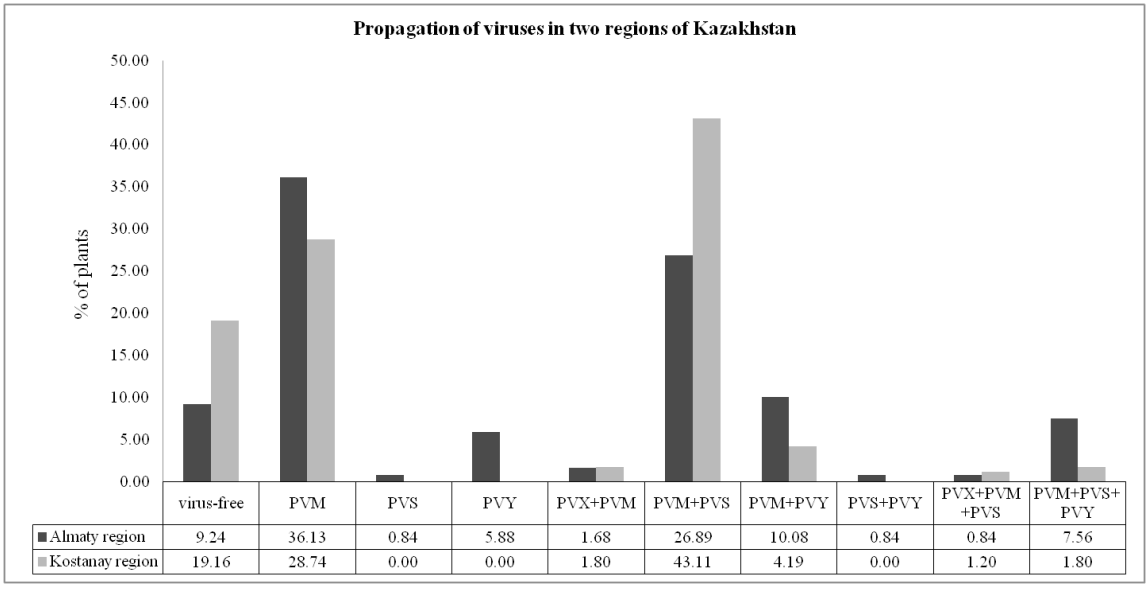
Individual and complex viral infection. When we were studying the potato virus diseases, we compared the prevalence of individual and complex infections in Almaty to Kostanay regions (Figure 1, B). The individual (monovirus) infections were caused by PVM, PVS or PVY as it can be seen at Figure1, B. By the literature information in the complex infection (when plants infected two or more types of viruses) were found up to five different types of viruses simultaneously [4].

Potato samples were found infected with three types of viruses at the same time. PVX was found only as part of a complex infection with PVM or in a mixture of PVM + PVS. The incidence of potato by monoviral infection was as follows: PVM – 36.13%, PVS – 0.84%, PVY – 5.88% for the southeast of Kazakhstan. In the northern region, PVM was the single example of monoinfection (28.74%). PVS and PVY were detected only as part of complex infection. The overall level of a single infection was 42.85% for the Almaty region and 28.74% for the Kostanay region.



	PVM	PVS	PVY	PVX	PLRV
Almaty region	84.03	36.97	24.37	2.52	0.00
Kostanay region	80.84	46.11	5.99	2.99	0.00

a



	virus-free	PVM	PVS	PVY	PVX+PVM	PVM+PVS	PVM+PVY	PVS+PVY	PVX+PVM +PVS	PVM+PVS+ PVY
Almaty region	9.24	36.13	0.84	5.88	1.68	26.89	10.08	0.84	0.84	7.56
Kostanay region	19.16	28.74	0.00	0.00	1.80	43.11	4.19	0.00	1.20	1.80

b

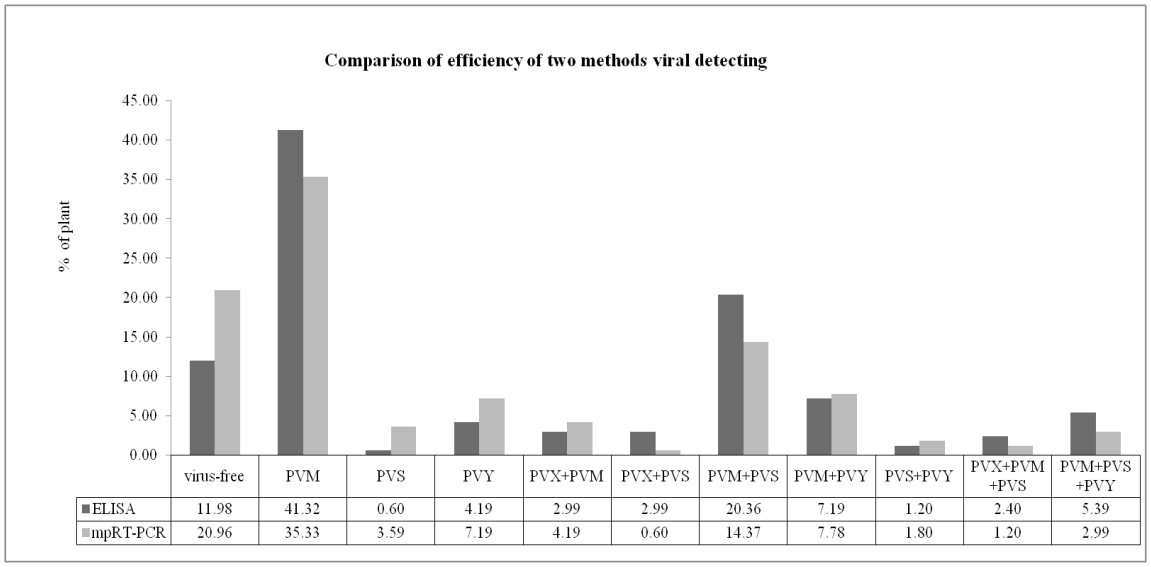
Figure 1 – Distribution of virus infections in Kazakhstan

The prevalence of complex infection varied between the two regions. It was 47.91% in Almaty area, while it was 52.1% in Kostanay area. Among the most common the complex infection was a combination of PVM + PVS: 26.89% in Almaty and 43.11% in Kostanay region. The ratio of mixed infection PVM + PVY was assessed as 10.08% in Almaty and 4.19% in Kostanay. Whereas the level of virus combination PVM + PVX was approximately the same for both regions 1.68% and 1.8%, respectively. The presence of PVS + PVY in samples was found only in the Almaty region (0.84%), and completely was absent in the Kostanay region. The main viruses (PVM and PVS) of the three-part infection belonged to carlaviruses, the third virus was either PVX or PVY. Complex infection of PVM + PVS + PVX was observed in samples from Almaty region (0.84%) and Kostanay region (1.2%) and was approximately at the same level. However, complex infection PVM + PVS + PVY was more distributed in Almaty region (7.56%) than in Kostanay (1.8%).

The total number of potato plant samples free from viral damage was 9.24% in Almaty region, and 19.16% in Kostanay region. Because in the south there are several periods of mass flying of aphids, which are the main vectors of viruses, the probability

of potato infection is increased in the these regions than in the north.

Thus, it was confirmed that potatoes grown in a cool climate are less susceptible to viral infection. The incidence of potato by PVX does not depend on the climatic conditions of the regions. The combination of PVM + PVS is currently the main type of viral infections of potatoes in the Republic of Kazakhstan.



	virus-free	PVM	PVS	PVY	PVX+PVM	PVX+PVS	PVM+PVS	PVM+PVY	PVS+PVY	PVX+PVM +PVS	PVM+PVS +PVY
ELISA	11.98	41.32	0.60	4.19	2.99	2.99	20.36	7.19	1.20	2.40	5.39
mpRT-PCR	20.96	35.33	3.59	7.19	4.19	0.60	14.37	7.78	1.80	1.20	2.99

Figure 2 – Comparison of efficiency of ELISA and mpRT-PCR

Variants of viruses and maximal values for each infection are indicated on abscissa.
Percentage of infected plant samples on ordinate

Comparison of the efficiency of ELISA and RT-PCR methods. Since the sensitivity of the analytic methods is a fundamental factor in the diagnostics of viral infections, we compared the efficiency of ELISA and mpRT-PCR methods to detect viruses in plant samples from Almaty and Kostanay areas (Figure 2).

There was shown from Figure 2, individual PVS or PVY virus was more effectively detected by mpRT-PCR (3.59% and 7.19%), in contrast to ELISA (0.6% and 4.19%, respectively). The ELISA method proved to be the more effective for PVM than mpRT-PCR (41.32% versus 35.33% respectively). PVM was not detected by mpRT-PCR if its ELISA reading was less than 0.4 units from negative control. Although according to the manufacturer recommendations of the ELISA kit, this value considered positive. Apparently, this was due to the peculiarities of the nucleotide sequence of genomic RNA (gRNA) and the amino acid sequence of the PVM isolate coat proteins, distributed in the territory of Kazakhstan. There is evidence that the efficiency of the ELISA kits is directly correlated with the amino acid sequence features of different PVM isolates, had various territorial distribution [5]. The primers for mpRT-PCR we used are complementary to the conservative regions of the gRNA encoding the PVM coat protein. Thus, PVM could more reliably detect by the ELISA both in single and complex infection.

The complex infections PVX + PVM, PVM + PVY and PVS + PVY were more accurately detected by mpRT-PCR method (4.19%, 7.78% and 1.8%, versus ELISA 2.99%, 7.19% and 1.2%, respectively). Diagnostics by ELISA was more effective for combinations of viruses PVX + PVS (2.99% vs. mpRT-PCR 0.6%), PVM + PVS (20.36% vs. 14.37%), PVX + PVM + PVS (2.4% vs. 1.2%) and PVM + PVS + PVY (5.39% vs. 2.99%).

It should be noted that the PVM was the dominant virus in complex infection. The low efficiency of the mpRT-PCR could be associated with the dominance of PVM gRNA compared to other virus RNAs in reaction mix. Other kinds of RT-PCR products could be found in minor amount. ELISA method permits to detect only one type of virus, so there is no competition for the reaction components. Also, this method allows using of frozen material for analysis, whereas it is preferable to investigate fresh one by mpRT-PCR.

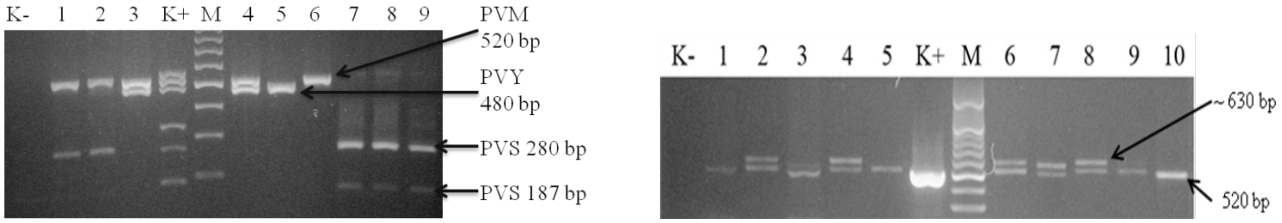
The discrepancy between the results of ELISA and mpRT-PCR often occurred in the diagnostics of PVS, while the presence of the virus was confirmed

only by the ELISA. In our opinion, this is due to the variety of PVS isolates common in Kazakhstan. Different isolates are characterized by the presence of features of the nucleotide composition of the gRNA, while the amino acid composition of proteins remains almost identical. Currently, there is no information on the diversity of PVS and PVM viruses isolates in the Republic of Kazakhstan. Therefore, to confirm viral diseases in the field, ELISA method is suitable because it is simple and not uses expensive equipment. The ELISA method does not allow the identification of different virus isolates, in which case the use of mpRT-PCR is more preferable. However, there were cases in our practice when the presence of a viral infection was confirmed only by the mpRT-PCR. Thus, it is sufficient to use ELISA for the analysis of potato plants for the presence of viral infections in the field. However, if it is necessary to confirm the purity of the plant material under study, we consider it expedient to use two diagnostic methods.

Variety of PVM and PVS isolates in the Republic of Kazakhstan. PVM and PVS are related viruses and belong to the Carlavirus group. According to NCBI GeneBank, there are many local populations of these viruses, distributed in various regions of the world and having the features of the nucleotide composition of the gRNA. PVS is subdivided into three subgroups: Andean (PVS^A), Ordinary (PVS^O) and Antioquia (PVS^P) [15]. Two subgroups of PVS^A and PVS^O are distributed around the world and are represented by a variety of different isolates. It is believed that the PVS^A subgroup is more aggressive with respect to the host plant. While PVS^O does not cause significant damage to potato plantings and is a moderate virus.

The hypothesis about the presence of different isolates of the Carlavirus group in our Republic was confirmed during the computer analysis of the gRNA sequences encoding the coat proteins of various PVS isolates. So, it turned out that the primers #PVS – Forward and #PVS – Reverse were used to identify the European PVS isolate (the size of the PCR-amplificated fragment was 187 bp [15], Table 1) had incomplete complementarity to the isolates first discovered in China and South America. When primers #PVS-new-Forward and #PVS-new-Reverse were used matched to the most conservative region of PVS gRNA (after sequencing), the efficiency of PVS detection significantly increased.

The results of analysis of some potato samples from the Almaty region were shown in the Figure 3 (A). Samples 1 and 2 contained DNA fragments of PVM (520 bp) and PVS (280 bp). In addition, there were no fragments of DNA 187 bp in size, which would indicate the presence of a European PVS isolate. Samples 7, 8 and 9 had both DNA fragments 187 bp specific for the European PVS isolate as well as 280 bp fragments, were universal for different isolates and identified in all samples.



a b

Figure 3 – Results of agarose gel electrophoresis of RT-PCR products. K – negative control, K+ positive control, M – marker DNA (GeneRuler™ 100 bp)

A – Results of electrophoresis in a 2% agarose gel after mpRT-PCR diagnostics of potato samples from the Almaty region. 1-9 – sample numbers, arrows indicate amplified DNA fragments for PVM (fragment size 520 bp), PVY (fragment size 480 bp), and PVS (fragment size 280 bp and 187 bp);

B – Results of electrophoresis in a 1.5% agarose gel of uniplex RT-PCR products in the diagnosis of PVM in samples from the Kostanay region. 1-10 – numbers of samples, arrows indicate the corresponding DNA fragments

Thus, we can detect the European isolate of PVS and distinguish it from other isolates.

When we analyzed some potato samples obtained from the Kostanay region, it was found differences during diagnostics of PVM by two methods. As a result it was decided to use the uniplex RT-PCR to PVM diagnostics. Two DNA fragments were identified in samples 2, 4, 6, 7, 8 and 9, expected fragment

of 520 bp in size, and a heavier one of 630 bp in size (Figure 3 (B)). Samples 1, 5 and 10 contained one DNA fragment corresponding to the expected size of 520 bp. It was found a shorter DNA fragment measuring ~ 500 bp in sample 3. Accordingly, there are three different PVM populations in the territory of the Kostanay area.

As a result, we detected two various PVS isolates and three PVM isolates on the territory of Kazakhstan.

Conclusion

There are PVX and PVY among the most dangerous viruses on the territory of Kazakhstan, which lead to death, as well as PVM and PVS, which provoke a secondary infection. Potato is less prone to viral infections in the northern regions. After comparing the degree of potato infection with viral diseases in two regions of Kazakhstan, the most common virus is PVM, the infection level of which reaches 84.03% in Almaty and 80.84% in Kostanay regions.

The ELISA method is advisable for diagnostics of potato viruses in the field conditions, when there is a need for an overall assessment of the level of contamination of potatoes. However, if necessary to analyze individual samples accurately, two supplementary methods ELISA and mpRT-PCR should be used. The efficiency of detecting viral infections by mpRT-PCR depends on the nucleotide sequence of genomic RNAs of the viruses being analyzed. The mpRT-PCR allows not only to detect a viral infection, but also to determine isolates of viruses.

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