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Research Article

Preparation of Polyclonal Antiserum for potato X virus

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Abstract

Recently, as a result of the development of agriculture, and the expansion of the international export of seeds and seedlings, pathogenic viruses that infect potatoes have spread to other regions, and changes in viruses under the influence of various climatic factors have led to the emergence of strong pathogenic isolates. The preservation of potato viruses in potato nodules and other plant organs creates several difficulties in their diagnosis. For this, it is advisable to use highly sensitive diagnostic methods. In this work, the results of the research on the preparation of polyclonal serum, which is the basis for obtaining antibodies necessary for the immunodiagnosis of potato X virus (PXV), are given. In this case, an isotonic solution of NaCl or cycloferon together with a purified virus antigen was injected between the muscles of the biceps and hind legs of rabbits. The delivered antigen dose was increased by the same amount for both rabbits. Before reimmunization, the titer of sera from the rabbit injected with cyclotron was 1:64, the titer from the rabbit injected with an isotonic solution of sodium chloride was 1:32, and after reimmunization, the titer of the serum isolated from the first rabbit decreased to 1:32, and from the rabbit injected with NaCl the resulting serum was found to be elevated to a titer of 1:256. The prepared serum was used in the diagnosis of PXV and its amount in plant tissue was determined.

Keywords: Potato X virus, antigen, isotonic solution, double-immunodiffusion, polyclonal antiserum

Introduction

Potato virus X (PVX) is one of the 4 most dangerous viruses among the 7 phytoviruses (Potato Leafroll Virus (PLRV), potato Y virus, potato X virus, potato A virus, potato S virus, potato M virus, Potato Aucuba Mosaic Virus (PAMV)) that are common in potato fields worldwide. PXV infection can reduce potato yield by more than 25-30% (Eskendirova *et al.,* 2014; *Fayziev et al.,*

2020). Diagnosis and detection of viruses is an important element in the field of protection of plants from diseases. In recent times, the globalization of agriculture, and the expansion of the international export of seeds and seedlings led to the introduction and spread of potato viruses in new areas. As a result of genetic recombination and mutation under the influence of biological and environmental factors, the high variability of viruses in nature causes the emergence of both new and aggressive isolates and the weakening of the properties of known strains and isolates (Anisimov *et al.,* 2009; Eskendirova *et al.,* 2014). At the same time, the spread of plant virus diseases depends not only on environmental factors such as the type of plant, and soil composition (Turaeva *et al.,* 2021, Shavkiev *et al.,* 2022), environmental discomfort affects important vital characteristics of the infected plant, including water transpiration, physiological indicators such as photosynthesis, which depends on the amount of pigment, has a negative effect and leads to a decrease in productivity (Ramazonov *et al.,* 2020; Fayziev *et al.,* 2020). This, in turn, requires the development of scientifically based ways of fighting against the virus, the use of biologically effective drugs synthesized from fungi and bacteria that do not harm the natural environment (Abdenaceur *et al.,* 2022; Turaeva *et al.,* 2023), planting virus-free seeds, using modern diagnostic methods for timely disposal of infected seeds, and at the same time requires the use of rapid methods for timely detection and identification of phytopathogenic viruses (Berzina *et al.,* 2013).

Today, various methods for the detection of viral diseases have been developed, most of them are immunological, based on the detection of a virus-specific antigen using antibodies prepared for it, or PCR methods based on the detection of certain fragments of the viral nucleic acid, which also have their equipment, laboratory conditions, necessary reagents and known from the researcher. requires a level of skill. In particular, the PCR method causes problems such as extremely sterile conditions, expensive reagents, and inapplicability in field conditions, while immunological methods have certain advantages due to their simplicity and the possibility of testing thousands of samples at the same time (Fayziev *et al.,* 2020). To date, several phytoviruses have been isolated in Uzbekistan: phytopathogenic viruses such as potato X virus necrotic isolate, alfalfa mosaic virus, plum pox virus, sorghum powdery virus have been isolated, their properties have been studied, purified preparations have been prepared and specific serum has been prepared for them, antiserum titer in serum has been studied and serum virus has been created, used in diagnostics (Fayziev *et al.,* 2020; Sobirova *et al.,* 2020; Khusanov *et al.,* 2020). In addition, the phylogeny of the necrotic isolate of the potato X virus was determined based on molecular genetic diagnostics (Fayziev *et al.,* 2023), scientific research is being conducted to create and identify virus-resistant varieties of wheat (Muminov *et al.,* 2023; Amanov *et al.,* 2020).

To date, plants such as D. *stramonium, N. burley*, and *N. bentamiana* have been used to obtain a purified preparation of potato virus X (Bikash *et al.,* 2012; Fayziev, 2019). In addition, the use of the protein coat of viruses (PXV-CP) in obtaining polyclonal antibodies against viruses such as PXV, PYV (Ahmed *et al.,* 2019) indicates the comprehensiveness of immunological methods. To date, the types of immunological methods based on precipitation, immunodiffusion, and targeted substances have been developed, all of which necessarily require antibodies prepared for the virus, and these antibodies were obtained by injecting viral antigens into various animals such as rabbits, rats, mice, guinea pigs under laboratory conditions (Kerstin *et al. al.,* 2010). The use of laboratory animals makes it easier to solve the problem of immunoreactivity. Large animals such as goats, rams, and horses are used to obtain a large amount of serum. It is more convenient to immunize male animals, because their immunogenic response is not much affected by hormonal changes (Egorov *et al.,* 1991). It is desirable to use pure antigen for specific sera, because antibodies specific to a few other compounds in the injected antigen can be formed. The level of the immune response depends on the amount of antigen administered, and immunological tolerance can occur at a certain concentration of the antigen, both high and low. Therefore, it is necessary to choose the optimal dose, taking into account the purity of the drug and its immunogenicity. In animals such as rabbits, a single dose is on average 100-300 micrograms (μg) per 2 kg of body weight, and in larger animals, this ratio varies from 0.25-10 mg (milligrams) (Egorov *et al.,* 1991). But if this proportion (in small animals) is 10-200 micrograms for strong immunogens (for example, PXV), it is 500-1000 micrograms for weak immunogens (Gnutova, 1987).

Methods of antigen administration (subcutaneous, intravenous, lymph node, intermuscular) and frequency affect the immunological activity of the serum. In addition, the antibodies in the serum obtained as a result of the first immunization and reimmunization also differ. A high titer serum can usually be obtained after several immunizations. Taking into account that changes in antibody properties determine the quality of serum, it is necessary to constantly monitor the quality of serum (Egorov *et al.,* 1991). Another feature of high-titer serum extraction is administration to the animal using adjuvants along with the antigen. Several researchers have studied the properties of immunomodulators, including cycloferon, which is used as an antiviral drug. During the experiment, it was found that when this drug is used, the immune system of the body increases in the fight against the virus, and the total amount of immunoglobulins is kept in the norm (Kharitonova *et al.,* 2018; Isakov et al., 2014, Isakov *et al.,* 2015). Based on these circumstances, the main goal was to obtain polyclonal serum against PXV, increase its titer, and study the effect of immunomodulators on this process.

Materials and methods

To experiment, initially, biologically purified potato virus X was propagated in D. stramonium and *D. tatula* L. plants. As an antigen, the preparation of the virus, which was propagated in the above-mentioned plants and purified by the gel filtration method in laboratory conditions, and for immunization and obtaining virus-specific polyclonal serum from 2 chinchilla male rabbits, and as an addition to the antigen, a 0.9% isotonic solution of sodium chloride and cycloferon was used.

Keeping and caring for animals. For each of the rabbits, a separate insulated iron cage adapted to the animals was selected. Rabbits were fed with special food twice a day. The average daily food mass for each rabbit was 120 gr. Clean water was provided for drinking. The animals were kept in a room with a room temperature of 23-24℃, air humidity of 55-60%, and 12-14 hours of light. Animals were under daily clinical observation. Their nutrition, regularity of breathing, appropriateness of their movements, skin, and ears were constantly checked.

Antigen preparation. Potato virus X was initially biologically purified in 3 passages according to the following scheme. As the initial natural source of the virus, the Gala variety of potatoes naturally infected with the virus was used. Isolation of the virus, biological purification, and reproduction in the collecting plant were carried out by the following scheme (Fig. 1).

Sample =>	Homogen..=>	*D.stramonium*, =>	*G. globosa* =>	*D. stramonium*
	0,02 M ФБ,	*D. tatula L.*	(necrosis)	*D. tatula L.*
	pH 7-8	(sis. mosaic)		(sis. mosaic)

Figure 1. Scheme of separation, biological purification and reproduction of PXV

After the virus was propagated in *D. stramonium* and *D. tatula* plants, the purified preparation was taken in laboratory conditions using the physicochemical and gel chromatography methods, and the degree of purity and concentration were determined and stored at +4℃.

A purified virus preparation was used as an antigen. An isotonic suspension of 0.9% NaCl and cycloferon were selected as excipients for obtaining polyclonal antiserum.

Identifying animals. Rabbits selected for the study were assigned an individual number. Rabbits were identified by ear lobe and their breed, sex, date of initiation of the experiment, and antigen adjuvant administered were recorded.

Immunization. The virus was administered by immunization of antigen between the two subscapularis and hind leg muscles of rabbits. The initial antigen dose was 160 μg, prepared separately with an isotonic solution of NaCl and cycloferon in a 1:1 ratio. The antigen dose was increased with each administration and the adjuvant accordingly. Injections were repeated 5 times with an interval of 3 days. Reimmunization was carried out 35 days after the last injection,

with antigen adjuvants administered intramuscularly in two subscapulars and hind legs of the rabbit.

Blood collection from experimental animals. The first blood was taken from the posterior ear vein of the rabbit after the 4th injection of the immunization procedure, the 2nd blood was taken 17 days after the last injection, and the 3rd blood was taken 26 days after the last injection. After reimmunization, 2 ml of blood was taken from each animal on days 6, 9, and 14 and from 50 milliliters on day 16.

Antiserum separation and storage. After keeping the collected blood in a thermostat (+37℃) for 2 hours, and +4℃ for 20 hours, the antiserum part was slowly put into another container using a sterile glass rod. 1500 a/min from the shaped elements of the antiserum hemolysis. It was cleared by centrifugation for 15 minutes (Egorov *et al.,* 1991) and stored in 2 ml test tubes at – 4 ℃ and – 20 ℃. All accessories were used in a sterile condition.

Preparation of gel for double-immunodiffision. Agarose and Difco agar gels of 25 ml each were prepared. For this purpose, 0.25 g of gel was placed in thermostable bottles containing 24,25 ml of 0.1 M sodium phosphate buffer pH 7.6. The agarose gel was melted in a microwave oven, and the Difco agar gel was melted in a water bath. After the gel was completely dissolved, 0.5 ml of streptomycin solution (1 g of streptomycin + 5 ml of 0.9% isotonic NaCl) was added to each container.

Determination of serum and antigen titer. Determination of the obtained serum titer and the titer of isolated antigen was performed using the double-immunodiffision method, micromodified by Zilber and Abelev, who developed Uxterlo. For this, 1% Difco agar was dissolved in 0.1 M sodium phosphate buffer pH 7.6, and 25 ml of liquid agar was spread evenly on a 9×12 cm glass plate. A 1% agarose gel was poured onto a second glass plate in the same manner. After the gel solidified on both plates, holes were made along a straight line using special stamps with a diameter of 3 mm and a gap of 4 mm. After adding 30 µl of AG and antiserum diluted to 1:2, 1:4, 1:8...1:1024 in the wells, the glass plates were placed in a desiccator with clean water and 10-20 µl of chloroform. Determination of antigen titer was also performed with antigen dilution as above. During 48 hours, diffusion occurred between antigen and antibody, and precipitation lines were formed from the specific collision of antibody and antigen**.**

Results

The interval between injections of the purified potato X virus preparation into chinchilla rabbits was 3 days, and the repetition was 5 times. In the course of immunization, cycloferon and isotonic solution were used as supplements. Injections were performed on days 1, 5, 9, 13, 17 using physiological isotonic solution and cycloferon (Table 1).

The duration of the immunization process in rabbits included 17 days. After 320 μg of antigen (PXV) was determined by spectrophotometry in 1 ml of viral sample, the amount of antigen was increased 4 times accordingly. Only the 5th injection dose was carried out in the same amount as the 4th dose (Fig. 2). The isolation of virus-specific serum took a total of 56 days, during which blood was drawn 7 times to study the titer. To determine the titer of antiserum, 2 ml and 10 ml of blood were taken, and the serum was separated from it. A final blood volume of 50 ml was collected from each rabbit for use in antibody isolation after serum titers were determined.

Table 1. Procedure for immunization of rabbits with KXV

Immunogenic	Days of injection	Immunization dose	Immunization scheme
A pure preparation of potato X virus	1	0,5 ml : 0,5 ml	PXV + NaCl PXV + cycloferon subscapularis, hind leg muscle
	2	0,7 ml : 0,7 ml	
	3	0,9 ml : 0,9 ml	
	4	2,0 ml : 2,0 ml	
	5	2,0 ml : 2,0 ml	

In rabbits, reimmunization 35 days after the time of immunization was also performed by injection under both shoulder blades and between the muscles of the hind legs. The process of blood collection from rabbits during immunization and the day of reimmunization was calculated and expressed in a tabular form (Table 2).

Table 2. Scheme of blood collection and serum titer parameters from rabbits

№	**Blood drawing scheme**	**A(AG+NaCl) titer**	**A (AG+C) titer**
1	16 days (from the 1st day of immunization)	1:32	1:64
2	17 days (from the last day of immunization)	1:32	1:32
3	27 days (from the last day of immunization)	1:64	1:32
4	6 days (from the 1st day of reimmunization)	1:64	1:32
5	9 days (from the 1st day of reimmunization)	1:64	1:32
6	14 days (from day 1 of reimmunization)	1:256	1:32
7	16 days (from the 1st day of reimmunization)	1:256	1:32

Note: A-antiserum, AG-antigen, C-cycloferon.

Blood samples were initially drawn 16 days after the first injection to determine the order in which the polyclonal serum titer changes. When the antiserum titer was determined by double-immunodiffusion before reimmunization, it was found that the serum titer for rabbit №1 was 1:64, and the serum titer for the 3rd time was 1:32, on the contrary, rabbit №2 for 1:32 was found

to be increased to 1:64. Even after reimmunization, the antiserum titer of rabbit №1 did not change from 1:32, an increase in titer was detected only in rabbit №2, according to which the initial antiserum titer was 1:64, but on the 14th day, it was found that the titer of antiserum increased to 1:256. The polyclonal serum titer at rest is shown in the graph below (Fig. 3)

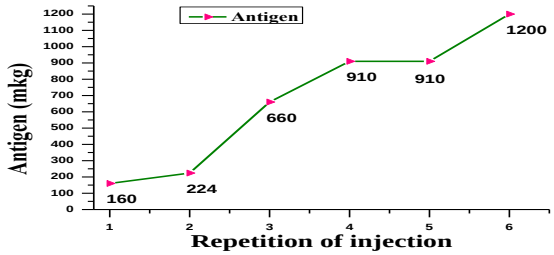

Figure 2. Antigen dose and immunization time.

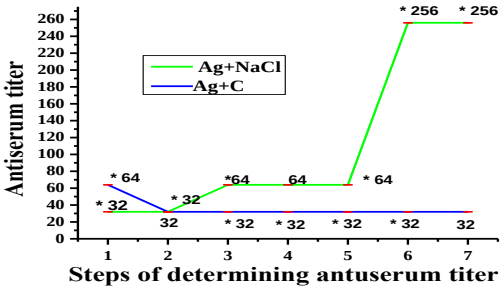

Figure 3. The dynamics of changes in the amount of antennae in the antiserum (determined using double-immunodiffision). Ag-antigen, C-Cycloferon, P≥0,05

Although both rabbits were injected with the same dose during immunization, it was observed that the polyclonal serum titers produced by the immunogen in the rabbits differed from each other. As shown in the graph (Fig. 3), the titer of the serum obtained from the rabbit injected with sodium chloride increased, and the titer of the serum isolated from the rabbit injected with cycloferon increased at first and then remained at the same level. Below is the serum titer obtained from a rabbit injected with PXV sodium chloride (Fig. 4).

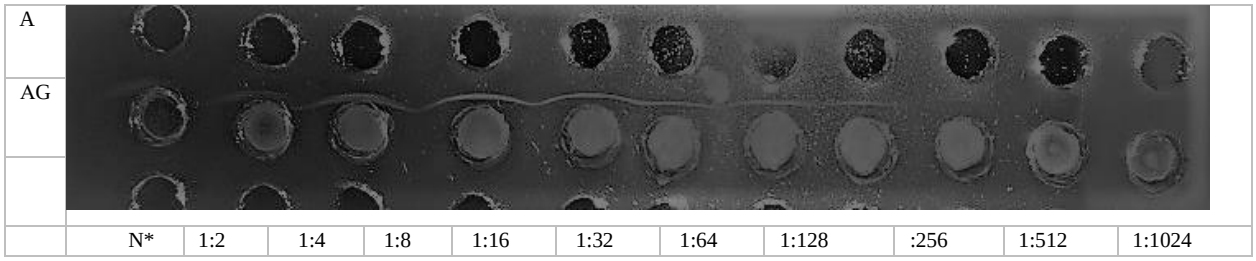

Figure 4. Image of determination of serum titer by double-immunodiffusion method (on 1% agarose gel). Pictured: A – antiserum prepared for PXV; AG - viral antigen; N* is the control, i.e., undiluted antiserum, and the remaining values are given in order of increasing serum dilution. In the course of the study, the titer of antigen (PXV) isolated from the *D. tatula* L. plant was also determined using the double-immunodiffusion method, and the precipitation lines formed between AG+NaCl and AG+Cycloferon were visually checked and the results were presented in the form of a table (Fig. 3).

Table 3. KXV titer isolated from *D. tatula* L. plant.

№	AG dilution rate	Determination of the concentration of the virus with the method of	AG+ NaCl precipitation line	AG+Cycloferon precipitation line

		immunodouble diffusion on samples, (µg/ml)		
1	Control	20,48	++++	++++
2	1:2	10,24	++++	++++
3	1:4	5,12	++++	+++
4	1:8	2,56	+++	+++
5	1:16	1,28	+++	+++
6	1:32	0,64	++	++
7	1:64	0,32	++	++
8	1:128	0,16	++	++
9	1:256	0,08	+	+
10	1:512	0,02	+	+
11	1:1024	-	-	-

The (+) sign in the table indicates the thickness of the quotation line.

In the study, the antigen was diluted up to 1024 times. Antiserum was placed in the wells of the first row (in mono), and AG was placed in the wells of the second row in the order of dilution. The amount of antigen was calculated in order of decreasing titer. Accordingly, if the control sample contains 9.6 µg of antigen, the serum at a titer of 1:256 contains 0.0375 or 3.75×10^{-2} µg, and at 1:512 it was calculated to be 18.75×10^{-3} µg. The result shows that the titer of PXV isolated from *D. tatula* L. was 1:512 when tested based on sera obtained from rabbits treated with AG+ NaCl and AG+Cycloferon. It was (+++++) at 1:2 and 1:4 dilutions, showed a weak precipitation line (+) at 256 and 512 dilutions, and showed no signs of precipitation at further dilution levels. Potato X virus is found in almost all potato plants and affects the yield and quality of the crop. The combination with other viruses causes an even higher incidence rate. The ELISA method is widely used in the diagnosis of the virus. Specific antibody acquisition is carried out using adjuvants. In the course of our study, virus-specific antiserum was obtained using isotonic NaCl (0.9%) solution and cycloferon. Taking into account that the titer of antiserum obtained using isotonic NaCl solution is 1:256, it is possible to obtain antiserum using this auxiliary substance. This will reduce the cost of virus detection. However, using cycloferon did not give a good result. During immunization, the initial titer was 1:64, then it decreased to 1:32. A small dose of KXVini for rabbits started at 160 mg, in reimmunization it was 1200 mg. Antiserum injections were administered every 3 days.

Discussion

In the experiments of Čeřovská et al., polyclonal antibodies to recombinant Potato virus X (PVX) coat protein (PVX-CP) were developed and their effectiveness was determined in different ELISA protocols. The PVX-CP gene was amplified by reverse transcription-polymerase chain

reaction, cloned, and expressed in Escherichia coli. For immunization, the CP fractions from bacterial lysate were purified either by simple fractionation or by excision from sodium dodecyl sulfate gels. The PVX-CP was injected into rabbits for antibody production. The PVX-CP antibodies reacted in an indirect plate-trapped antigen enzyme-linked immunosorbent assay and immunoblot assay and were useful for the detection of a broad spectrum of isolates of PVX.

Potato virus M (PVM) is one of the most common and economically important potato viruses in potato-growing regions worldwide. To investigate and control this viral disease, efficient and specific detection techniques are needed. In this study, PVM virions were purified from infected potato plants and used as the immunogen to produce hybridomas secreting PVM-specific monoclonal antibodies (Zhang et al., 2020).

DAS-ELISA test was performed to determine the prevalence of Potato virus Y (PVY), Potato leaf roll virus (PLRV), and Alfalfa mosaic virus (AMV). All samples were also tested by RT-PCR using specific primers to confirm the results DAS-ELISA. The results revealed that 54 % of samples were infected with PVY, PLRV, and AMV. PVY was the most common virus in the survey areas with a ratio of 50 %, followed by PLRV (5.5%) and AMV (1.38%) in all tested samples, respectively. Mixed infections also were detected within collected plants (Topkaya et al., 2020). From the analysis of the results of the experiment, it was found that a higher rate of anti-viral antibodies was recorded from the 0.9% isotonic solution of NaCl than cycloferon. Based on the authors' experiments on cycloferon and the results of our research, it is appropriate to use this immunomodulation to extract immunoglobulin E from the antiserum formed against the antigen.

Conclusion

From the analysis of the results of the conducted experiment, it became clear that a higher index was recorded when using an isotonic solution of NaCl with 0.9% for the extraction of antibodies against the virus than cycloferon. From the authors' experiments on cycloferon and the results of our research, it was concluded that it is appropriate to use this immunomodulator in the isolation of immunoglobulin E from the antiserum formed against an antigen.

During the experiment, it was assumed that the density of the gel increased and prevented the precipitation line from rising because the room temperature was not kept normal when using the double-immunodiffision method and the temperature was around 18-20℃. And in this line, taking into account the property of keeping the body's immune system at a standard, when using cycloferon, it was thought that it is necessary to choose an additional immunomodulator or other analogues. This means continuing the research more extremely.

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