Serological and Molecular Detection of Alfalfa Mosaic Virus in the Major Potato Growing Areas of Saudi Arabia

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ABSTRACT

Potato (*Solanum tuberosum* L.) is considered one of the most important and potential vegetable crops in Saudi Arabia. *Alfalfa mosaic virus* (AMV), genus *Alfamovirus*, family *Bromoviridae* is among the broad spread viruses in potato. Several potato fields in four major growing areas (Riyadh,Qassim, Hail and Hard) were inspected for the presence of AMV, during spring and fall growing seasons of potato in of 2015 and 2016. The presence of AMV was detected in potato leaf samples using ELISA. RT-PCR was conducted to validate the sensitivity of ELISA test and dot blot hybridization was established as alternative method of ELISA. The highest occurrence of AMV was observed as 18.6% of yellow leaves in Qassim followed by Riyadh with 15.2% while, the lowest infection rates were recorded in Hard and Hail, 8.3 and 10.4%, respectively. The sequences of seven isolates of AMV obtained in this study were determined and the sequences were aligned with the other sequences available in the GeneBank database. Analyses confirmed the low variability among AMV isolated in this study, which means that all AMV isolates may originate from the same source. Due to high incidence of AMV, other economic susceptible crops may become affected by high incidence of this virus in potato crops. This requires accurate indexing of imported potato seed tubers to prevent further spread of the virus in Saudi Arabia. The obtained results indicated that the hybridization and ELISA are suitable techniques in the routine detection of AMV in a large number of samples while RT-PCR is more sensitive and essential for molecular characterization of AMV.

Key Words: *Alfamovirus*, AMV, Dot blot hybridization, ELISA, PCR, Potato.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is considered one of the most important and potential vegetable crops in Saudi Arabia. The potato cultivation is being increased in Saudi Arabia due to strict governmental regulations encouraging short duration crops to conserve water resources (Chikh-Ali *et al*. 2016). Potato crop is susceptible to many plant pathogens including fungi, bacteria, nematodes, phytoplasma, viroid and viruses. Potato plant is a host for more than 40 viruses (Wale *et al*., 2008).

Alfalfa mosaic virus (AMV) belongs to the genus *Alfamovirus* (family, *Bromoviridae*) infects ~600 host plants species mostly belong to *Solanaceae* family (Bol, 2003). The infection inflects a broad range of symptoms including bright yellow mosaics and leaf lesions with chlorosis mostly damaging the chloroplast tissues (Balasubramaniam *et al*., 2014). The viral genome of AMV is tripartite, capped positivesense single stranded tripartite RNA encapsidated into baciliform particles and encodes four proteins. The RNA1 encodes P1 protein, which contains the putative methyltransferase (MT) and helicase (HEL) domains, while RNA2 encodes P2 protein, which contains the polymerase (POL) domain. Whereas, the movement protein (MP) is encoded by the RNA3 component of AMV genome. The coat protein (CP) of AMV is translated through an additional non-replicating sub-genomic RNA4 (sgRNA4), which is synthesized by RNA3 during its replication (Bol, 2005). The interactions between CP and different host factors activate certain plant defenses in the infected plants (Aparicio and Pallas, 2017).

An early detection and identification of plant viruses needs a reliable and sensitive diagnostic method for an effective management of viral infection in potato cultivation. Dot blot hybridization is a reliable and sensitive method used for detection of plant viruses in large number of samples (Alfaro-Fernández *et al*., 2016). AL-Saleh, *et al.,* 2014 conclud-

ed that dot hybridization is more sensitive than ELISA and has been proved to be an alternative to the serological methods on large-scale screening of potato viruses. Moreover, simultaneous detection of multiple viruses is possible by mixing the probes or using specific polyprobes (Sánchez-Navarro, *et al*., 1999, Lin, *et al*., 2011; Peiró, *et al*., 2012) (Alfaro-Fernández, *et al*., 2016).

This study was carried out for testing the incidence of AMV in four different regions in the Southern and Eastern of Saudi Arabia using ELISA test, comparison of the nucleotide sequences of the coat protein gene of isolated AMV with other isolates available in GenBank and establishment of dot blot hybridization for detection of the incidence of AMV.

MATERIALS AND METHODS

Survey of the major potato growing areas in Saudi Arabia for incidence of AMV using ELISA method:

Leaf samples of the potato plants showing yellow blotch symptoms and asymptomatic plants were collected from Riyadh, Qassim, Hail and Hard regions, Saudi Arabia, during spring and fall growing seasons, 2015-16. All the collected samples were serologically examined for the presence of AMV using double antibody sandwiched enzyme-linked immunosorbent assay (DAS-ELISA) (Bioreba, Switzerland) according to manufacturer's instructions. The Colour development was measured at 405/492 using dual filter reader (Awareness Technology, Inc. USA). The ELISA test was carried out with three replicates, including positive and negative controls supplied with kits.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):

RNA extraction and purification from potato leaf samples was carried out using RNeasy Mini Kit (QIAGEN, Cat No. 74106) according to the manufacturer´s instructions. RT-PCR was carried out in 25 µl reaction volume using the specific primer pair (AMVF, 5`-CCATCATGAGTTCTTCACAAAAG-3` and AMVR, 5`- TCGTCACGTCATCAGTGAGAC-3`, Xu and Nie 2006) and ''iScript One Step qRT-PCR Kit '' (Biomatik, Cat No. A4223). Each reaction contained 1 µl of the RNA extract (40 ng of total RNA), 12.5 µl iGreen Mastermix, 1.5 µl of 10 µM of each primer pair, 0.5 µl of 50x qRT-PCR Enzyme Mix and the reaction was completed to 25 µl with Nuclease-free water. RT-PCR was conducted using the ESCO Swift Maxi Thermal Cycler. Synthesis of cDNA was done at 42°C for 30 min followed by one cycle of 95°C for 10 min, 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final cycle of 72°C for 10 min. PCR product aliquots of 5 µl were loaded in 1.5% agarose gels with 100 bp DNA ladder (Biomatik Cat No. M7123) and pictures were taken under UV Light with digital imaging system gel doc (Syngene Bio Imagins, IN Genius).

Nucleotide Sequence and Phylogenetic Analyses

The amplified PCR products were completely sequenced using Sanger sequencing (Macrogen, Korea). The obtained nucleotide sequences were initially blasted in NCBI GenBank database. The pair-wise nucleotide sequence comparison was carried out using Muscle algorithm available in species demarcation tool (SDT) (Muhire *et al*., 2014) software. The phylogenetic tree was constructed using Neighbor-Joining (NJ) algorithm with Kimura-2 parameter model available in Mega7 (Kumar *et al*., 2016) using the mostly closely related sequences retrieved from NCBI.

Dot blot hybridization and membrane preparation:

The presence of the virus in potato plants and validity of the prepared probes were confirmed with non-radioactive hybridization. Five µl of each extracted RNAs and RT-PCR products were dot onto the positively charged nylon membrane. The hybridization experiments were carried out

using Gene Images AlkPhos and Chemiluminescent Detection System signal generation and detection with CDP-Star (Amersham, Biosciences, UK Limited). Dotted membrane lyses was done using 3 layer of Whatman paper steeped with 0.5 M sodium hydroxide for 5 min in a glass tray. The membranes were held in 1M Tris (pH 7.4), then 2x SSC (300 mM sodium chloride, 30 mM sodium citrate), then by 90% ethanol for 5 min each. Incubation was performed with slight shaking. Membranes were allowed to air dry and then used directly for hybridization. Pre-hybridization, hybridization, chemiluminescent signal generation and detection with CDP- Star were done using Gene Images AlkPhos Direct Labeling and Detection System (Amersham, Biosciences UK Limited).

Probe preparation and hybridization

Twenty µl of cross-linker solutions diluted with 80 µl water to give the appropriate working concentration. The RT- PCR product from AMV positive sample was diluted to a concentration of 10 ng/µl using distilled water for labeling. Ten µl of the diluted DNA sample was placed in a micro centrifuge tube and denatured by heating for 5 min in a vigorously boiling water bath; the DNA was immediately cooled on ice for 5 min and spin briefly to collect the contents at the bottom of the tube. Ten µl of the reaction buffer was added to the cooled DNA, and 2 µl labeling reagent and 10 µl of the cross-linker working solution were added, mixed gently and incubated for 30 min at 37ºC.

Pre-hybridization and hybridization were carried out at 55ºC. Membranes were placed in hybridizer tube (each membrane of each virus was placed in separate tube). The required volume (0.25 ml/cm^2) of prepared AlkPhos Direct hybridization buffer was pre heated to 55ºC and added in each tube. The membranes placed into the hybridization buffer and pre-hybridized for at least 15 min at 55ºC in hybridizer oven. Labeled probe of each virus (5-10 ng/ml of hybridization buffer) was added onto the pre-hybridized membranes for hybridization at 55ºC overnight on hybridizer oven. The hybridized membranes were washed two times using 2-5 ml/cm^2 at 55ºC for 10 min of primary wash buffer consisted of 2 M Urea, 0.1% (w/v) SDS, 50m M Na phosphate (pH 7), 150mM NaCl, 1 mM $MgCl_2$, and 0.2% (w/v) Blocking reagent,. The membranes were placed in a clean container and excess of secondary wash buffer (1M Tris base and 2M NaCl (pH 10.0) was added with gentle agitation, for 5 min at room temperature, fresh secondary wash buffer were performed for further wash at room temperature for 5 min (Juan *et al*., 2007).

Chemiluminescent signal generation and detection with CDP-Star:

The excess secondary wash buffer drained from the membranes and placed them on a clean non-absorbent flat surface, detection reagent were pipetted on to the membranes (30-40 $\mu l/cm^2$) and left for 2-5 min then the excess of the detection reagent were drained. The membranes wrapped in Saran Wrap and placed in the film cassette, the lights switched off and a sheet of autoradiography film (X-OMAT TM AR Kodak) was placed on the top of the membrane, the cassette was closed and exposed for 3 h at room temperature. The film was removed and developed using developing buffer (Janczur et al., 2006).

RESULTS AND DISCUSSION

Survey of the major potato growing areas in Saudi Arabia for incidence of AMV:

Field inspection of potato fields in four regions, Riyadh, Qassim, Hail and Hard was carried out to detect the presence of AMV infection using serological analysis.

A total of 1215 potato plant samples showing leaf mosaic, yellowing, and yellow blotching were collected along with the symptomless samples (Figure 1).

Figure 1. Diseased potato plants with yellow blotching symptoms of AMV (A) compared with healthy plants (B) during collecting of samples.

Through the screening of AMV using ELISA, the highest infection rate 18.6% was found in Qassim followed by Riyadh where infection rate was 15.2% while the lowest infection rates were recorded in Hard and Hail, 8.3 and 10.4%, respectively, It has been observed that viral infection rate in potato crops planted by local seed tubers ranged from 9.5% to 29 % in the inspected fields. While the other potato crops planted by imported seed tubers "Class; Elite" had low infection or free from viral infection. Due to low price of local seed tubers comparing with seed tubers class "Elite", some potato growers divided the cultivated area into two parts, the first one is cultivated by local seed tubers where most of production is sold for local consumption and processing while they cultivated Elite tubers to produce seed tubers for plantation in the fall season, which explained that imported seeds that had been planted next to the local seeds were developed viral symptoms and ELISA test of these samples revealed that infection rate ranged from 6.5% up to 7.7 % in Qassim and Riyadh respectively (Table 1).

Table 1: Detection of Alfalfa Mosaic Virus in potato crop fields at Riyadh, Qassim, Hail and Hard regions using ELISA test.

Location	No of collected samples	Local seeds (LS)/ infected	Elite seeds (ES) / infected	Infected samples	Infection %
Riyadh	165	100/20	65/5	25	15.2
Qassim	370	200/58	170/11	69	18.6
Hail	450	350/42	100/5	47	10.4
Hard	230	200/19	30/0	19	8.3
Total	1215	850/139	365/21	160	13.2

In Saudi Arabia, AMV has been reported as one of the most widespread potato viruses (Al- Shahwan, 2003, Al-Saleh, 2014). This has increased the need for accurate, reliable

and cost-effective identification method of the virus.

RT-PCR was used to test the sensitivity of ELISA. Degenerate virus-specific primers AMVF and AMVR were used to amplify a 351 bp fragment from the coat protein gene (CP) of AMV. Obtained results revealed that RT-PCR gave the same results with all positive samples in ELISA. Some of samples that gave negative result with ELISA test showed positive results with RT-PCR. Data in Table (2) shows there are 6 out of 40 samples gave positive results with AMV, 3 samples from Hard, 2 samples from Hail and one samples from Riyadh. These results demonstrated that RT-PCR is more accurate and sensitive as it can detect viral infection at low concentration. This data was in agreement with previous findings by Spiegel and Martin, 1993 and Dietzgen, 2002; they reported that despite the high cost of RT-PCR but it is more sensitive 100 times than ELISA.

Table 2: Detection of ELISA tested - potato samples using RT-PCR

Region	*(+)AMV	*(-)AMV
Riyadh	10	1
Qassim	10	0
Hail	10	2
Hard	10	3
Total	40	6

Ten positive samples obtained from ELISA test (AMV (+)*) and ten negative samples obtained from ELISA test (AMV(-)*).

Based upon serological and RT-PCR analysis. The nucleotide (nt) sequences of the coat protein (CP) of the seven isolates of AMV were determined and deposited in the NCBI GenBank database to get an accession number (Table 3).

Table 3. The accession numbers of the sequenced fragments of AMV isolates in this study

Location	Accession number
Riyadh	KX458466
Hail	KX458467
Hail	KX458468
Hard	KX458469
Hard	KX458470
Qassim	KX458471
Qassim	KX458472

The obtained nt sequenced were aligned with the selectively retrieved sequences of related sequences using SDT software. The nt sequence comparison showed that the mutual nt sequence identity of the obtained sequences was 97% while, they showed highest nt sequence identity at 96% with the AMV isolates from Italy, Brazil and Australia (Figure 2). The phylogenetic dendrogram was constructed using Mega7 software. The phylogenetic analysis placed AMV isolates in this study with other isolates from Italy, Brazil and Australia in a separate well-supported clade (Figure 3).

In the phylogenetic, analysis the previously two AMV isolates from Saudi Arabia (Al-Saleh, *et al*., 2014) from Hail and Wadi Al-Dawasser were located in the same clade. High similarity levels between isolates from different regions indicated that all isolates may have the same origin and easily transmitted by infected seed tubers, mechanical transmission or aphid transmission (Bol, 2008).

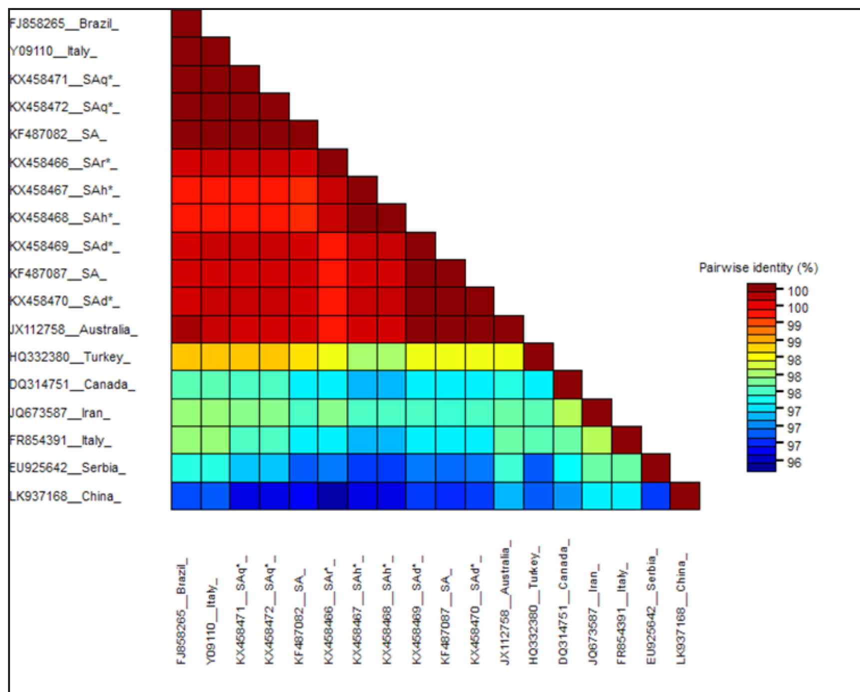

Figure 2. Pair-wise nucleotide sequence identities of the core CP sequences of AMV using SDT analysis. SA* = In this study. SAq = Qassim isolate, SAh = Hail isolate, SAr = Riyadh isolate, SAd = Hard isolate.

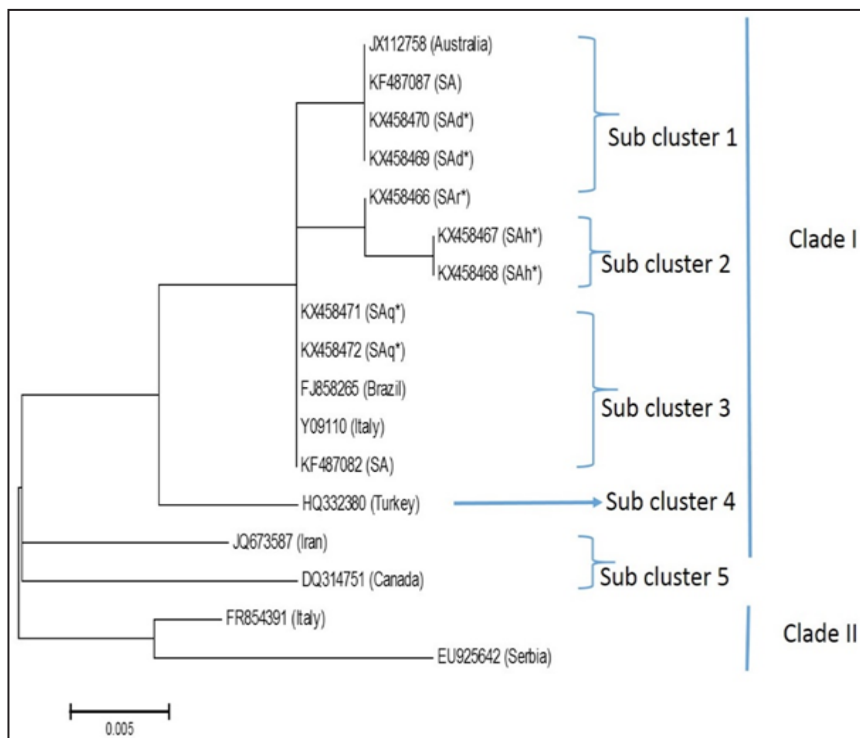

Figure 3. Phylogenetic tree based upon the core CP sequences to show the evolutionary relationships between the identified AMV isolates in this study and other isolates selected from GenBank database.

DNA non-radioactive Dot Blot Hybridization

Dot blot hybridization was developed for the detection of potato viruses from leaf tissues as an alternative to ELISA technique. The results in Figure 4 Showed that the dot blot of

PCR product and RNA extraction of AMV gave positive signals with the specific probe. The results also demonstrated that healthy plants used as a negative control didn’t result in any reaction.

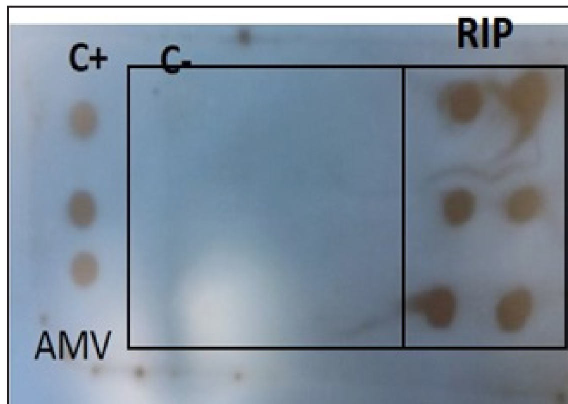

Figure 4. Dot blot hybridization with specific non-radioactive DNA labeled probe with Alk-Phos and Chemiluminescent Detection System for potato samples infected with AMV. C+ = positive control (RNA from infected samples), C- = RNA extracted from healthy plants as negative control and RIP= RNA extracted from infected plants.

On the other hand, the sensitivity of the dot blot hybridization was investigated with different concentrations of RT- PCR products (200 ng, 150 ng, 100ng, 10 ng, 1 ng, 100 pg, 50pg, 10 pg, and 1 pg,). The results showed the sensitively of the prepared probes was 50 pg, which indicates the capability of dot blot hybridization to detect potato viruses at a very low concentration (Figure 5).

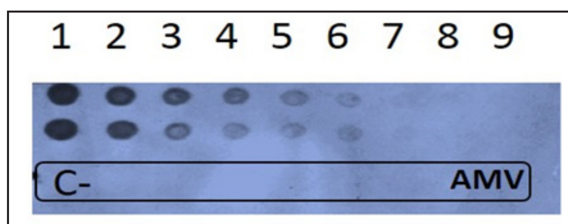

Figure 5. Dot blot hybridization with specific non-radioactive DNA labeled probe with Alk-Phos and chemiluminescent detection system for potato samples infected with AMV. C- = RNA extracted from healthy plants as negative control and different dilution of RT-PCR products of each virus. 1= 200 ng, 2 = 150ng, 3 = 100ng, 4 = 10 ng, 5 = 1 ng, 6 = 100 pg, 7 = 50pg, 8 = 10 pg, and 9 = 1 pg,).

Improvement of nucleic acid hybridization method for detection and diagnosis of potato viruses is essential to achieve the goal of developing virus-free potato material. Hybridization is a sensitive, economic and high throughput technique (Hopp, *et al*., 1991, AL-Saleh, et al., 2014). Thus, dot blot hybridization was developed for the detection of AMV from leaf tissues as an alternative to ELISA technique. DNA/RNA hybridization methods with specific and general probes have been reported for Potato-infecting viruses (Hopp, *et al*., 1991). Obtained results showed that the dot blot of PCR product and RNA extraction of AMV gave positive signals with the specific probe at a very low concentration. These findings were in agreement with AL-Saleh, *et al*., 2014, who reported that dot blot hybridization was more sensitive than ELISA and many negatively tested samples by ELISA gave positive results by dot blot hybridization so that this method was recommended for the detection of potato viruses (Peiró, *et al*., 2012). Some symptomatic plants showed negative results which suggested that these samples may be infected with other potato viruses, there are more than 40 viruses infect potato leads to appearance of different symptoms including disturbance in plant growth, distortion, green mottling, yellow blotches , stunt, leaf roll, mosaic, local or necrotic lesions that end with the death of plant (Salazar, 1996). The development of symptoms during infection is a complex process has not been understood especially when it interacts with the metabolism of the plant at various stages (Aranda and Maule, 1998). AMV is one of the most prevalent potato viruses during the last decade in Saudi Arabia. The wide spread of this virus through mechanical and/or aphid transmission can severely affect potato production as well as other susceptible economic crops. A timely detection of AMV in the field is highly needed for an accurate screening for infection.

These findings prompt us to use at least one of the three detection methods in this study

to be implemented in Saudi quarantine to import virus-free seed tubers and to check the widespread occurrence across the borders into Saudi Arabia to avoid an epidemiological occurrence of AMV.

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الكشف السيرولوجي والجزيئي عن فيروس تبرقش البرسيم في المناطق الرئيسية لزراعة البطاطس في المملكة العربية السعودية

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الملخص

تعد البطاطس من أهم محاصيل الخضر في المملكة العربية السعودية. فيروس تبرقش البرسيم من بين أكثر الفيروسات انتشارًا في البطاطس. تم إجراء عدة زيارات ميدانية في مناطق زراعة البطاطس الرئيسية (الرياض – القصيم – حائل – حرض) خلال فصلي الربيع والخريف من موسم زراعة البطاطس في عامي 2015 و 2016. تم الكشف عن وجود الفيروس في العينات باستخدام اختبارات الإليزا. تم عمل تفاعل النسخ العكسي وتفاعل البلمرة العكسي للتحقق من حساسية اختبار إليزا، بينما تم عمل طريقة التهجين كبديل لاختبار الإليزا. وقد لوحظت أعلى نسبة للعدوى بالفيروس بنسبة 18.6% في حقول القصيم، تليها الرياض بنسبة 15.2%، في حين سجلت أدنى معدلات الإصابة في كل من حقول حرض وحائل 8.3 و10.4% على التوالي. تم تحديد التتابعات النيكليوتيدة لسبع عزلات من فيروس تبرقش البرسيم التي تم الحصول عليها في هذه الدراسة، والتي تمت مقارنتها بالتتابعات المتاحة في قاعدة بيانات بنك الجينات. وأكدت التحليلات انخفاض التباين بين سلالات فيروس تبرقش البرسيم في هذه الدراسة، مما يعني أن جميع العزلات لهذا الفيروس قد تكون نشأت من المصدر نفسه. ونظراً لارتفاع معدل الإصابة بفيروس تبرقش البرسيم، فقد تتأثر المحاصيل الاقتصادية الأخرى القابلة للإصابة بهذا الفيروس نتيجة ارتفاع نسبة الإصابة في محصول البطاطس. وهذا يتطلب فحصًا دقيقًا لدرنات البطاطس المستخدمة كتقاوي لمنع انتشار الفيروس في المملكة العربية السعودية. وأشارت النتائج التي تم الحصول عليها إلى أن طريقة التهجين للحمض النووي واختبار الإليزا هي تقنيات مناسبة في الكشف الروتيني لفيروس تبرقش البرسيم في عدد كبير من العينات، في حين أن تفاعل النسخ العكسي– تفاعل البلمرة المتسلسل هو أكثر حساسية وأساسي للتوصيف الجزيئي لفيروس تبرقش البرسيم .

الكلمات المفتاحية: الإليزا، البطاطس، تفاعل البلمرة المتسلسل، تهجين الحمض النووى، الفاموفيرس، فيروس تبرقش البرسيم.