Use of Rapid Serological and Nucleic Acid- based Methods for Detecting the *Soybean mosaic virus*

A. Ahangaran[1*], Gh. Mosahebi Mohammadi[1], M. Koohi Habibi[1], S. Khezri[1], and N. Shahraeen[2]

ABSTRACT

Soybean mosaic virus (SMV) which belongs to the virus family Potyviridae, causes a disease in soybean that is present in soybean-growing areas of the world, and is widely distributed in northern Iran. Detection of SMV is very important for disease management. In the present study several serological and molecular (nucleic acid- based) methods of rapid virus detection were compared. Serological studies including DAS-ELISA, DAC-ELISA, TPIA and DIBA were optimized and compared to identify the virus by using a polyclonal antibody. Among the serological methods, TPIA and DIBA are simple and TPIA is rapidly and easily applicable in the field. However, TPIA was found to be preferable. TPIA is time-saving, not requiring conventional sap extraction and also nitrocellulose membranes used for printing can be used in the field and stored for a long time or transported to other laboratory to be processed. RT-PCR and Immunocapture RT-PCR (IC-RT-PCR) were performed as molecular methods for detecting SMV using a pair of primers designed to amplify a fragment in the coding region of the SMV coat protein. To extract total RNA for RT-PCR, two methods including RNAWIZ and phenol-chloroform were used. A part of the coat protein genome of SMV was converted to cDNA using a reverse transcription (RT) reaction. For IC-RT-PCR method, virus partial purification was carried out by solid-phase (0.2 ml microfuge tube) adsorbed polyclonal antibody, and then the RT reaction was carried out in the tube. In both methods cDNAs were amplified by PCR. Both methods amplified the expected fragment in virus-infected plants. Whereas RT-PCR requires total RNA extraction, ICRT- PCR do not have total RNA extraction problems. Our findings suggest that TPIA and IC- RT- PCR can be routinely used for SMV detection, with high efficiency.**

Keywords: DIBA, IC-RT-PCR, RT-PCR, SMV, Soybean, TPIA.

INTRODUCTION

Soybean mosaic virus (SMV) is one of the common viral diseases of soybean and is found throughout most soybean production areas in the world. SMV is a member of the large and economically important plant virus family, the Potyviridae [3, 12, 18]. Plants grown from SMV-infected soybean seeds provide the primary inoculum source. Secondary virus spread within and between fields is mediated by several aphid species, with transmission accruing in a non-persistent manner. SMV can significantly reduce soybean yields, and yield losses as high as 50% have been reported [1, 2, 6, 12]. The first report of this virus from Iran was in 1978 [7]. Then several studies were conducted on it, and reported throughout most soybean production areas in Iran [8, 10]. Detection of SMV is very important for disease management [13]. Serological methods are sensitive techniques and have been widely used in the detection of plant viruses [4, 11, 14]. The polymerase chain reaction (PCR) is an extremely sensitive and

[1] Department of Plant Protection, Faculty of Agriculture, Tehran University, Karaj, Islamic Republic of Iran.
* Corresponding author, e-mail: akbarahangaran@yahoo.com
[2] Department of Plant Virology, Plant Pest and Diseases Research Institute, Tehran, Islamic Republic of Iran.

specific technique, and reverse transcription-PCR (RT-PCR) has been used for the detection of RNA plant viruses [5, 19, 20, 23)] RT-PCR has also been used for the differentiation of different strains of the same virus [9, 16, 17]. IC-RT-PCR, a method combining immunocapture and PCR amplification, was performed for the detection of plant viruses and subviral pathogens [15].

In this study several serological and molecular (nucleic acid-based) methods including DAS-ELISA, DAC-ELISA, TPIA, DIBA, RT-PCR, and IC-RT-PCR were optimized and compared with each other for the rapid detection of SMV.

MATERIALS AND METHODS

Collection of SMV-infected Samples

Leaf samples were collected from field-grown soybeans in the North of Iran during June and July 2004, at the trifoliate stage. Leaf samples were kept in plastic bags in the refrigerator for further investigation.

DAS-ELISA

Double antibody sandwich-ELISA (DAS-ELISA) is used routinely to detect and diagnose viral diseases [4, 14]. This test was carried out using a polyclonal antibody (DSMZ, Germany). ELISA microplates were coated by incubating for 3 hours at 37ºC with the polyclonal antibody diluted (1:1000) in coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 in 1 L water pH 9.6). The plates were washed and incubated with extracts from healthy and infected plants, overnight at 4ºC. Extracts were prepared by grinding the leaf samples at a ratio of 1:10 (w/v) in extraction buffer (phosphate buffer saline, pH 7.4, 0.05% Tween 20, 2% PVP). The plates were washed and incubated for 3 hours at 37ºC with conjugate antibody diluted (1:1000) in conjugate buffer (PBST, 2% PVP, 0.2% egg albumin). After washing, the plates were incubated for 10 minutes at room temperature with 1µl ml^{-1} of p-nitrophenyl phosphate (pNPP) in substrate buffer (97 ml diethanolamine in 1 L water pH 9.8). Results were measured by an ELISA-reader (Statefax-2100) at 405 nm.

DAC-ELISA (direct antigen coating-ELISA)

In this test, microplates were coated and incubated overnight at 4ºC with extracts from leaf samples in coating buffer and then the plates were washed and incubated for 30 minutes at 37ºC with a blocking solution (2% skimmed milk in PBST). After washing, the plates were incubated for 3 hours at 37ºC with conjugate antibody, then washed and incubated for 10 minutes with substrate (pNPP) in substrate buffer. Results were measured by ELISA-reader (Statefax-2100) at 405 nm.

DIBA and TPIA Tests

Dot immunobinding assay (DIBA or Dot-Blot) and tissue print immunoassay (TPIA) were performed for SMV detection [11]. For both tests nitrocellulose membrane was used. These membranes were cut to an appropriate size and marked on a grid of 1×1 cm squares with a soft pencil. For TPIA, samples were rolled and cut with a scalpel and fresh sections of healthy and infected plant materials were imprinted onto each square. For DIBA the nitrocellulose membranes were immersed in PBS buffer for 15 minutes and were dried on filter paper for 15 minutes and, then, 10 µl of extracts (from healthy and infected plants) were dotted onto each square of nitrocellulose membranes. In both tests the membranes were blocked by incubation in blocking solution (2% skimmed milk in PBST) for 1 hour, and then incubated for 2 hours in a 1:1000 dilution of IgG-conjugate. After incubation, the membranes were washed three times with PBST for 15 minutes. Then the membranes were incubated for 10

minutes in substrate solution. NBT/BCIP tablets in distilled water or Fast red solution (containing 0.2 M Tris-HCl buffer and 2 mM $MgCl_2$, pH 7.8) were used as substrate solution. The processed membranes were washed, dried, and examined under a binocular microscope.

Total RNA Extraction from Plants

Two methods were used to extract total RNA from healthy and SMV-infected leaves. The first method was performed using a RNAWIZ solution (DSMZ, Germany). In this method 100 mg of leaf material was ground in liquid nitrogen and then mixed with 1 ml RNAwiz solution including 200 µl chloroform, and incubated for 5 minutes at room temperature. The homogenate was transferred to a 1.5 ml microfuge tube and centrifuged for 15 minutes at 13000g. The supernatant was collected and the total RNA was precipitated with isopropanol. In the second method total RNA was isolated by phenol/chloroform extraction [21, 22]. 50 mg samples of tissue were ground to a fine powder in liquid nitrogen with a small mortar and pestle. The samples were transferred to 1.5 ml microfuge tubes, and 300 µl of extraction buffer (containing 0.1 M Glycine, 0.1 M NaCl, 0,01 M EDTA, 1% sodium dodecyl sulfate(SDS), 1% mercaptoethanol, pH 9.0) was added and the samples were vortexed for 15 seconds. Then 250 µl of phenol and 250 µl of chloroform were added, the samples were vortexed for 15 seconds and centrifuged for 5 minutes in an Eppendorf microfuge, and water phase was transferred to a new tube. Total RNA was precipitated with ethanol. In both methods, total RNA was suspended in 50 µl of sterile H_2O.

RT-PCR (reverse transcription-polymerase chain reaction)

A pair of primers, -a forward (SMV-cpf: 5´-CAA GCA GCA AAG ATG TAA ATG-

3´) and a reverse (SMV-cpr: 5´-GTC CAT ATC TAG GCA TAT ACG-3´)- was designed for amplification of a conserved region (a fragment of 469-bp) in the coding region of SMV coat protein. For the RT reaction, 3 µl of total RNA was denatured for 2 minutes at 72ºC and immediately chilled on ice. The samples were added to 17 µl of reaction mixture (4 µl of 5x RT reaction buffer, 1 µl of 0.1 M dithiothreitol [DTT], 0.5µl of RNasin [40 u µl^{-1}], 1 µl of dNTPs [10 mM each], 1 µl of reverse primer [100 pmols µl^{-1}], 0.5µl of MMLV reverse transcriptase [200 u µl^{-1}], and 9 µl of H2O) and incubated for 1 hour at 42ºC. Five microliters of cDNA were added to 45 µl of PCR reaction mixture (5 µl of 10x PCR buffer, 2 µl of MgCl2 [50 mM], 1 µl of dNTPs [10 mM each], 1 µl of forward primer [100 pmols µl^{-1}], 1 µl of reverse primer [100 pmols µl^{-1}], 0.5 µl of Taq DNA polymerase [5 u µl^{-1}] and 34.5 µl H2O). The thermal cycling conditions were: 94ºC for 2 minutes, 35 cycle of 94ºC for 1 minutes, 55ºC for 1 minute, 72ºC for 2 minutes and a final extension at 72ºC for 10 minutes.

Seven micro liters of PCR products were analyzed by electrophoresis through 1% agarose gel followed by staining in ethidium bromide and the visualization of DNA bands using gel documentation.

IC- RT- PCR (Immunocapture- RT- PCR)

In this method, virus partial purification was carried out by solid-phase (0.2 ml tube) adsorbed polyclonal antibody, in such a manner that the PCR tube was coated by incubating for 3 hours at 37°C with polyclonal antibody diluted in coating buffer. The tubes were washed and incubated overnight at 4°C with extracts from healthy and infected plants. The tubes were washed and the RT reaction was then carried out in the tubes in the same way that was described previously. Then cDNAs were amplified by PCR. Amplification products were analyzed by electrophoresis in 1% agarose gel as previously mentioned.

RESULTS AND DISCUSSION

Results of DAS-ELISA and DAC-ELISA after incubation for 10 minutes with substrate (pNPP) solution were a change of yellow color in the wells that contained SMV or the positive control, which were not observed in the wells containing healthy samples or the negative control. The results were measured by ELISA-reader (Statefax-2100) at 405 nm.

In the DIBA and TPIA methods, after incubation of the membranes for 10 minutes in substrate solution NBT/BCIP, prints or blots of infected tissues turned into dark violet (Figures 1 and 2). In the substrate solution Fast red, prints or blots of infected tissues turned into red, whereas prints or blots of healthy ones did not show any color changes in either staining. Under a binocular microscope color changes were more clearly observed (Figure 3).

DIBA, as did ELISA, detected virus in infected plants and little equipment is needed. It is based on the use of membranes instead of plates, and an ELISA-reader is not needed. TPIA may not always achieve the same sensitivity as ELISA and DIBA but, as with DIBA it can be preformed with little equipments. In addition, tissue imprinting can provide data on virus localization within plant organs.

DIBA and TPIA are rapid and simple, and of course, the TPIA method is rapidly and easily applicable in the field. However, TPIA and DIBA have some advantages over the other methods. DIBA is time-saving in comparison to ELISA, while TPIA is very time-saving since there is no need for conventional sap extraction. In TPIA, nitrocellulose membranes used for printing can be used in the field and stored for a long time or transported to an other laboratory for processing.
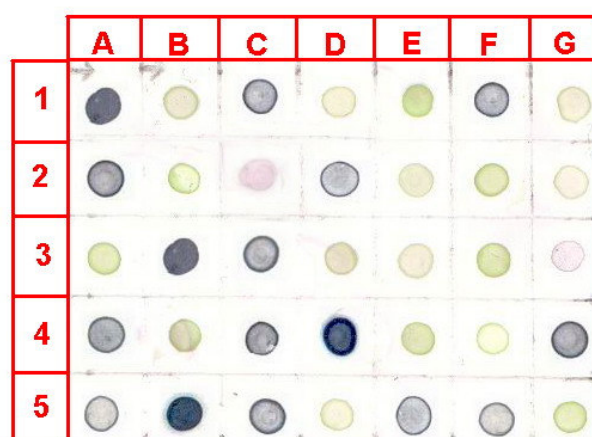

Figure 1. Results of DIBA with substrate solution NBT/BCIP, F5: Positive control, and F3: Negative control.
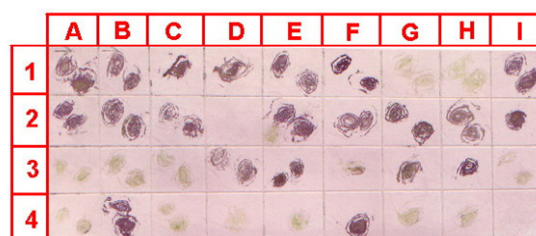

Figure 2. Results of TPIA with substrate solution NBT/BCIP, A1 and B1: Positive controls, and H4: Negative control**.**
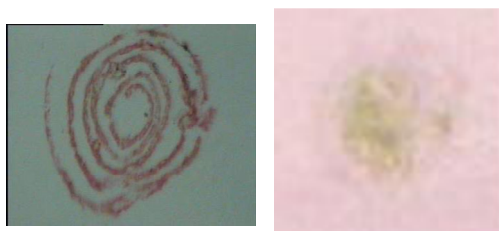
Figure 3. Results of TPIA under a binocular microscope (magnification 10X), Left: Print of SMV- infected sample, and Right: Print of healthy sample.

Both RNAWIZ and phenol/chloroform extraction methods yielded total RNA of appropriate quality for RT-PCR.

Both RT-PCR and IC-RT-PCR amplified a 469-bp fragment in virus-infected plants, while this band was not present in similarly treated healthy plants (Figure 4). Whereas RT-PCR required total RNA extraction or virus purification, the IC-RT-PCR method close not have any total RNA extraction problems.

In an other study we detected four strains (G1, G3, G4, and G5) of soybean mosaic virus in North Iran. All serological methods (using polyclonal antibody) and molecular methods (using universal primer) detected SMV strains in infected plants as mentioned.

Our findings suggest that TPIA and IC-RT-PCR can be used routinely and with high efficiency for SMV detection.
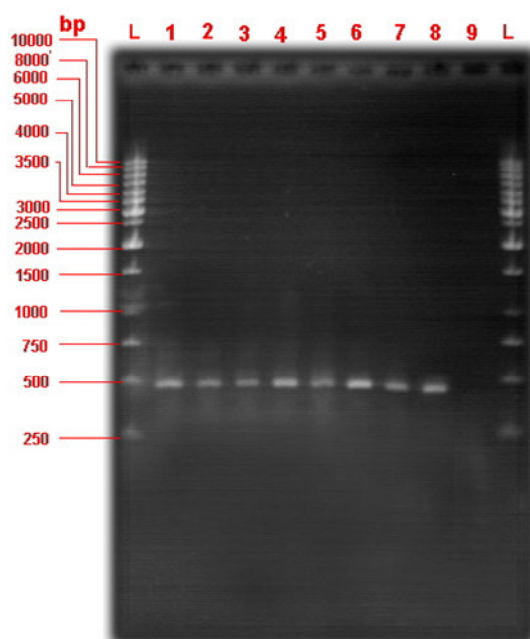

Figure 4. Results of RT-PCR and IC-RT-PCR using of SMV specific primers (cpf and cpr). L: Gene Ruler TM 1 kb DNA ladder; Lanes 1, 2, 4, 7: Results of RT-PCR, Lanes 3, 5, 6, 8: Results of IC- RT- PCR that both amplified a 469-bp fragment in virus-infected plants and Lane 9: result of IC- RT- PCR from healthy plant.

95

REFERENCES

1. Bowers, G. R. Jr. and Goodman, R. M. 1979. Soybean Mosaic Virus: Infection of Soybean Seed Parts and Seed Transmission. *Phytopathology*, **69**: 569- 572.
2. Bowers, G. R. Jr. and Goodman, R. M. 1991. Strain Specificity of Soybean Mosaic Virus Seed Transmission in Soybean. *Crop Sci.*, **31**: 1171-1174.
3. Cho, E. K., Chung, B. T. and Lee, S. H. 1977. Studies on Identification and Classification of Soybean Virus Diseases in Korea. II. Etiology of a Necrotic Disease of *Glycine max. Plant Dis. Rept.*, **61**: 313-317.
4. Clark, M. F. and Adams, A. N. 1977. Characteristic of the Microplate Method of Enzyme-linked Immunosorbent Assay for the Detection of Plant Viruses. *J. Gen. Virol.*, **34**: 475-483.
5. Colinet, D., Kummert, J., Lepoivre, P. and Semal, J. 1994. Identification of Distinct Potyviruses in Mixedly-infected Sweet Potato by the Polymerase Chain Reaction with Degenerate Primers. *Phytopathology*, **84**: 65- 69.
6. Dragoljob, D., Sutic, D., Ford, R. E. and Tosic, M. 1999. *Hand Book of Plant Virus Diseases*. CRC Press, Boca Raton. 553 PP.
7. Eskandari, F. 1978. Studies on soybean mosaic in Iran. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz*, **85**: 686- 688.
8. Farzadfar, Sh., Golnaraghi, A. R. and Pourrahim, R. 2002. *Plant Viruses of Iran*. Saman Co., Tehran, Iran. 203 PP.
9. Gillings, M., Broadbent, P., Indsto, J. and Lee, R. 1993. Characterization of Isolates and Strains of Citrus Tristeza Closterovirus Using Restriction Analysis of the Coat Protein Gene Amplified by the Polymerase Chain Reaction. *J. Virol. Methods*, **44:** 305-317.
10. Golnaraghi, A. R., Shahraeen, N., Pourrahim, R., Farzadfar, Sh. and Ghasemi, A. 2002. First Report of the Natural Occurrence of Eight Viruses Affecting Soybean in Iran. *Plant Pathol. New Dis. Rept.*, **51**: 794.
11. Hampton, R., Ball, E. and Boer, S. D. (Eds.). 1990. *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens*. APS Press. 389 PP.
12. Hill, J. H. 1999. Soybean Mosaic Virus. In: "*Compendium of Soybean Diseases*". 4th Ed., Hartman, G. L., Sinclair, J. B. and Rupe, J. C. (Eds.). The American Phytopathological Society, St. Paul, Minnesota. PP. 70-71.
13. Hill, J. H., Lucas, B. S., Benner, H. I., Tachibana, H., Hammond, R. B. and Pedigo, L. P. 1980. Factors Associated with the Epidemiology of Soybean Mosaic Vvirus in Iowa. *Phytopathology*, **70**: 536- 540.
14. Lister, R. M. 1978. Application of the Enzyme-linked Immunosorbent Assay for Detecting Viruses in Soybean Seed and Plant. *Phytopathology*, **68**: 1393-1400.
15. Nolasco, G., Blas, C. D., Torres, V. and Ponz, F. 1993. A Method Combining Immunocapture and PCR Amplification in a Microtiter Plate for the Detection of Plant Viruses and Subviral Pathogens. *J. Virol. Methods*, **45**: 201-218.
16. Omunyin, M. E., Hill, J. H. and Miller, W. A. 1996. Use of Unique RNA Sequence-specific Oligonucleotide Primers for RT-PCR to Detect and Differentiate Soybean Mosaic Virus Strains. *Plant Dis.*, **80**: 1170-1174.
17. Rizos, H., Gunn, L. V., Pares, R. D. and Gillings, M. R. 1992. Differentiation of Cucumber Mosaic Virus Isolates Using Polymerase Chain Reaction. *J. Gen. Virol.*, **73**: 2099- 2103.
18. Shukla, D. D., Ward, C. W. and Brunt, A. w. 1994. *The Potyviridae*. CAB International, Wallingford, UK.
19. Smith, G. R. and Van de Velde, R. 1994. Detection of Sugarcane Mosaic Virus and Fiji Disease Virus in Diseased Sugarcane using the Polymerase Chain Reaction. *Plant Dis.*, **78**: 557- 561.
20. Thomson, K. G., Dietzgen, R. G., Gibbs, A. J., Tang, Y. C., Liesack, W., Teakle, D. S. and Stackebrandt, E. 1995. Identification of Zucchini Yellow Mosaic Potyvirus by RT-PCR and Analysis of Sequence Variability. *J. Virol. Methods*, **55**: 83- 96.
21. Wadsworth, G. J., Redibaugh, M. G. and Scandalios, J. G. 1988. A Procedure for the Small-scale Isolation of Plant RNA Suitable

for RNA Blot Analysis. *Anal. Biochem.*, **172**: 279-283.

22. Wang, R. Y. and Ghabrial, S. A. 2002. Effect of Aphid Behavior on Efficiency of Transmission of Soybean Mosaic Virus by the Soybean-colonizing Aphid, *Aphis glycines. Plant Dis.*, **86**: 1260-1264.

23. Zerbini, F. M., Koike, S. T. and Gilbertson, R. L. 1995. Biological and Molecular Characterization of Lettuce Mosaic Potyvirus Isolates from the Salinas Valley of California. *Phytopathology,* **85**: 746- 752.

استفاده از سریعترین روشهای سرولوژیکی و مولکولی برای شناسایی ویروس موزائیک سویا (SMV)

ا. آهنگران، غ. مصاحبی محمدی، م. کوهی حبیبی، ص. خضری و ن. شهرآیین

چکیده

ویروس موزائیک سویا (SMV) از خانواده Potyviridae در تمام مناطق کشت سویا در دنیا وجـود دارد. ایـن ویروس به طور وسیعی در مناطق سویا کاری شمال ایران گسترده شـده اسـت. شناسایی ایـن ویـروس در مـدیریت بیماری از اهمیت ویژهای برخوردار است. در این تحقیق چندین روش شناسایی سرولوژیکی و مولکـولی در جهـت تعیین سریعترین روش شناسایی این ویـروس بـا هـم مقایسـه شـدند. روشهای سـرولوژیکی شـامل DAS-ELISA، DAC-ELISA، TPIA و DIBA با کاربرد آنتی بادی چندهمسانه، جهت شناسایی ویروس استفاده و باهم مقایسه شدند. در میان روشهای فوق، دو روش TPIA و DIBA ساده و سریع بـوده ولی روش TPIA سـریعتر اسـت و بـه آسانی میتوان آن را در مزرعه استفاده کرد. از مزایای دیگر TPIA می توان صرفه جویی در زمان را نام بـرد چـون نیازی به انجام عصاره گیری از نمونه ها نیست و همچنین کاغذ نیتروسلولزی را که در ایـن روش اسـتفاده مـی شـود می‌توان در مزرعه با نمونه‌های مورد نظر اثرگذاری (پرینت) کرده و برای مدت زمان طولانی تـا انجـام بقیـه پروسـه نگهداری کرد و یا به آزمایشگاه دیگری ارسال نمود. RT-PCR و ایمیونوکپچر (IC- RT- PCR)،RT- PCR بـه عنوان روشهای مولکولی شناسایی استفاده شدند. در هر دو روش از یک جفت آغازگری اسـتفاده شـد کـه موجـب تکثیر قطعه‌ای در ناحیه ژنوم پروتئین پوششی SMV می‌شوند. برای انجام RT- PCR ابتدا RNA کل بهوسیله یکی از دو روش، استفاده از بافر RNAWIZ یا فنل – کلروفرم اسـتخراج شـد. سپس بخشی از ژنـوم پـروتئین پوشـشی SMV با استفاده از آزمون نسخه‌برداری معکوس (RT) به DNA مکمل (cDNA) تبدیل شد. در مورد روش IC- RT-PCR، خالص‌سازی نسبی ویروس بـا اسـتفاده از آنتی‌بـادی چندهمـسانه‌ای کـه درون تیـوپ 0.2ml پوشـش گذاری شده بود، انجام شد. به دنبال آن واکنش RT درون تیوپ صورت گرفت. در هر دو روش فـوق cDNA هـا بهوسیله واکنش زنجیره‌ای پلیمراز تکثیر شدند. هر دو روش قطعه مورد نظر را در گیاهـان آلـوده بـه ویـروس تکثیر کردند. روش RT-PCR نیاز به استخراج RNA کل دارد ولی IC-RT-PCR مشکلات مربوط به اسـتخراج RNA را ندارد. نتایج نشان داد که دو روش TPIA و IC-RT-PCR می تواننـد بـه طـور معمـول بـا کـارایی بـالایی بـرای شناسایی SMV استفاده شوند.