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(54) Title: DIAGNOSTIC KIT FOR DETECTION OF CARNATION MOTTLE VIRUS

(57) Abstract: The present invention relates to primers useful for detection of Carnation mottle virus in plants. More particularly, this invention relates to a method for detection of Carnation mottle virus in plants by using the said primers useful for detection of Carnation mottle virus in plants. The present invention also relates to a diagnostic kit useful for detection of coat protein of Carnation mottle virus in plants.



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DIAGNOSTIC KIT FOR DETECTION OF *CARNATION MOTTLE VIRUS***Field of invention:**

The present invention relates to primers useful for detection of *Carnation mottle virus* in plants. More particularly, this invention relates to a method for detection of *Carnation mottle virus* in plants by using the said primers useful for detection of *Carnation mottle virus* in plants. The present invention also relates to a diagnostic kit useful for detection of coat protein of *Carnation mottle virus* in plants.

Background of invention:

Carnation mottle virus (CarMV), a type member of Carmo virus group, has a wide host range and infects members of about nine families. It infects number of floriculture crops (carnations, begonia, periwinkle, saponaria, gomphrena, Petunia, Celosia, Daphne). CarMV is the most widespread virus in Carnations and more than 90% of tested commercial stocks of many cultivars were found to have almost 100% incidence of infection when tested by enzyme linked immunosorbent assay (ELISA) at Institute of Himalayan Bioresource Technology (IHBT) Palampur. Specific gene sequences of CarMV were detected in total RNA extracts of plants by initially transcribing viral RNA into cDNA and then amplifying by PCR. The different primer pairs were designed (EMBL Nucleotide Sequence Accession Numbers: AJ585097, AJ585096, AJ585095, AJ585094, AJ566194, AJ566193, AJ566192 and AJ566191) based on the available gene sequences and were used successfully for identification and characterization of an Asian strain of CarMV. Coat protein and both the movement protein (p7 and p9) genes were amplified, cloned and sequenced. The sequences thus obtained were submitted to the nucleotide database (Raikhy, G., Hallan, V., Kulshrestha, S., Ram, R. and Zaidi, A. A. 2005. Multiplex PCR and genome analysis of *Carnation mottle virus* Indian isolate; EMBL Nucleotide Sequence Accession Numbers: AJ844587, AJ844586, AJ844585, AJ844584, AJ844553, AJ844552, AJ844549, AJ811998, AJ584843, AJ584842, AJ549954). Complete genome of Asian strain of CarMV has been cloned and sequenced (EMBL Nucleotide Sequence Accession Number AJ811998). Commercially available antibodies (Bio Rad, USA) against CarMV coupled with RT-PCR have been successfully used for CarMV coat protein gene detection through Immunocapture RT-PCR (Singh, H., Hallan, V., Raikhy, G., Kulshrestha, S., Ram, R., Garg, I. D. and Zaidi, A. A. 2005.

Characterization of an Indian isolate of *Carnation mottle virus* infecting Carnations. Current Science, In Press).

Carnation is one of the most important commercial ornamental crops of the world. Presence of magnificent varieties with vivid colours has attracted its cultivation
5 throughout the world. It ranks among top five cut flower crops (Hudson, D. and Griffin, E. 2004. Market potential for "Mississippi Grown" cut flowers. Bulletin 1140: Mississippi Grown Cut Flowers, 1-9).

Carnation is being propagated both through seeds and vegetatively. Viral infection tends to be carried from one generation to another by cuttings. Quality of germplasm
10 and minimizing the infection of viruses to different cultivars, proper diagnosis and control for viral diseases are not only highly desirable but also essential for improving crop productivity.

The identity of the virus in carnation was first established as CarMV from UK (Kassanis, B. 1955. Some properties of four viruses isolated from carnation plants.
15 Annals of Applied Biology. 43: 103-116). CarMV although causes mild mottling on carnation leaves but it renders plants susceptible for subsequent viral and fungal infections. High titer of virus causes reduction in flower quality and quantity (Hollings, M. and Stone, O. M. 1964. Investigations of carnation viruses I *Carnation mottle*. Annals of Applied Biology. 53: 103-118). CarMV isolates separated in time and space
20 show a very high genetic stability. They can be segregated in two main groups namely PK and AN based on the covariation of the amino acid sequence for coat protein between $\overrightarrow{\text{Pro164}}$ (located at the S domain) and $\overrightarrow{\text{Lys331}}$ (within the P domain) by which a change $\text{Pro}^{164} \rightarrow \text{Ala}$ correlated with a change $\text{Lys}^{331} \rightarrow \text{Asn}$ (Canizares, M. C., Marcos, J. F. and Pallas, V. 2001. Molecular variability of twenty-one geographically
25 distinct isolates of *Carnation mottle virus* (CarMV) and phylogenetic relationships within the Tombusviridae family. Archives of Virology. 146: 2039-2051).

Traditional diagnosis of plant viruses requires bioassaying through an indicator plant, symptom observation, host range determination and virus particle morphology and vector relations. However progress in molecular biology, biochemistry and
30 immunology has led to the development of many new accurate, rapid and less labour intensive methods of virus detection and determination. There are various diagnostic techniques available in the field of virology like precipitation tests, agglutination tests, fluorescent antibody test, enzyme-linked immunosorbant assay, dot immunobinding

- assay, tissue blotting assay, Western blotting, nucleic acid hybridization with radio labelled and non radio labelled probes and polymerase chain reaction based detection. Agar gel double diffusion test has been successfully used for the detection of CarMV from carnations. Agar gel test was found to be the most reliable. The agar gel double
- 5 diffusion test is comparable with the bioassay on *Chenopodium quinoa*. (Oertel, C., 1977. On the reliability of routine tests on virus diseases in ornamental plants. Archiv fur Phytopathologie und Pflanzenschutz 13: 163-176; Martinez, L. G., La Rotta, M. C.F., Huertas, C. A., Luque, C. S. 1974. *Carnation mottle virus* in Colombia. Fitopatologia. 9: 2, 105-109).
- 10 Enzyme linked immunosorbant assay (ELISA) and other forms of ELISA have been extensively used for the detection of CarMV from carnations. Using ELISA CarMV has been reported from the different parts of the world (Lisa, V. 1995. Carnation. In: Loebenstein, G., Lawson, R. H., Brunt, A. A. (Eds): *Virus and virus like Diseases of Bulb and Flower Crops*. John Wiley & Sons, UK, pp. 385-395). ELISA was used
- 15 routinely for the indexing of CarMV (Severin, C. and Gonzalez, M. 1996. Survey of *Carnation mottle virus* (CMV) and *Carnation latent virus* (CLV) in Carnation in southern Sante Fe province, Argentina. Fitopatologia 31: 1, 84-86). This test demonstrated that CarMV is the most widespread virus among commercial Carnations. A double antibody sandwich ELISA procedure was developed. Under optimum
- 20 reaction conditions 5 ng of purified virus or less could be detected. The method was used for testing meristems from different Carnation cultivars at initial stages of growth (Eskenazy, M., Jankulova, M., Bakardjieva, N., Bachvarova, R. and Chavdarov, I. 1983. Quantification of *Carnation mottle virus* (CarMV) by ELISA. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz. 90(1): 73-82).
- 25 A sequential batch testing procedure can be used in conjunction with ELISA to estimate levels of virus incidence in cut flower carnations. Plant Improvement Program has been instrumental in reducing the incidence of *Carnation mottle virus*. Growers who purchased cuttings from specialist propagators had lower levels of virus than propagators who implemented little or no quality control (Rodoni, B. C., Hepworth, G.
- 30 Richardson, C. and Moran, J. R. 1994. The use of a sequential batch testing procedure and ELISA to determine the incidence of five viruses in Victorian cut flower Sim Carnations. Australian-Journal-of-Agricultural-Research. 45(1): 223-230).

Radial immunodiffusion, immuno-enzyme analysis (ELISA) and agglutination of bacterio-viruses (ABV) were all successfully used in large-scale diagnosis of the virus but when different techniques were compared ELISA and ABV were found to be the most sensitive (Odinets, A. G., Kulinich, A. V., Chirkov, S. N., Kozitskii, Yu N. and
5 Atabekov, I. G. 1983. Sel'skokhozyaistvennaya-Biologiya. 1983, No.5, 37-41
Comparative testing of immunological methods for mass diagnosis of *Carnation mottle virus*). Serological latex and ELISA tests when compared, latter was found to be more sensitive but the former was recommended being simpler and cheaper (Polak, J. 1983. Comparison of the sensitivity of the serological latex and ELISA tests. Biologia-
10 Plantarum. 25(1): 33-35).

The high sensitivity of the urease form of ELISA system allowed the use of pooled samples for CarMV detection with greater sensitivity than the phosphatase system. The urease-ELISA was used for a field assessment of the effectiveness of a Plant Improvement Programme (PIP) for carnations for the CarMV control (Moran, J. R.,
15 Wilson, J. M., Garrett, R. G. and Smith, P. R. 1985. ELISA indexing of commercial Carnations for *Carnation mottle virus* using a urease-antibody conjugate. Plant-Pathology 34(4) 467-471).

ELISA was routinely used for the CarMV detection in meristem. More than half of plants grown from infected cuttings were infected by *Carnation mottle virus*. The
20 isolation of meristems from healthy cuttings, checked by ELISA, markedly decreased infection (Zhola, I. and Ushkaura, A. 1983. The use of ELISA test for the detection of mottle virus in meristem Carnation. Trudy-Latviiskoi-Sel'skokhozyaistvennoi-Akademii. No.207, 36-38).

On comparison of Indirect ELISA when compared with double-antibody sandwich
25 ELISA for the detection of *Carnation mottle virus* in carnations, it was observed that Indirect ELISA was more sensitive for purified virus and also for detecting virus in crude extracts. The main advantage was its simplicity for routine virus detection. Interference and enhancement phenomena were observed when indirect ELISA was used with crude plant extracts (Lommel, S. A., McCain, A. H. and Morris, T. J. 1982.
30 Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses. Phytopathology 72(8): 1018-1022).

A survey in USA showed CarMV was ubiquitous and significant temporal spread was observed. An effective indirect ELISA indexing programme and appropriate sanitation

measures should effectively control these viruses in commercial plantings (Lommel, S. A., Stenger, D. C. and Morris, T. J. 1983. Evaluation of virus diseases of commercial Carnations in California. *Acta-Horticulturae*. No.141, 79-88).

A direct blotting on nitro-cellulose membrane by infected tissues was developed in order to make dot-ELISA more simple and practical. Using this technique, *Carnation mottle carmovirus* (CarMV) was readily detected from leaves, stems and incompletely opened flowers of carnations. Comparison tests show that the direct tissue blotting immunoassay was as sensitive as the commonly used ELISA and dot-ELISA for the detection of CarMV (Zhang, A. P. 1999. Direct tissue blotting ELISA for detection of Carnation virus in Carnation. *Acta-Agriculturae-Shanghai*. 15(2) 84-86).

A non-isotopic molecular hybridization (NIMH) technique for the detection of Carnation viruses (*Carnation mottle carmovirus* (CarMV), *Carnation etched ring caulimovirus*, *Carnation vein mottle potyvirus*, *Carnation ringspot dianthovirus*, *Carnation latent carlavirus* and *Carnation yellow fleck virus* [*Carnation necrotic fleck closterovirus*]) in carnations was used with great success. This technique can be divided into 3 steps: the preparation of samples and their application onto a nylon membrane; the incubation of the membranes with a hybridization solution containing the appropriate probe; and following hybridization and blocking steps, the detection of bound probes by high affinity antibody Fab-fragments coupled to alkaline phosphatase. Two alternative detection methods can be applied, the more sensitive chemilluminiscent method or the simpler colorimetric one (Pallas, V., Sanchez Navarro, J. A., Canizares, M. C. and Cano, E. A. 1999. A new test for detecting Carnation viruses. *FloraCulture International*. 9(5): 32-34).

Similar to ELISA, Immunosorbant Electron Microscopy (ISEM) also revealed easy detection of CarMV from carnations (Song, R. L., Chen, H., Wei, X. X. and Wu, R. J. 1999. Identification and elimination of *Carnation mottle virus* in Fujian of China. *Journal of Fujian Academy of Agricultural Sciences*. 14: 3, 26-31; Danesh, D. and Gavgani, A. M. 1979. Occurrence of *Carnation mottle virus* on carnation in Iran. *Plant-Disease-Reporter*. 63(11): 940-944).

Another mean of producing purified coat protein is cloning and expression of coat protein gene in *E. coli* followed by purification of coat protein (Zhang, A. P., Yue, Y., Ye, R. and Yu, S. Q. 1999. Cloning and expression of coat protein gene of *Carnation*

mottle virus Shanghai isolate in *E. coli*. Journal of Fudan University (Natural Science Edition). 38(5): 489-492).

Since last decade, RT-PCR has been widely used with varying degree of modification for detection of viral genome in infected plants. The lowest detected RNA content by RT-PCR was 5 ng, proved that the detection of CarMV by RT-PCR was feasible (Kong, B. H., Cai, H., Liu, J.Y. and Chen, H. R. 2002. Identification and detection of *Carnation mottle virus* on Carnation by RT-PCR. Plant-Protection. 2002, 28(1): 22-28). Complete nucleotide sequence of CarMV has been worked out and found to be ~4000nucleotide in length and functions of different ORFs have been worked out (Canizares, M. C., Marcos, J. F. and Pallas, V. 2001. Molecular variability of twenty-one geographically distinct isolates of *Carnation mottle virus* (CarMV) and phylogenetic relationships within the *Tombusviridae* family. Archives of Virology. 146, 2039-2051).

Northern hybridization has been used successfully for CarMV detection. DNA complementary to CarMV RNA synthesized using the reverse transcriptase of avian myeloblastosis virus as enzyme, and an hydrolysate of calf thymus DNA as primer. The DNA transcript hybridized to at least 92% of the CarMV-RNA sequences. CarMV-cDNA hybridized completely to a crude RNA preparation from CarMV-infected carnation leaves, but not to a comparable RNA preparation from healthy leaves, thus permitting the use of CarMV-cDNA to detect and to quantify the CarMV (Kummert, J. 1980. Synthesis and characterization of DNA complementary to *Carnation mottle virus* RNA. Virology. 105(1): 35-40; Carrington, J. C. and Morris, T. J. 1984. Complementary DNA cloning and analysis of *Carnation mottle virus* RNA. Virology. 139(1) 22-31).

Combining of PCR with molecular hybridization can detect even picogram quantities of virus and this combination is 4-5 order superior to direct molecular hybridization (Sanchez-Navarro, J. A., Cano, E. A. and Pallas, V. 1996. Non-radioactive molecular hybridization detection of *Carnation mottle virus* in infected Carnations and its comparison to serological and biological techniques. Plant Pathology. 45(2): 375-382).

Thus DAS-ELISA, DTBIA, ISEM, RT-PCR and IC-RT-PCR are the suitable techniques for the detection of CarMV infecting carnations. RT-PCR and nucleic acid hybridization are the sensitive tools to detect the virus but they require sophisticated and costly instruments. So, until now ELISA/DTBIA have been extensively used for

diagnosis of virus infecting carnations and other crops also because these are quick methods, easy to perform, can be used even in field conditions and are cost effective also. So, these can be exploited in the form of diagnostic kits.

5 **Objectives of the invention:**

The main objective of the present is to provide primers useful for detection of *Carnation mottle virus* in plants.

Another objective of the present invention is to provide a method for detection of *Carnation mottle virus* in plants by using designed primers useful for detection of
10 *Carnation mottle virus*.

Still another object of the present invention is to provide a diagnostic kit useful for detection of coat protein of *Carnation mottle virus* in plants.

Summary of the invention:

15 The present invention related to a method for detection of *Carnation mottle virus* in plants using designed primers of

Sequence ID 1: upstream primer GGGGATCCGTATGGAAAATAAAGGAGA

Sequence ID 2: downstream primer

AACTGCAGTCACATCCTATAAACAACCCATT;

20 It also relates to a diagnostic kit useful for detection of coat protein of *Carnation mottle virus* in plants comprising:

- a) polyclonal antibodies against *Carnation mottle virus* coat protein in plants
- b) conjugate labeled with alkaline phosphatase;
- c) coating buffer;
- 25 d) extraction buffer;
- e) ECI buffer;
- f) PNP buffer.

Detailed description of the invention:

30 Accordingly the present invention relates to a set of primers represented by SEQ ID NO: 1 and SEQ ID NO:2 useful in detecting *Carnation mottle virus*.

Present invention also relates to a method for detection of *Carnation mottle virus* in test sample, said method comprises the steps:

amplifying coat protein gene of CarMV by using primers as of claim 1;

expressing the said amplified gene in transformed *E. coli* and obtaining CarMV coat protein;

obtaining Polyclonal antibodies against said coat protein; and

detecting CarMV in a test sample using conventional detection methods.

- 5 In the present invention the expression of coat protein is carried out in the presence of an inducer in a concentration in the range of 0.5 to 1.0mM at 28°C for a period in the range of 3 to 4 hrs, wherein the inducer is IPTG.

In another embodiment of the invention, the polyclonal antibodies are obtained by using immunization techniques, wherein immunization is carried out in an animal
10 wherein the animal is rabbit.

In another embodiment of the invention, immunization is carried out intramuscularly, subcutaneously or intravenously.

In one embodiment of the invention, immunization is carried out with coat protein optionally with an adjuvant.

- 15 In another embodiment of the invention, the adjuvant is selected from the group comprising Freund's complete adjuvant, Freund's incomplete adjuvant, Montanide adjuvant, Ribi's adjuvant, Hunter's TiterMax, Gerbu adjuvant.

In an embodiment of the invention, the ratio of coat protein and adjuvant is 1:1.

In another embodiment of the invention, the polyclonal antibodies are obtained after 14
20 to 15 days of immunization.

In an embodiment of the invention, the coat protein is purified coat protein.

In one embodiment of the invention, the detection method is selected from the group comprising ELISA, indirect sandwich ELISA, DAS-ELISA, immunodiffusion assay, precipitation test.

- 25 In another embodiment of the invention, the detection method is preferably DAS-ELISA, wherein in DAS-ELISA the microtitre plates are coated with polyclonal antibodies, diluted in a coated buffer in a ratio ranging from 1:500 to 1:1000 followed by 4-5 times washing with PBS-T.

In an embodiment of the invention, the test sample is plant sample.

- 30 In another embodiment of the invention, the plant sample is macerated plant sample, wherein the test samples are prepared in microtitre plates by macerating infected leaf tissue from plant with extraction buffer followed by dilution from 1x – 1/60x of the original antigen.

In one embodiment of the invention, in DSA-ELISA the antibody conjugated or labeled with alkaline phosphatase in ECI buffer is added in the ratio ranging between 1:500 to 1:1000 for a period of 2 hrs at 37° C followed by washing with PBS-T and adding 100µl of about 1 mg/ml p-nitrophenyl phosphate solution in PNP buffer.

- 5 In another embodiment of the invention, the reaction is terminated by adding about 50µl of about 3M NaOH after 15-20 min to obtain yellow colour and the absorbance of the colored product is measured at 405nm to detect the *Carnation mottle virus* (CarMV).

- Further the present invention also provides a diagnostic kit useful for detection of coat protein of Carnation mottle virus (CarMV) comprising:
- 10 polyclonal antibodies against Carnation mottle virus coat protein in plants;
conjugate labeled with alkaline phosphatase;
coating buffer;
extraction buffer;
- 15 ECI buffer;
PNP buffer; and
instruction manual

- For the development of a diagnostic kit for the detection of CarMV, coat protein gene was amplified using the especially designed primers having restriction enzyme sites compatible for directional and inframe cloning in pGex-2Tk vector. PCR product was
- 20 gel purified, ligated in pGex-2Tk vector and transformed in BL21 competent cells.

- Expression of coat protein gene was performed in transformed *E. coli* cells grown in YT medium. Expression conditions were standardized against IPTG concentration, time of incubation, growth conditions, aeration and method of cell disruption. Growing
- 25 the transformed *E. coli* having volume 5 times than the total YT volume, induction using 1mM IPTG final concentration at 0.5 OD₆₀₀ for 3 hrs, along with disruption of cell using both lysozyme (100mg/ml) and sonication (pulser on for 9.5 sec. and pulser off for 4 sec.) gave the maximum expressed recombinant coat protein yield in soluble form.

- 30 The antisera against the recombinant CarMV coat protein were prepared in rabbit by both subcutaneous and intramuscular injections of 100 µg of the purified coat protein in three-week intervals. The purified coat protein was emulsified with an equal volume of complete Freund's adjuvant for the first injection and with the Freund's incomplete

adjuvant for subsequent two injections (Meenu Katoch, A. A. Zaidi and Raja Ram. 2002. Development of diagnostic kit for the detection of *Bean yellow mosaic virus*. Patent file no 76/NF/2002). The rabbits were bled two weeks after the last injection. The serum fraction was collected and stored at -20°C until used. The conjugate was developed by tagging the antibodies with alkaline phosphatase.

Accordingly the present invention provides primers useful for the detection of *Carnation mottle virus* (CarMV) in plants, comprising the following sequence:

Sequence ID 1: upstream primer GGGGATCCGTATGGAAAATAAAGGAGA

Sequence ID 2: downstream primer

AACTGCAGTCACATCCTATAAACAACCCATT;

Further, the present invention also provides a method for detection of *Carnation mottle virus* (CarMV) in plants, wherein the said method comprising the steps of:

a) providing a purified coat protein of CarMV by using designed primers of

Sequence ID 1: upstream primer GGGGATCCGTATGGAAAATAAAGGAGA

Sequence ID 2: downstream primer

AACTGCAGTCACATCCTATAAACAACCCATT;

b) preparing polyclonal antibodies against CarMV coat protein obtained from step (a);

c) performing direct antibody sandwich enzyme linked immunosorbent assay (DAS ELISA) for detection of CarMV.

In an embodiment of the present invention, the complete coat protein of CarMV is amplified using designed primers having a

Sequence ID 1: upstream primer GGGGATCCGTATGGAAAATAAAGGAGA

Sequence ID 2: downstream primer

AACTGCAGTCACATCCTATAAACAACCCATT;

In another embodiment of the invention, the primers were used successfully for the amplification of complete coat protein gene of *Carnation mottle virus*. An amplicon of ~1040 bp was obtained through RT-PCR using the above mentioned self designed primers.

In another embodiment of the present invention, the complete coat protein of CarMV comprising sequence ID having number CAH59633

MENKGEKIAMNPTVQTLAQKGDKLAVKLVTRGWASLSTNQKRRRAEMLAGYT
PAILAFTPRRPRMTNPPPRTSRNSPGQAGKSMTMSKTELLSTVKGTTGVIPSFED

WVVSPRNVAVFPQLSLLATNFNKYRITALTVKYSPACSFETNGRVALGFNDDA
 SDTPPTTKVGFYDLGKHVETAAQTAKDLVIPVDGKTRFIRDSASDDAKLVDFG
 RIVLSTYGFDKADTVVGELFIQYTIVLSDPKTAKISQASNDKVS DGPTYVVPVS
 NGNELQLRVVAAGKWCIIVRGTVEGGFTKPTLVGPGISGDVDYESARPIAICEL

- 5 VTQMEGQILKITKTAEQLQWVVYRM is cloned in pGex-2TK followed by transformation using E. coli strain BL 21.

Further In another embodiment of the present invention, the optimal expression of CarMV coat protein is checked with 0.5-1.0mM IPTG concentration at about temperature 28 degree C for 3.5-4.0 h.

- 10 Still in an embodiment of the present invention, obtained coat protein of CarMV is sequenced by known sequencing methods.

Still in an embodiment of the present invention, the purification of CarMV coat protein is carried out by the known method.

- Still an embodiment of the present invention, the immunization in rabbits are carried
 15 out three times with purified coat protein of CarMV and Freund's complete adjuvant in the ratio of 1:1 at weekly intervals.

Still in another embodiment of the present invention, the route for immunization may be intramuscularly, subcutaneously or intravenously.

- Yet in another embodiment of the present invention, the rabbits are bled after 14 to 15
 20 days to obtain polyclonal antibodies against CarMV coat protein.

Yet in another embodiment of the present invention, the polyclonal antibodies against CarMV coat protein are purified from the serum by known methods.

- Yet another embodiment of the present invention, the microtitre plates are coated with polyclonal antibodies diluting in a coating buffer in a ratio ranges from 1:500-1:1000
 25 followed by 4-5 times washing with PBS-T.

Yet another embodiment of the present invention, the test samples are prepared in microtitre plates by maceratin infected leaf tissue from plant with extraction buffer followed by dilution from 1x-1/60x of the original antigen.

- Yet in another embodiment of the present invention, the microtitre plate is incubated
 30 overnight at about 37°C followed by washing to allow coating of antigen in the wells.

Yet in another embodiment of the present invention, the antibody conjugate in ECI buffer is added in the ratio ranges between 1:500 to 1:1000 for a period of 2hrs at 37°C followed by washing with PBS-T.

Yet in another embodiment of the present invention, about 100µl of about 1mg/ml p-nitrophenyl phosphate solution in PNP buffer is added in the mix.

Yet in another embodiment of the present invention, the reaction is terminated by adding about 50µl of about 3M NaOH after 15-20 min to obtain yellow color product.

- 5 Yet in another embodiment of the present invention, the color product is antigen and antibody conjugate.

Yet in another embodiment of the present invention, the absorbance of the colored product is measured at 405nm for detection of Carnation mottle virus (CarMV).

- 10 Further the present invention also provides a diagnostic kit useful for detection of coat protein of Carnation mottle virus (CarMV) comprising:

- a) polyclonal antibodies against Carnation mottle virus coat protein in plants;
- b) conjugate labeled with alkaline phosphatase;
- c) coating buffer;
- d) extraction buffer;
- 15 e) ECI buffer;
- f) PNP buffer,
- g) instruction manual.

- 20 A part of antibodies and conjugate were kept in refrigerator (4°C-10°C), whereas the other parts were kept at room temperature (20°C-35°C) to be used for the studies later on. Results were found positive every time in first part, whereas using other part, results were positive only upto 2-3 months. This indicates that activity of the kit remains as such for 2-3 months and whole year at room temperature and in refrigerator at 4°C respectively.

- 25 For present studies, the carnation plants were collected from experimental fields of IHBT wherein these are growing since last 14 years and these were originally procured from Solan, Himachal Pradesh.

In another embodiment of the present invention, the plant used is carnation (*Dianthus caryophyllus*).

- 30 In another embodiment of the present invention, young leaves of *Dianthus caryophyllus* are used.

Further for the present invention different sequences of Carnation mottle virus was obtained through GenBank and subjected to multiple alignments. 5' and 3' end of the consensus sequence thus obtained was used for primer designing. The primers thus

designed were used for amplification of different Carnation mottle virus isolates. The different amplicons were confirmed by sequencing.

The following examples are given by way of illustration and therefore should not be constructed to limit the scope of the present invention.

Example 1

Detection of *Carnation mottle virus* from Carnations:

Different varieties of carnations were checked using DAS-ELISA as described below. At the same time they were also checked by a reference kit Agdia (Agdia, USA).

10

Direct Antibody Sandwich ELISA:

- 1 Plates (Nunc Immuno TM plate, Denmark) were coated with 100µl of polyclonal antibodies (diluted 1:500-1000) in coating buffer and incubated overnight at 4°C in a humid box.
- 15 2 The plates were washed five times with PBS-T.
- 3 Test samples were prepared by macerating infected leaf tissue 1g/2ml in extraction buffer. Several dilutions were made corresponding to 1x - 1/160x dilution of the original antigen and 100 µl of the diluted antigen was pipetted into the wells of microtiter plate as per the loading diagram and incubated overnight at 37°C in a humid box to allow coating of antigen in the wells.
- 20 4 Washings steps were repeated and conjugate (diluted 1:500-1000) in ECI buffer was added in the wells (100 µl/ well). Plates were incubated for 2 hrs at 37°C in a humid box.
- 5 5 After washing the plates with PBS-T, the wells were filled with 100 µl solution of 1mg/ml p-nitrophenyl phosphate made in PNP buffer.
- 25 6 After appropriate colour development (15-20 min.) the reaction was terminated by adding 50 µl of 3M NaOH to each well.
- 7 7 Positive and negative controls were also made on the same plate. Absorbance at 405 nm was measured for complete ELISA plate with a flow ELISA micro plate reader. The reaction was considered positive if absorbance was observed to be greater than 0.1, which was at least three times the background of the healthy control.
- 30

Results were positive in the positive control sample, where as negative in the negative control sample.

Coating buffer (0.05M per liter): 1.59g sodium carbonate and 2.93g sodium bicarbonate, pH 9.6

PBS buffer: 20mM sodium phosphate pH 7.4 and 150mM NaCl

PBS-T buffer: 20mM sodium phosphate pH 7.4; 150mM NaCl and 0.05% (v/v)

5 Tween 20

Extraction buffer: 1.3g sodium phosphate (anhydrous), 20g Polyvinylpyrrolidone (PVP) MW 24-40,000, 0.2g sodium azide, 2.0g powdered egg albumin grade II and 20.0g Tween-20 were dissolved in 1000ml 1xPBST and pH was adjusted to 7.4

10 ECI Buffer: 2.0g BSA, 20.0g PVP 24-40,000 and 0.2g sodium azide were dissolved in 1000ml 1xPBST and pH was adjusted to 7.4

PNP buffer: 0.1g magnesium chloride, 0.2g sodium azide and 97ml diethanolamine were dissolved in 800ml distilled water, pH was adjusted to 9.8 and volume was made to 1000ml.

15

Table 1 ELISA Results of Different Cultivars of Carnation Using Reference Kit and Test Kit

SN.	Carnation cultivars	ELISA Using Reference Kit	ELISA Using Test Kit
1.	Acca pola	+	+
2.	Aicardi	+	++
3.	Alima	+	+
4.	Aliso	+	+
5.	Antille	+	+
6.	Ariane	+	+
7.	Arthur Sim	+	++
8.	Astrid	+	+
9.	Awanti no-3	+	+
10.	Blade Pink	+	+
11.	Bottom Nibera	+	+
12.	Bottom Norman	+	+
13.	Bright Levdevory	+	+
14.	Cabret	+	+
15.	Candy	+	++
16.	Castellero	+	+
17.	Charmint	+	+
18.	Charmour	+	++
19.	Cherrio	+	+

20.	Cherry Solar	-	-
21.	Dalila	+	+
22.	Delphe White	+	+
23.	Dessio	+	++
24.	Dona Brecas	+	+
25.	Dusty Pink	+	+
26.	Eastern Light	+	+
27.	Equinoxe	+	+
28.	Espana	+	+
29.	Etope	+	++
30.	Firato	-	-
31.	Flair	-	-
32.	Forka	+	+
33.	Garbo	+	+
34.	Indalo	+	+
35.	Irma	+	++
36.	Jack	+	+
37.	Josh	+	+
38.	Laspama	+	+
39.	Lavender Lace	+	+
40.	Leon	+	+
41.	Liberty	+	+
42.	Loris	+	+
43.	M. Collette	+	+
44.	Malaga	+	+
45.	Mastu	+	+
46.	Mercia	+	+
47.	Michelle	+	+
48.	Liberty	+	+
49.	Loris	+	+
50.	M. Collette	+	++
51.	Malaga	+	+
52.	Mastu	+	+
53.	Mercia	+	+
54.	Michelle	+	+
55.	Middle Sand	+	+
56.	Mistral	+	+
57.	Monalisa	+	+
58.	New Espana	+	+
59.	Nordika	-	-
60.	Oasis	-	-
61.	Orange Triumph	+	+
62.	Paraoo Fancy	+	+
63.	Peachy Mambo	+	+
64.	Petra Alekptera	+	+
65.	Pink Aicardi	+	+

66.	Pinkdona	+	+
67.	Pintoo	+	+
68.	Pleasure	-	-
69.	Prinidello	+	+
70.	Purple Chopin	+	+
71.	Red Arhradite	+	+
72.	Red Carso	+	+
73.	Roggio-de-sole	+	+
74.	Saleya	+	+
75.	Salmanca	+	+
76.	Scania	+	+
77.	Shocking Pink	+	++
78.	Solar	-	-
79.	Sorriso	+	+
80.	Sunrise	+	+
81.	Super Green	+	+
82.	Talima	+	+
83.	Tasman	+	++
84.	Tikal	+	+
85.	Top	+	+
86.	Top Hidalgo	+	+
87.	Top solar	+	+
88.	Trendy	+	+
89.	White Candy	+	+
90.	White King	+	+
91.	White Pink	+	+
92.	White Wedding	+	+
93.	Yellow Candy	+	+

(-) = Negative reaction; (+) = Moderately positive reaction; (++) = Strongly positive reaction

5 **Example 2**

Raising of antisera

Purified recombinant coat protein was used as antigen for immunization of rabbit. Healthy white New Zealander male albino rabbits approximately six months old were used to raise the hyperimmune sera each against CERV. Antigen (about 100 µg per injection) was mixed with Freund's adjuvant (1:1) and injected by two routes viz. intramuscularly and sub-cutaneously into the thigh muscles of rabbits separately. First two injections were given along with Freund's complete adjuvant at the interval of one week. Similarly third and fourth injections were given along with Freund's incomplete adjuvant (1:1) at the interval of one week. After two-week immunization schedule, the

animals were bled from the marginal ear vein. The blood was collected in a glass tube and allowed to clot at room temperature for an hour. Subsequently the glass tube containing the clotted blood was kept at 4°C overnight. The serum was collected by using pasture pipette and centrifuged at 5000 rpm for 10 min at 2-6°C. The supernatant was collected and stored at 4°C after adding sodium azide to a concentration of 0.02% (w/v). To collect more serum, booster injections were given 5, 12, 16 and 22 weeks after the initial injection.

For reference and serological testing, antibodies for CERV and CarMV were procured from Bio-Rad, USA.

Purification of antibody (Separation of IgG from whole serum)

A) By Ammonium sulphate precipitation

- 1) Distilled water (9 ml) was added to 1 ml of crude serum.
- 2) Slowly drop wise 10 ml of neutralized saturated ammonium sulphate (Sigma) was added with continuous stirring.
- 3) After stirring, it was kept at room temperature for about 1 h. The resulting solution should appear viscous and cloudy because of precipitation of antibodies i.e. IgG.
- 4) Solution was centrifuged at 9,000 g for 15 min and precipitate was washed with 2 ml of half-strength PBS. Washing step was repeated three times to remove the traces of ammonium sulphate.
- 5) Finally precipitate was dissolved in 1 ml of half-strength PBS.
- 6) O.D. was measured at a wavelength of 280 nm.
- 7) The antibodies were diluted in a way that final concentration became 1 mg/ml (O.D. reading 1.4 = 1 mg/ml).
- 8) Aliquots of 1 ml along with 0.02% w/v sodium azide were stored at -20°C for further use.

PBS (100 ml): $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ – 5.8 g; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ – 1.0 g; NaCl – 8.76 g.

B) By Affinity Chromatography:

- 1) Protein-A sepharose (Sigma) was swelled and packed in a column.

- 2) Column was washed with equilibration buffer.
 - 3) Serum was diluted and passed through the column with a regulated flow.
 - 4) Unbound proteins were washed with PBS until no more protein leaves the column (it was monitored by spectrophotometer).
 - 5 5) Bound protein (IgG) was eluted with elution buffer.
 - 6) pH was neutralized with Tris-HCl.
 - 7) Column was regenerated by washing alternatively with equilibration buffer and storage buffer. Then, the column was stored at 4°C.
 - 8) Elute was dialyzed thrice against PBS and stored at -20°C until used further.
- 10 PBS (100 ml): $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ – 5.8 g; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ – 1.0 g; NaCl – 8.76 g.
- Equilibration buffer (5x): Tris – 0.05 M; NaCl – 0.15 M, pH 8.6.
- Storage buffer: Na_2HPO_4 0.05 M, Thiomersol – 0.05%, pH 6.0.
- Elution buffer: CH_3COONa – 0.05 M, NaCl – 0.15 M, pH 4.5.

15 **Preparation of antibody-enzyme conjugate (using alkaline phosphatase)**

- (i) 1 mg of alkaline phosphatase (Sigma) was dissolved in 2 ml of purified antibodies.
- (ii) Fresh glutaraldehyde (25% stock) was added to the solution in a way to make the final concentration 0.05% and mixed well.
- 20 (iii) It was incubated at room temperature for 4 h. A faint brown colour was developed.
- (iv) After 4 hrs, it was centrifuged at 9,000 g for 20 min.
- (v) The precipitate was washed twice with half strength PBS and finally dissolved in 2 ml of half strength PBS.
- 25 (vi) Bovine serum albumin (BSA) to 5 mg/ml and sodium azide to 0.02% w/v were dissolved in it to enhance its shelf life. It was stored at 4°C till further use.

Evaluation of alkaline phosphatase conjugate

Activity of conjugate was checked by DAS-ELISA as described in the examples given in complete specifications of patent using known positive and negative samples and titrated too.

5 Example 3

Detection of CarMV from other floriculture crops:

To detect CarMV from other floriculture crops, samples were checked using DAS-ELISA as describes above in example 1. Extraction of sample was similar to the steps used for carnation samples as given in Example 1. At the same time they were also
10 checked by a reference Bio Rad kit (Bio Rad, USA) and results as depicted in Table 2.

Table 2 ELISA Results of Other Floriculture Crops Using Reference Kit and Test Kit

SN.	Floriculture Crops	ELISA Using Reference Kit	ELISA Using Test Kit
1	Petunia	+	+
2	Periwinkle	+	+
3	Saponaria	+	+
4	Gomphrena	+	+

Advantages:

15 **The Main Advantages of the Present Invention are:**

1. Carnation is among the top ten cut flower crop in domestic as well as in the international floriculture trade. Since it is severely gets affected by CarMV which reduces its vigour in subsequent years and renders plants susceptible to other pathogen infection. To develop the disease free propagating material and
20 selection of healthy plants by using indigenous diagnostic kit are absolutely essential as it helps in utilizing the proactive strategies for the plant virus management.
2. The kit can detect all CarMV strains including the Asian strains of CarMV.
3. The kit can detect the CarMV in different carnation cultivars giving more strong
25 reaction signals compared to the reference kit (Table 1).
4. All the components of the kit can be stored at 4°C without any appreciable loss in activity while few components of the reference kit need to be stored at -20°C.
5. This being an indigenous diagnostic kit, is cost effective too.

6. Since it is based on polyclonal antibodies, it also detects other strains of CarMV.
7. The kit can detect CarMV from floriculture crops (carnation, petunia, periwinkle, saponaria and gomphrena).
- 5 8. The kit along with its components can be used for detection of CarMV in virus elimination program and screening of tissue culture raised plants.
9. The kit along with its components can be used for detection of CarMV for Plant Quarantine Program.
- 10 10. Kit is useful/ will be used in carnation industry for quality carnation rooted cutting mass production schedules.
11. It will improve the quality of the planting material and subsequently its bloom.
12. The kit along with its components can be used for detection of CarMV during disease epidemiology and forecasting.
13. The kit along with its components will be helpful in development of proactive measures for CarMV disease management.
- 15 14. The cloned/ expressed coat protein of CarMV in *E. coli* BL21 strain can be used for the regular supply of desired protein at one's own will. This will also ensure about quality of antigen and minimizes chances of batch to batch variation.

We claim:

1. A set of primers represented by Seq ID No. 1 and Seq ID No. 2.
2. A method for detection of *Carnation mottle virus* in test sample, said method
5 comprises the steps:
 - a) amplifying coat protein gene of CarMV by using primers as of claim 1;
 - b) expressing the said amplified gene in transformed *E. coli* and obtaining
CarMV coat protein;
 - c) obtaining Polyclonal antibodies against said coat protein; and
 - 10 d) detecting CarMV in a test sample using conventional detection methods.
3. A method as claimed in claim 2, wherein expression of coat protein is carried
out in the presence of an inducer in a concentration in the range of 0.5 to 1.0mM
at 28°C for a period in the range of 3 to 4 hrs.
4. A method as claimed in claim 3, wherein the inducer is IPTG.
- 15 5. A method as claimed in claim 2, wherein polyclonal antibodies are obtained by
using immunization techniques.
6. A method as claimed in claim 5, wherein immunization is carried out in an
animal.
7. A method as claimed in claim 6, wherein the animal is rabbit.
- 20 8. A method as claimed in claim 5, wherein immunization is carried out
intramuscularly, subcutaneously or intravenously.
9. A method as claimed in claim 5, wherein immunization is carried out with coat
protein optionally with an adjuvant.
10. A method as claimed in claim 9, wherein the adjuvant is selected from the
25 group comprising Freund's complete adjuvant, Freund's incomplete adjuvant,
Montanide adjuvant, Ribi's adjuvant, Hunter's TiterMax, Gerbu adjuvant .
11. A method as claimed in claim 9, wherein the ratio of coat protein and adjuvant
is 1:1.
12. A method as claimed in claim 5, wherein the polyclonal antibodies are obtained
30 after 14 to 15 days of immunization.
13. A method as claimed in claim 2, wherein the coat protein is purified coat
protein.

14. A method as claimed in claim 2, wherein the detection method is selected from the group comprising ELISA, indirect sandwich ELISA, DAS-ELISA, immunodiffusion assay, precipitation test.
15. A method as claim in claim 14, wherein the detection method is DAS-ELISA.
- 5 16. A method as claimed in claim 15, wherein in DAS-ELISA the microtitre plates are coated with polyclonal antibodies, diluted in a coated buffer in a ratio ranging from 1:500 to 1:1000 followed by 4-5 times washing with PBS-T.
17. A method as claim 2, wherein the test sample is plant sample.
18. A method as claimed in claim 17, wherein the plant sample is macerated plant
10 sample.
19. A method as claimed in claim 18, wherein the test samples are prepared in microtitre plates by macerating infected leaf tissue from plant with extraction buffer followed by dilution from 1x – 1/60x of the original antigen.
20. A method as claimed in claim 17, wherein the plant sample is carnation plant
15 sample (*Dianthus caryophyllus*).
21. A method as claimed in claim 15, wherein in DSA-ELISA the antibody conjugated or labeled with alkaline phosphatase in ECI buffer is added in the ratio ranging between 1:500 to 1:1000 for a period of 2 hrs at 37° C followed by washing with PBS-T and adding 100µl of about 1 mg/ml p-nitrophenyl
20 phosphate solution in PNP buffer.
22. A method as claim in claim 22, wherein the reaction is terminated by adding about 50µl of about 3M NaOH after 15-20 min to obtain yellow colour.
23. A method as claimed in claim 16, wherein the absorbance of the colored product is measured at 405nm to detect the *Carnation mottle virus* (CarMV).
- 25 24. Use of primers represented by SEQ ID NO. 1 and SEQ ID NO. 2 for detection of Carnation mottle virus (CarMV) in plants
25. A diagnostic kit useful for detection of coat protein of Carnation mottle virus (CarMV) comprising:
 - 30 a) polyclonal antibodies against Carnation mottle virus coat protein in plants;
 - b) conjugate labeled with alkaline phosphatase;
 - c) coating buffer;
 - d) extraction buffer;
 - e) ECI buffer;
 - f) PNP buffer; and
 - 35 g) instruction manual

SEQUENCE LISTING

<110> COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH
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<120> DIAGNOSTIC KIT FOR DETECTION OF CARNATION MOTTLE VIRUS

<130> PCT0607

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INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2006/002019

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SINGH H P ET AL: "Characterization of an Indian isolate of Carnation mottle virus infecting carnations" CURRENT SCIENCE (BANGALORE), vol. 88, no. 4, 25 February 2005 (2005-02-25), pages 594-601, XP009075710 ISSN: 0011-3891 cited in the application the whole document	1,24
Y	—/—	2-23

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Date of the actual completion of the international search

5 December 2006

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27/12/2006

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INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2006/002019

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	-& DATABASE EMBL [Online] 11 June 2003 (2003-06-11), "Artificial oligonucleotide primer sequence (AOS-CarMVCPu) for Carnation mottle virus coat protein analysis" XP002410351 retrieved from EBI accession no. EM_VI:AJ566191 Database accession no. AJ566191 -& DATABASE EMBL [Online] 11 June 2003 (2003-06-11), "Artificial oligonucleotide primer sequence (AOS-CarMVCPd) for Carnation mottle virus coat protein analysis" XP002410352 retrieved from EBI accession no. EM_VI:AJ566192 Database accession no. AJ566192	
X	US 2004/241776 A1 (GEISTER REBECCA L [US] ET AL) 2 December 2004 (2004-12-02) claims 28,31,32 page 7, paragraph 146 page 10, paragraph 310 page 11, paragraph 328 - paragraph 329	25
Y	HÉLIAS VALÉRIE ET AL: "Production of recombinant Potato mop-top virus coat protein in Escherichia coli and generation of antisera recognising native virus protein." JOURNAL OF VIROLOGICAL METHODS. 9 JUN 2003, vol. 110, no. 1, 9 June 2003 (2003-06-09), pages 91-97, XP002410234 ISSN: 0166-0934 the whole document	2-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2006/002019

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