# Characterization of cucumber mosaic virus isolate infecting *Gladiolus* cultivars and comparative evaluation of serological and molecular methods for sensitive diagnosis

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**Severe mosaic, leaf stripe and colour-breaking symptoms were observed in five gladiolus cultivars. The causal agent of the disease was identified as an isolate of Cucumber mosaic virus (CMV) on the basis of aphid transmission in a non-persistent manner, presence of 28–29 nm isometric particles, 26 kDa coat protein subunits, four species of RNA and serological relationship with a CMV strain. The serological (ELISA, ISEM, WBA) and molecular (RT–PCR and Southern hybridization) methods were evaluated for the sensitive detection of CMV in gladiolus leaf and corms. Among them, RT–PCR followed by Southern hybridization test was found to be the most sensitive system for reliable detection of CMV from both the tissues of gladiolus.**

*GLADIOLUS psittacinus* of Iridaceae family is an important ornamental plant grown for beautiful flowers, bouquets, floral baskets and cut flowers. The floriculture industry is being affected due to infection/infestation of various diseases, resulting in drastic reduction in quality and quantity of gladiolus flowers. Among various pathogens, viruses play an important role in the deterioration of ornamental quality of blooms/flowers, which ultimately affects the floriculture trade in India. The use of infected, propagating material (corms) is to be considered as the main source for the virus movement and spreading of diseases.

Literature survey revealed the natural occurrence of pea mosaic virus[1], tomato spotted wilt virus[2], tomato black ring spot virus[3], arabis mosaic virus[4] and tobacco streak virus[5] on gladiolus. Besides this, bean yellow mosaic virus (BYMV)[6–10] and cucumber mosaic virus (CMV)[11–19] are also recorded to affect gladiolus crop severely. CMV is one of the most important systemic pathogens which affects the gladiolus crop in India and abroad. Although natural occurrence of CMV in gladiolus has been detected earlier[11–19], not much information is available on its identification and characterization. In this communication we report characterization of CMV isolate causing severe mosaic, leaf stripe and colour-breaking disease of gladiolus cultivars based on virus transmission, particle morphology, molecular weight of viral protein subunits and nucleic acid analyses. Serological as well as molecular detection methods were also evaluated and compared for sensitive detection of CMV in gladiolus leaves and corms.

A survey of gladiolus fields near Lucknow and experimental plots at the National Botanical Research Institute (NBRI) gardens was conducted in three subsequent years (1997–2000) for natural occurrence of the viral diseases. The natural infection of severe mosaic, leaf stripe and colour-breaking disease in various gladiolus cultivars was observed (Table 1). Attempts were made to transmit the disease by mechanical inoculations and aphid transmission tests from naturally infected gladiolus to a number of plant species. For mechanical inoculations, leaf tissues of gladiolus (H12 cultivar) showing severe mosaic and stripe symptoms were ground in 1 : 3 (w/v) 0.1 M phosphate buffer, pH 7.0 containing 1.0% sodium sulphite. Crude sap was inoculated mechanically on a number of plant species of different families (Table 2), and inoculated plants were observed for one month for the appearance of symptoms, if any. For aphid transmission tests, *Aphis gossypii* (Glove) and *Myzus persicae* (Sultz) were used with a preacquisition starvation period of 2 h, acquisition period of 2 min and an inoculation period of 24 h on 8 plants of *Nicotiana tabacum* cv. White Burely.

**Table 1.** Disease incidence in different hybrids/cultivars of gladiolus during 1999–2000

| Hybrid | Cultivar | Infected/total | Percentage |
|---|---|---|---|
| Snow White × Fidalio | H5 | 20/80 | 25 |
| Snow White × Oscar | H7 | 11/60 | 18.3 |
| Friendship × My Love | H12 | 27/98 | 27.5 |
| Friendship × Tambri | H13 | 15/113 | 13.2 |
| Kajal (Pure) | Kajal | 35/220 | 15.9 |

**Table 2.** Symptoms induced on various plant species by virus isolate from H12 cultivar of gladiolus

| Host species | Local symptom | Systemic system |
|---|---|---|
| *Nicotiana glutinosa* | – | – |
| *N. tabacum* cv. White Burley | NLL | SM, GB, LD |
| *N. rustica* | NLL | SM, GB, LD |
| *N. tabacum* cv. Samsun NN | – | – |
| *Lycopersicon esculentum* | CLL | SM |
| *N. benthamiana* | CLL | SM |
| *Physalis minima* | CLL | SM |
| *Vicia faba* | NLL | – |
| *Chenopodium amaranticolor* | NLL | – |
| *C. qunoa* | – | – |
| *C. album* | – | – |
| *Amaranthus tricolor* | – | – |
| *A. hypochondriachus* | NLL | SM |
| *A. cruentus* | – | – |
| *Cucumis sativus* | – | SM |

NLL, Necrotic local lesions; CLL, Chlorotic local lesions; SM, Systemic mosaic; GB, Green blisters; LD, Leaf deformations; –, No symptoms and no virus detected by back inoculation tests.

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The virus isolate was propagated on *N. rustica* plants by mechanical inoculation for purification of virus particles. The inoculated leaves (100 g) were harvested after 4–5 days of virus inoculation and ground in 0.1 M citrate buffer, pH 6.5 in the ratio 1 : 3 (w/v). Purification of the virus was done by following the procedure of Lot *et al.*[20] and final pellet of the virus was suspended in 2 ml borate buffer, pH 9.0. UV spectrum of purified preparation was done in a spectrophotometer. Infectivity of purified preparation was tested on *N. rustica* plants by mechanical inoculations. Purified preparations were further observed under electron microscope at Central Institute of Medicinal and Aromatic Plants, Lucknow. Uranyl acetate (2.0%, pH 4.2) was used for negative staining of virus particles.

Antiserum was raised against the virus isolate by three intramuscular injections (200 µg/200 µl virus + 200 µl incomplete adjuvant) given to a rabbit at 10 days interval. Blood was collected 7 days after of the last injection, and the antiserum was separated from blood by a low-speed centrifugation. The titer of antiserum obtained was tested by gel double-diffusion test.

The molecular weight of the viral coat protein subunits was identified by SDS–PAGE. Viral coat protein subunits were disrupted by addition of an equal volume of 0.5% SDS, 0.1% 2-mercaptoethanol and 0.02% bromophenol blue to the purified virus preparation and boiling the mixture for 3 min in a water bath. Molecular weight of coat protein was estimated after performing SDS–PAGE, as described by Laemmli[21]. The broad range prestained protein marker (NEB, Germany) was also loaded on gel in a side lane for comparison with viral coat protein.

In order to determine the genome organization of the virus isolate, its nucleic acid was extracted from purified particles by disrupting the virus particles on addition of 1% SDS followed by extraction with an equal volume of phenol–chloroform and precipitation by ethanol containing one-tenth volume of 3 M sodium acetate. The nucleic acid was finally suspended in DEPC-treated, RNAase-free sterile water and stored at – 20°C. The nucleic acid (10 µg) preparations were mixed separately with DNAase (20 U) and RNAase (20 µg/ml) in appropriate amount, incubated for 1 h at 37°C, and inoculated on *N. rustica* plants for infectivity tests. In order to separate the RNA species of virus isolate, agarose gel electrophoresis of the extracted nucleic acid was performed in 1.2% TBE as described by Sambrook *et al.*[22]. RNA markers (0.28–6.58 kb), Promega, Madison, USA were also loaded on the gel for comparison and estimation of molecular weight of viral RNA species.

The various serological methods adopted were ELISA, ISEM and Western blot assays (WBAs) for sensitive detection of virus in leaf and corm samples of five gladiolus cultivars, namely H5, H7, H12, H13 and Kajal. Three samples of leaf and corm from plants showing symptoms of the disease from each cultivar were pooled, ground in buffer for respective tests and used for virus detection.

ELISA was performed by pre-coating of wells with antiserum of CMV-S (PVAS-242a, ATCC, USA) at 1 : 1000 dilutions in carbonate buffer, pH 9.6 and incubated at 37°C for 3 h. Crude sap from leaf and corm samples from various gladiolus cultivars prepared in extraction buffer containing PBS-T (phosphate-buffered saline containing 0.05% Tween 20) pH 7.6 + 2% PVP and 2.25% DIECA was used for binding of antigen at three dilutions (1 : 100, 1 : 500 and 1 : 1000), and kept overnight at 4°C. Goat anti-rabbit IgG labelled with alkaline phosphatase at 1 : 10,000 (Sigma) dilutions in PBS-TO (PBS-T + 0.2% ovalbumin) was used as secondary antibody. Washing of wells after each step was done with 200 µl of PBS-T. Para nitrophenyl phosphate tablets (Sigma) were used as substrate for developing colour and reaction was stopped after 30 min using 3 N NaOH. The absorbance was recorded by reading the plate at 405 nm using Bio-Rad ELISA reader. ISEM was performed in leaf and corm samples of gladiolus using antibodies to CMV-S (PVAS-242a, 1 : 500) as described earlier for electron microscopy of purified preparations.

WBA was performed in leaf and corm samples. After SDS–PAGE, the proteins were transferred to nitrocellulose membrane and processed with antibodies to CMV-S, as described earlier by Renart and Sandoval[23]. The membrane was washed in blocking buffer TTBS (Tris 50 mM, NaCl 150 mM, Triton X-100, 0.1% and Tween-20, 0.05%) containing 5% non-fat, dry milk powder for 2 h at room temperature and then transferred to fresh blocking buffer containing 1 : 1000 diluted CMV-S antiserum for 3 h. After three subsequent washings with TTBS, the blot was transferred to anti-rabbit IgG alkaline phosphatase conjugate (1 : 10,000 dilution) and incubated at 4°C overnight. Finally, the protein bands were elucidated by colour-development reaction on adding BCIP/NBT (Sigma) in dark. The reaction was terminated by adding sterile water and the blot was dried.

The molecular methods applied for sensitive detection and diagnosis of virus infection in various cultivars under study were RT–PCR and Southern hybridization tests. CMV infection was detected by reverse transcription polymerase chain reaction (RT–PCR) amplification. RT–PCR was carried out as described earlier by Raj *et al.*[18] using the primers from the conserved region of CMV-RNA3 (see ref. 24). In brief, 100 mg leaf tissue/corm sample was ground in 10 volumes extraction buffer (50 mM Tris, pH 8.0; 10 mM EDTA; 2% SDS and 1% BME). The plant sap was extracted once with phenol: chloroform (1 : 1 ratio), precipitated by 2.5 volume of ethanol (containing 1/10 volume 3 M Na acetate). The nucleic acid pellet obtained after spinning the mixture at 12,000 rpm for 5 min was washed with 70% ethanol, dried and resuspended in 100 µl RNAase-free sterile water. The cDNA synthesis was carried out using AMV

RTase (10 U) at 42°C for 90 min, in a total volume of 20 µl containing RNAase inhibitor (25 U), 1 mM each dNTP and downstream primer (15 pmol). The up- and downstream primers used were from the conserved region of CMV-RNA3, as described earlier by Blas *et al.*[24]. For PCR, 3 µl of the cDNA was taken as template using Taq DNA polymerase (3 U). The reaction mix finally consisted of 200 µM each dNTP, upstream primer (15 pmol) and 1.5 mM $MgCl_2$. The PCR cycles set were, initial one cycle of denaturation at 94°C of 3 min followed by denaturation at 94°C (30 s), annealing at 52°C (1 min) and extension at 72°C for 1.30 min. The final extension at 72°C was given for 5 min. After completing 25 cycles of PCR, a part of the PCR amplification product was electrophoresed in 1.2% agarose gel as described earlier[22], in 1X TBE. DNA marker Lambda DNA/*Eco*RI and *Hin*dIII double digest (Bangalore Genei Pvt Ltd) was also loaded in a side lane for comparison.

For Southern hybridization, the RT–PCR gel containing the bands was denatured (30 min), neutralized (30 min) and DNA transferred to nylon membrane (Amersham Pharmacia) according to the manufacturer's instructions. The DNA sample was fixed to the membrane by UV cross-linking for 7 min. Radio-labelled probe was prepared using CMV coat protein cDNA[25] by random primer extension method, as described by Fienberg and Vogelstein[26]. Prehybridization and hybridization steps were carried out at 42°C for 14–16 h in the buffer containing 5X SSC, 5X Denhardt's solution and 0.5% SDS. The blot was washed twice for 15 min each in 2X SSC, 1X SSC and 0.1X SSC containing 0.1% SDS. The blot was then exposed to X-ray film and the radiogram developed subsequently.

Our three-year survey (1997–2000) of various cultivars of gladiolus from fields nearby Lucknow and experimental plots at NBRI gardens, revealed various symptoms of the viral disease in H5, H7, H12, H13 and Kajal cultivars. Naturally infected plants exhibited severe mosaic, dark green stripes on leaves (Figure 1 *a*) colour-breaking of petals and stunting symptoms compared to healthy plants (Figure 1 *b*). Poor blooming was also noticed in affected plants of H5 and H12 cultivars. The severity of disease symptoms observed during 1999–2000 is shown in Table 1, which reveals a variation from 13.2 to 27.5% among five cultivars. The cultivars H12 (27.5%) and H5 (25%) were found highly infected compared to H7 (18%), Kajal (15%) and H13 (13%).

Mechanical inoculations resulted in a successful transmission of the virus isolate from H12 cultivar of gladiolus to a number of plant species (Table 2). Necrotic local lesions, systemic mosaic, green blisters and leaf deformations on *Nicotiana rustica* and *N. tabaccum* cv. White Burley were scored. Various types of local and systemic symptoms were also observed on *Amaranthus hypochondriachus*, *Chenopodium amaranticolor*, *Cucumis sativus*, *Lycopersicon esculentum*, *N. benthamiana* and *Physalis minima* (Table 2). However, neither local nor systemic symptoms appeared on inoculated *A. cruentus*, *A. tricolor*, *C. album*, *C. quinoa*, *N. glutinosa* and *N. tabacum* cv. Samsun NN even after 30 days of virus inoculation. During aphid transmission by *Myzus persicae*, only one out of eight *N. tabacum* cv. White Burley plants could develop systemic mosaic (Figure 1 *c*). However, *Aphis gossypii* could not transmit the virus on any of the test plants. The poor aphid transmission was also supported by earlier workers where CMV from gladiolus showing stripe symptoms could infect *N. glutinosa*, *Gomphrena globosa* systemically and *C. amaranticolor*, fababean, cowpea locally, but could not be transmitted by *M. persicae*[16].

Purified virus preparations obtained from *N. rustica* using the procedure of Lot *et al.*[20], showed UV spectrum characteristic of nucleoprotein and were found infectious when tested on *N. rustica* and *C. sativus*. During electron microscopy of purified preparations, plenty of isometric virus particles of 28–29 nm diameter with a central core could be observed (Figure 2), which resembled CMV described by Francki *et al.*[27]. The antiserum of virus iso-

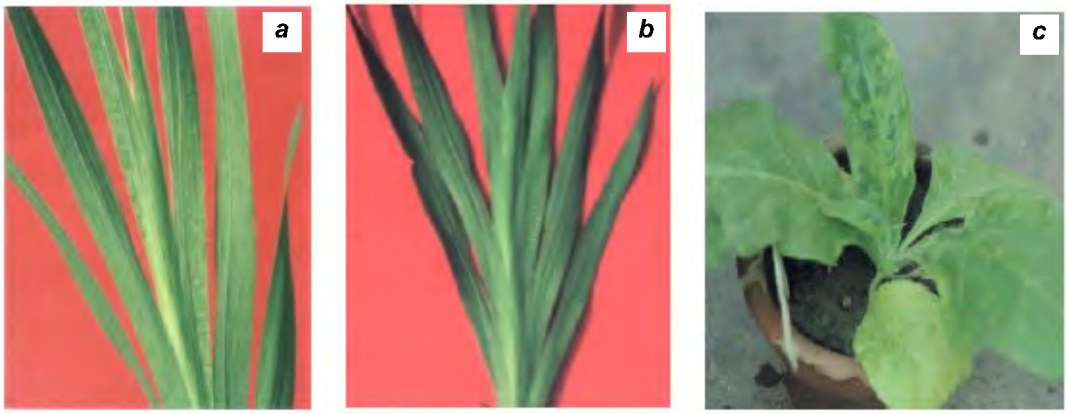


**Figure 1.** ***a***, Infected leaf of gladiolus showing mosaic and dark-green stripe symptoms; ***b***, healthy leaf compared; and ***c***, *N. tabacum* cv. White Burley showing mosaic symptoms after aphid transmission.

late raised in rabbit was tested by double-diffusion tests that revealed a low titer (1 : 128).

During ISEM studies, a few isometric virus particles of 28–29 nm diameter with a central core were observed in leaf samples of H5 and H12, but not in other cultivars (Table 3). The shape and size was identical to virus particles observed from purified preparations. However, several attempts by us failed to detect the virus particles from any of the corm samples.

SDS–PAGE of purified virus preparations revealed two bands corresponding to 26 kDa and 52 kDa of capsid protein. The 26 kDa protein band closely matched the molecular weight of CMV coat protein[28]. The other band of 52 kDa seems to be a dimer of the 26 kDa protein (Figure 3). Such bands were also detected during Western blot immunoassay using CMV-S antibodies.

WBA revealed the presence of 26 kDa and 52 kDa viral proteins with CMV-S antibodies in leaf samples of H5, H12 and Kajal cultivars out of five cultivars tested. However, we could neither detect CMV in corms of the above nor in leaf and corms of other cultivars during WBA (Table 3).

ELISA using CMV-S antibodies could detect CMV in leaf and corm samples of H5, H7, H12, H13 and Kajal cultivars of gladiolus, except corms of H5 (Figure 4). The virus concentration was higher in H12 (corm and leaf), H13 (corm) and Kajal (leaf) compared to others. The 1 : 500 and 1 : 1000 dilutions of antigen and antibodies respectively, were found ideal for optimum ELISA reaction in both leaf and corm tissues. However, at higher (1 : 1000 and above) and lower (below 1 : 500) dilutions of crude sap, we could not detect the virus from any of the samples, which may be due to the low concentration of the virus at higher dilution, and/or presence of inhibitors interfering with the ELISA reaction at higher concentration of the sap. A direct correlation between severity of symptoms on leaves and virus detection by ELISA was observed.

The nucleic acid preparations were found infectious when inoculated on *N. rustica*, and infectivity was abolished after treatment with RNAase but not with DNAase (data not included). Agarose gel electrophoresis of nucleic acid preparation extracted from purified particles revealed a clear-cut separation into three RNA species with a subgenomic RNA 4 (Figure 5). The separation of the three genomic and a subgenomic species was identified to those observed earlier in case of CMV–Amaranth strain[28]. However, there was no evidence of satellite RNA.

RT–PCR followed by agarose gel electrophoresis of the PCR products revealed the expected size fragment of about 540 bp in gladiolus leaf samples of all five cultivars of gladiolus (Figure 6 *a*). In corm samples, the bands of amplification product were faint compared to leaf samples (Table 3). Southern hybridization using cDNA clone of CMV-P as probe showed positive signals in all the gladiolus samples at the same size. Signals were strong in leaf compared to corm samples, which may be due to low amount of PCR product transferred on membrane (Figure 6 *b*).

CMV was first identified as the major disease of gladiolus cultivars in 1948 in Tasmania. At present it is a serious problem in India for production of cut flowers and corms. CMV-infected gladiolus plants show colour-breaking and can vary from a few streaks to various forms of floral or whole plant deformations[1,12]. It is suggested that the intensity of symptoms depends on the cultivars and rate of virus accumulation in the plant.

**Figure 2.** Electron micrograph of purified preparation of virus isolate showing isometric particles with a central core.

**Table 3.** Comparative evaluation of diagnostic procedures for detection of cucumber mosaic in leaf and corm of five gladiolus cultivars

| Diagnostic method | H5 | | H7 | | H12 | | H13 | | Kajal | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf | Corm | Leaf | Corm | Leaf | Corm | Leaf | Corm | Leaf | Corm |
| ELISA | + + + | – | + + | + | + + + | + + + + | + + | + + + | + + + | + + |
| ISEM | + | – | – | – | + | – | – | – | – | – |
| WBA | + + | – | – | – | + + | + | – | – | + | – |
| RT–PCR | + + + | + | + + + | + | + + + + | + + + | + + | + + | + + + | + + |
| Southern hybridization | + + + + | + | + + + + | + + | + + + + | + + + | + + + | + + + | + + + + | + + |

+ + + +, Very strong; + + +, Strong; + +, Moderate; +, Weak; and –, No reaction.

The virus under study that causes severe mosaic, leaf stripe and colour-breaking disease in various gladiolus cultivars was characterized as an isolate of CMV based on the presence of isometric particles of 28–29 nm diameter, 26 kDa capsid protein subunits, aphid transmission in a non-persistent manner, host range study, positive ISEM, ELISA and WBA tests using antibodies to CMV-S. The virus also resembled the shape and size of the CMV reported earlier by Francki *et al.*[27]. The characteristics of nucleic acid and three RNA species were found identical to CMV-Amaranth[28]. In addition, based on the RT–PCR amplification of an expected size (540 bp) fragment from specific primers designed from conserved region (RNA 3) of many CMV strains[24] and positive Southern hybridization with a CMV cDNA probe[25], the virus under study has been confirmed as an isolate of CMV and identified as CMV-G (G stands for gladiolus).

Serological detection methods (ISEM, ELISA and WBA) could detect CMV in leaves of H5 and H12 cultivars of gladiolus (Table 3), but not in corm samples (except by ELISA). It may be due to the presence of some inhibitors in corms which interfere with the serological reactions, as evidenced by several workers in case of BYMV[8,9]. ELISA was found most sensitive among three serological tests.

Molecular-based tests, viz. RT–PCR and Southern hybridization tests have successfully detected CMV in leaf and corm samples of all gladiolus cultivars. RT–PCR was able to detect even at 1 : 10,000 dilutions of leaf sap; however, serological tests could not work beyond the limit of 1 : 500 dilutions. These findings also support an earlier report of Takamatsu *et al.*[17], who observed the detection limit of CMV by RT–PCR as 10–100 ng of CMV particles present in 10 mg of fresh leaf weight. The high sensitivity may be due to the amplification of the targeted molecules. Moreover, RT–PCR in combination with Southern hybridization was found to be more sensitive and specific because of annealing of CMV-specific primers to the template and amplification of the cDNA fragment of an expected size, which hybridizes selectively to the probe from CMV cDNA clone.

The comparative evaluation of serological and molecular detection methods revealed that RT–PCR followed by Southern hybridization proved to be the most sensitive and specific among all the above techniques evaluated for detection of CMV in gladiolus. This technique also works for CMV detection in corm, where the virus may persist in latent form or be present in very low concentration. Hence the technique may be useful for indexing of nuclear materials to be used for mass propagation by tissue culture, breeding programme and industrial production of

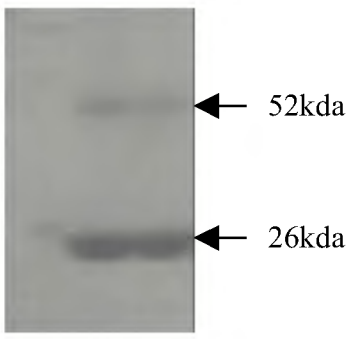


**Figure 3.** SDS–PAGE of purified preparation of virus isolate showing 26 kDa band of coat protein and its 52 kDa dimer.

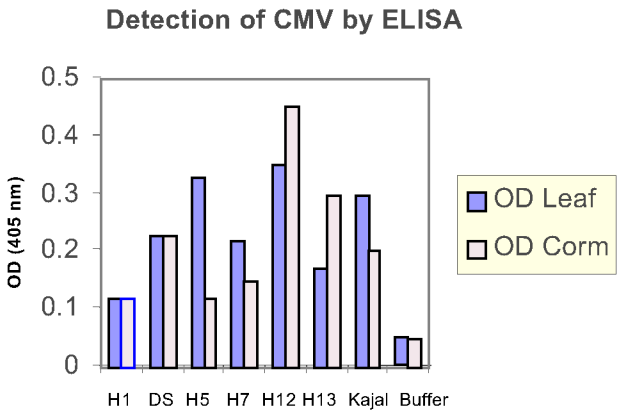


**Figure 4.** Detection of CMV by ELISA in various cultivars of gladiolus based on data of absorbance at 405 nm; Antibodies, CMV-S (PVAS-242a, ATCC, USA), dilution of 11000; Antigen, Crude sap from leaf and corm samples (dilution of 1 : 500) from H5, H7, H12, H13 and Kajal gladiolus cultivars; Hl, Sap from uninoculated; and DS, inoculated *N. rustica* plants taken as negative and positive controls.

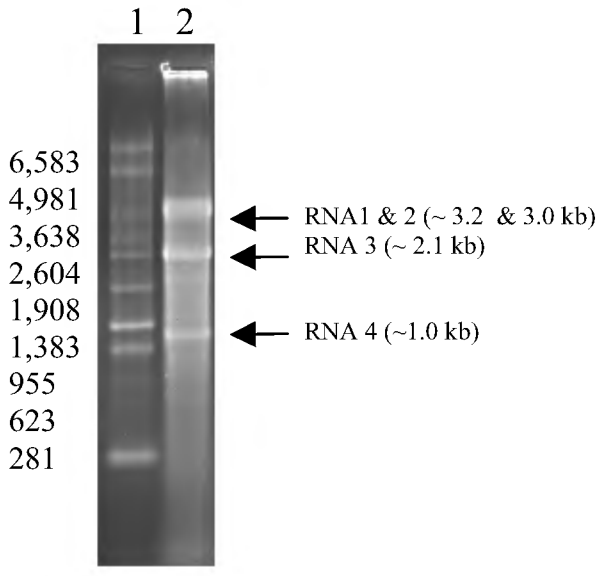


**Figure 5.** Agarose gel electrophoresis of RNA preparation extracted from virus particles of virus isolate from H12 cultivar of gladiolus. Lane 1, RNA markers (0.28–6.58 kb), Promega, Madison, USA; lane 2, RNA preparation from CMV isolate showing separation of RNA species (RNA 1 and 2, RNA 3 and RNA 4) of CMV isolate.

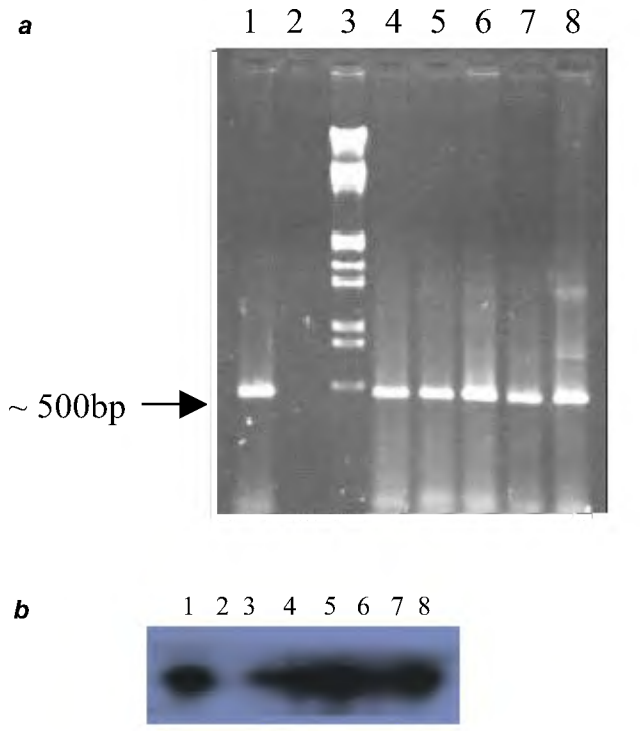


**Figure 6.** ***a***, Agarose gel electrophoresis of RT–PCR products from leaf extracts of gladiolus cultivars using primers specific for CMV. Lanes 1 and 2, PCR product from CMV-inoculated and healthy *N. rustica* taken as (+)ve and (–)ve control; lane 3, DNA marker Lambda DNA/*Eco*RI and *Hin*dIII double digest, Bangalore Genei Pvt Ltd; lanes 4–8, PCR products from H5, H7, H12, H13, and Kajal cultivars showing an amplification of ~ 500 bp. ***b***, Southern hybridization signals of the RT–PCR products (DNA bands of Figure 6 *a* transferred on membrane) with the cloned CMV coat protein gene probe.

corms for floriculture industry. Furthermore, the antibodies to the CMV-G isolate raised indigenously will be useful for indexing of CMV in various gladiolus cultivars and in searching of the alternate host/reservoir of CMV.


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