

Biological and Molecular Characterization of *Cucumber mosaic virus* Subgroup II Isolate Causing Severe Mosaic in Cucumber

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Abstract *Cucumber mosaic virus* (CMV) has a wide host range causing severe damage in many important agricultural and ornamental crops. Earlier reports showed the prevalence of CMV subgroup I isolates in India. However, some recent reports point towards increasing incidence of subgroup II isolates in the country. The complete genome of a CMV isolate causing severe mosaic in cucumber was characterized and its phylogenetic analysis with other 21 CMV isolates reported worldwide clustered it with subgroup II strains. The genome comprised of RNA 1 (3,379 nucleotides), RNA 2 (3,038 nucleotides) and RNA 3 (2,206 nucleotides). The isolate showed highest homology with subgroup II isolates: 95.1–98.7, 87.7–98.0, and 85.4–97.1 % within RNA1, RNA2, and RNA3, respectively. RNA1 and RNA2 were closely related to the Japanese isolate while RNA3 clustered with an American isolate. Host range studies revealed that isolate showed severe mosaic symptoms on *Nicotiana* spp. and *Cucumis* spp. The isolate induced leaf deformation and mild filiform type symptoms in tomato. To best of our knowledge this is the first report of complete genome of CMV subgroup II isolate from India.

Keywords *Cucumber mosaic virus* · Subgroup II · Host range

A. A. Zaidi—deceased.

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Introduction

Cucumber mosaic virus (CMV) is a devastating plant virus having worldwide distribution in temperate and tropical areas. The virus was first reported in 1916 [11, 22] and since then reported to cause disease in a variety of economically important agricultural and ornamental crops. The virus has widest host range infecting over 1,200 species from 100 plant families. CMV is an icosahedral virus approximately 28–30 nm in diameter and belongs to the genus *Cucumovirus* in the family *Bromoviridae*. The virus is rapidly transmitted by more than 80 aphid species in a non-persistent manner [31].

CMV is a tripartite virus having three plus sense, single stranded RNA molecules encased in separate particles. RNA1 and RNA2 encodes for the protein 1a and 2a, respectively which forms the replicase complex [31]. N-terminal region of 1a protein contains putative methyltransferase domain [39] and C-terminal region shows sequence similarity to viral helicases [16]. RNA 2 encodes for another protein 2b, which is expressed from the subgenomic RNA 4A and acts as suppressor of gene silencing [4, 5, 27]; plays a role in long distance movement of the virus [9, 23, 43, 54] and also behave as pathogenicity determinant [10, 41, 43]. A recent finding suggested that this suppressor protein is indirectly involved in aphid transmission [56]. A study on mechanism of 2b action showed that the suppressor specifically recruits AGO4 small RNAs and directly interacts with PIWI and PAZ domains of AGO4 affecting its slicer activity, causing hypomethylation of its loci [18]. RNA3 encodes for two proteins: movement protein (MP) located at 5' end and coat protein which is expressed from another ORF through subgenomic RNA 4. MP plays an important role in cell to cell movement of the virus. While coat protein is required

for encapsidation of the genomic RNAs into virus particles, important in aphid transmission [7, 30] and affecting symptom expression [42, 49]. CMV strains are divided into two subgroups I and II [3, 31, 33] and subgroup I is subdivided into IA and IB based on nucleotide sequence of the 3' untranslated region of RNA3 [37]. Nucleotide sequence similarity among IA and IB subgroup strains is 92–94 %. Subgroup IA and II have worldwide distribution and IB is restricted to Asia only [38].

Broad host range of CMV from subgroup I and II have been identified in India, which includes: *Chrysanthemum* [45]; *Ocimum sanctum* and *Zinnia elegans* [34]; *Amaranthus tricolor*, *Datura innoxia* and *Hyoscyamus muticus* [44]; *Gerbera* [53]; *Vanilla* [28]; *Pelargonium* [52]; *Piper longum* and *Piper betel* [19]; *Rouwolfia serpentina* [35]; *Jatropha curcas* [36]; *Catharanthus roseus* [40]; *Daucus carrota* [1]; Banana [24, 29]; *Lycopersicon esculentum* [15, 25, 47]; *Gladiolus* [13]. Although many CMV strains were isolated from India, but incomplete information is available at the genome level about these isolates. Till date only one complete genome from subgroup I have been reported [25] in India. Here we report molecular and biological characterization of the CMV subgroup II isolate, further information obtained can be utilised for the development of improved diagnostics and finding out symptom and virulence determinants.

Materials and Methods

Mechanical Transmission and Host Range Studies

Young infected leaves of Cucumber plants (*Cucumis sativus*) showing symptoms of chlorosis and mottling were crushed in chilled 0.1 M potassium phosphate buffer, pH 8.0 in a sterile mortar. The crushed sap was filtered through muslin cloth and then filtrate was mechanically inoculated by making gentle abrasion on the leaves of *Nicotiana glutinosa* using carborundum powder. Then plants were maintained at 22–25 °C under glass house conditions and after 1 week young leaves were analysed for presence of virus. For further study, pure culture of the virus was also maintained on the *N. glutinosa* plants by single lesion transfer and aphid transmission.

Host range studies were carried out by mechanical inoculation of pure culture of the virus on *N. glutinosa*, *N. clevelandii*, *N. benthamiana*, *N. rustica*, *Chenopodium amaranticolor*, *C. quinoa*, *L. esculentum* cv. Pusa ruby, different varieties of *C. sativus* and *Capsicum annum* plants. The plants were observed for symptom development 3 weeks post inoculation.

Cloning and Sequencing of the Genome

Total RNA was extracted from infected leaf tissue (100 mg) of *N. glutinosa* by using TRIZOL reagent

(Invitrogen, Thermo Fisher Scientific, USA). The integrity and quality of the total RNA was checked on 1 % agarose gel and quantified by nanodrop2000 (Thermo Fisher Scientific, USA).

For the amplification of RNA1, 2 and 3 specific primers were designed by multiple alignments of sequences of different isolates of CMV (Supplementary Table 1) available in NCBI database. First strand cDNA synthesis was carried out with ~4 µg total RNA. Total RNA was denatured along with 1.0 µl reverse primer (10 pmol/µl) at 72 °C for 2 min, followed by addition of 5 µl 5× first strand buffer, 0.2 µl ribonuclease inhibitor (40 U/µl), 1.5 µl 40 mM dNTPs and 0.5 µl MMLV-RT (200 U/µl) in a total reaction of 25 µl. Reaction was performed at 42 °C for 60 min followed by incubation at 80 °C for 5 min.

Amplification was done using Taq DNA polymerase (Bangalore Genei, Bengaluru, India) in a thermocycler (G-storm, Somerton Biotechnology centre, Somerset, UK) with initial denaturation of 1 min at 95 °C followed by 35 cycles of denaturation at 94 °C for 40 s, annealing and extension temperature being specific for particular amplicon and finally an extension time of 10 min was given at 68 °C (Supplementary Table 1). PCR reaction contained 5 µl of 10× Taq buffer A, 2 µl of forward primer and reverse primer each (10 pmol/µl), 2.5 µl of dNTPs, 1 µl cDNA and 3 U of Taq polymerase (3 U/µl) in total reaction of 50 µl. PCR products were ligated into pGEMT-easy vector (Promega, Madison, USA) according to the manufacturer's instructions and then transformed into *E. coli* DH5α. Recombinant colonies were screened by restriction digestion. Positive clones were sequenced in automated DNA sequencer (ABI PRISM 3130xl genetic analyzer) using ABI prism Big Dye Terminator v3.1 ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) by primer walking. For each amplicon, at least three clones were sequenced. Assembled sequences of RNA1, 2 and 3 were submitted to the GenBank database (HE650150, HE613667, and HE583224 respectively) and the isolate was named as CMV-SG.

Phylogenetic Analysis

Sequences of 32 isolates of CMV belonging to subgroup I and II were retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and compared using MEGA 5.05 version (<http://www.megasoftware.net>). Phylogenetic relationships between these isolates were inferred from the nucleotide sequence alignment by maximum likelihood (Juke canter and 1,000 non-parametric bootstrap replicates). To check the possibility of recombination within CMV isolates we used RDP3 program with default settings. Sequence identity percentage was calculated using Bioedit sequence alignment editor version 7.1.3.0 [17].

Results

CMV-SG isolate developed mosaic symptoms on the inoculated cucumber (Summer Beauty, Summer Green and Veer varieties; Fig. 1b, d), *N. glutinosa* (Fig. 1m, n; mock inoculated and infected) and *N. rustica* plants 4–5 days post inoculation (dpi). Concentric chlorotic lesions were found in *N. clevelandii* (Fig. 1o). Plants showed severe stunting in all tested varieties of cucumber (Fig. 1a, c). In case of tomato, mosaic pattern appeared 5–6 days post inoculation which later became systemic and newly emerging leaves showed downward leaf curling and mild filiform like symptoms (Fig. 1e mock inoculated, f, g infected). In case of *Chenopodium quinoa*, chlorotic lesions were observed after 3 dpi which later became necrotic (Fig. 1k, l; mock inoculated and infected) whereas in chilli, mild mosaic symptoms appeared 7 dpi and plant showed stunting (Fig. 1h–j; mock inoculated and infected). Cryo-electron microscopy of the purified virus revealed the presence of ~28 nm isometric particles typical of CMV (Supplementary Fig. 1). SDS-PAGE and western blot analysis of purified viral particles showed the presence of dimeric ~52 kDa and monomeric ~26 kDa coat protein (CP) as major band (Supplementary Fig. 2).

The sequence analysis revealed that RNA1, 2 and 3 comprised of 3,379, 3,038, and 2,206 nucleotides, respectively. Location of various ORFs on various RNAs was established. It can be seen that the ORF 1a codes for 993 amino acids (aa) protein, known to be involved in replication. RNA2 consisted of two overlapping ORFs, 2a and 2b. 2a encodes for RNA dependent RNA polymerase whereas 2b protein functions as viral suppressor. RNA3 codes for 280 aa movement protein (3a) and 219 aa long 3b (CP).

Complete genome sequences of 31 different isolates were selected worldwide and compared with CMV-SG isolate (Supplementary Table 2–4). It showed highest homology with subgroup II isolates viz. 95.1–98.7, 87.7–98.0, and 85.4–97.1 % with RNA1, RNA2, and RNA3, respectively whereas it showed 71.3–75.6, 67.5–69.6, and 65.0–72.0 % homology with subgroup I isolates for respective RNAs. RNA1 of CMV-SG (HE650150) showed maximum nucleotide identity 98.7 and 98.6 % with American (AF416899), Japanese (AB176849) and Australian isolates (AF198101) from subgroup II. RNA2 also shared maximum identity of 98 % with American (AF416900) and Japanese (AB176848) isolates. Similarly, RNA3 shared 97 % sequence similarity with Japanese (AB176847) and Chinese (EF202397) isolates. It was observed that the 3' UTR of viral RNAs were conserved and showed 96 % identity in case of RNA1 and 2, 88 % in RNA1 and 3 and 87 % identity in RNA2 and 3. Comparison with complete RNA3 sequences of Indian

isolates showed that CMV-SG isolate showed 95.8 and 94.3 % nucleotide identity with EU642567 (carrot) and JF279605 (Tss-In) isolates, respectively. Based on coat protein sequence analysis, CMV-SG isolate showed 99 and 96.9 % identity with isolates from geranium (AJ866272) and lily (AJ585086), respectively and 98.1 % identity with Aligarh isolate from *Ocimum sanctum* (EU600216). Phylogenetic analysis of 22 CMV isolates revealed that RNA1 (Fig. 2a) and RNA2 (Fig. 2b) clustered with Japanese isolate while RNA3 clustered with American and Australian isolates from subgroup II (Fig. 2c). RDP analysis didn't show any recombination event among CMV isolates. Amino acid sequence comparison of 1a protein with other isolates showed some unique changes at following positions: serine at 223; tyrosine at 460; phenylalanine at 624 and glutamic acid at 904 when compared to members of subgroups I and II. In 2a protein: glycine at 53 position; arginine at 206; glycine at 451; cysteine at 594; asparagine at 600; proline at 657; cysteine at 688 and alanine at 788 positions. In 2b protein histidine is substituted with glutamine at aa 14 and asparagine at position 32 with serine (Fig. 3). There were two substitutions in case of coat protein at aa 10 serine with glycine and at aa 106 histidine with arginine. 3a protein also showed three substitutions at aa 42 (glycine with serine), at aa 158 (phenylalanine with tyrosine) and at aa 253 (asparagine with serine). The isolate showed maximum changes in RNA2 (8 substitutions in 2a protein and 2 substitutions in 2b protein). Nuclear localization signal motif (NLS1), KRRRRR; NLS2, RRAR and putative phosphorylation motif KSPSE were conserved in 2b. The substitution at aa 14 histidine with glutamine is unique among subgroup II isolates but represented by subgroup I isolate and other substitution at position 32 is present before NLS2 sequence. The 3'UTR were conserved among RNA1, 2 and 3.

Discussion

In India, CMV isolates have been reported from several regions. Mainly, subgroup I isolates has been reported and there are few reports of subgroup II isolates [1, 15, 47, 53]. The isolate under study showed up to 99 and 76 % similarity to the subgroup II and I isolates, respectively. Depending on the host and virus strain, CMV is known to cause variable symptoms, including necrotic or chlorotic lesions, mild to severe mosaic, stunting, leaf deformation and shoestring formation [14]. Subgroup I strains shows severity in terms of symptom and disease development on tobacco [55]. The present isolate (CMV-SG) induced chlorotic lesions becoming necrotic in local lesion assay host, *Chenopodium quinoa* 3 dpi. Mosaic symptoms and systemic infection was observed in all *Nicotiana* species

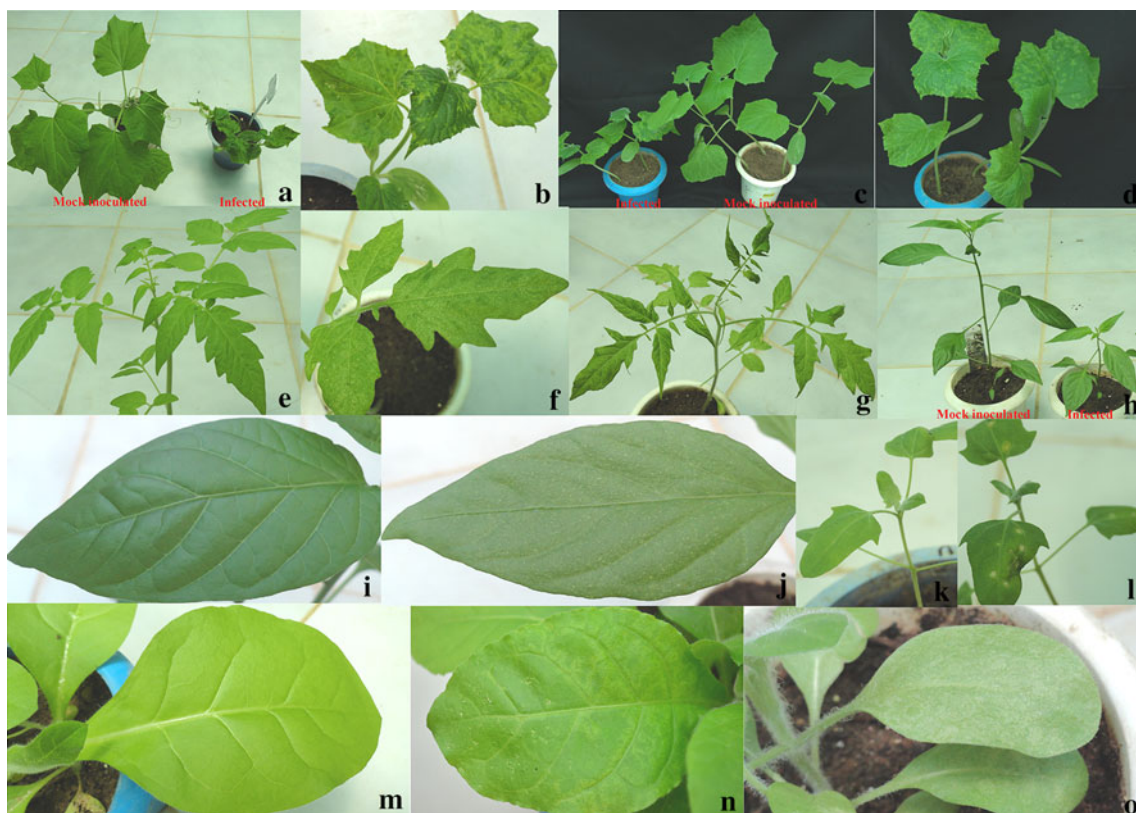


Fig. 1 Symptoms induced by the CMV on different host plants. *Cucumis sativus* (a, c) stunting shown by infected plants of *C. sativus* var. veer and var. summer green respectively in comparison to control. b, d chlorosis and mosaic on leaves after 2 weeks post inoculation; *Lycopersicon esculentum* (var. Pusa Ruby) e mock inoculated, infected f chlorotic patches on leaves, g leaf deformation

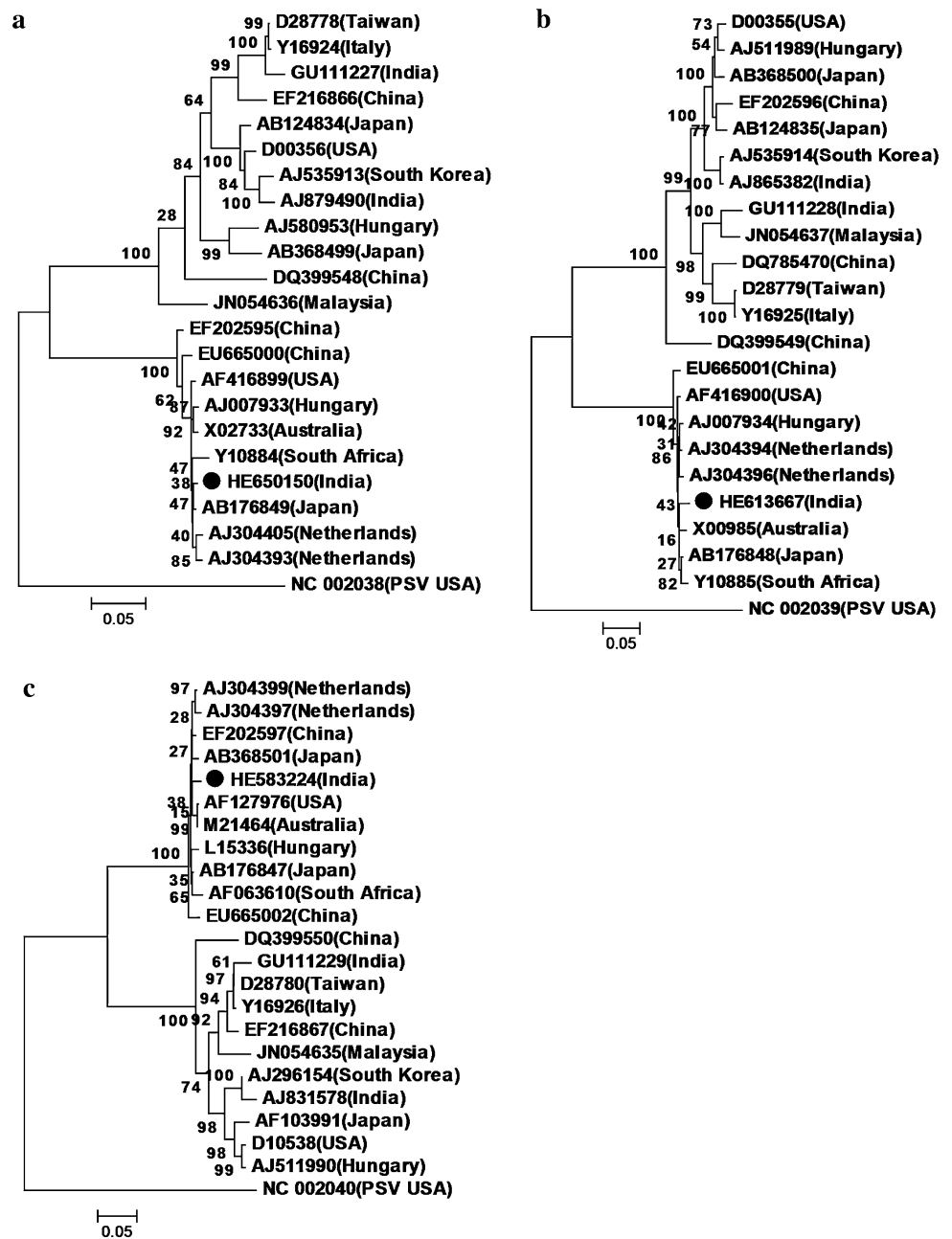
and curling; *Capsicum anuum* h stunted growth in infected plant, i mock inoculated leaf, j infected leaf showing chlorotic patches; *Chenopodium quinoa* k mock inoculated plant, l chlorotic necrotic lesions on leaves; *Nicotiana glutinosa* m mock inoculated leaf, n leaf showing mosaic pattern and lesions; *Nicotiana clevelandii* o infected leaf showing presence of concentric chlorotic lesions

(*N. glutinosa*, *N. clevelandii*, *N. benthamiana* and *N. rustica*). Lesions and concentric chlorotic rings were observed on *N. glutinosa* and *N. clevelandii* respectively. Severe chlorotic, mosaic and stunting symptoms developed on various cucumber varieties (Fig. 1b, d). In newly developing tomato leaves, viral isolate induced symptoms ranging from severe chlorotic patches, downward curling and reduced leaf lamina to mild filiformism. Previously, shoestring like leaves symptoms were reported only with some subgroup I strains [2, 20, 32, 46, 48]. From India also there was report of mosaic symptoms on tomato by subgroup II strain [47] and now in a recent observation it was found that shoestring symptoms were not restricted to subgroup I. A subgroup II isolate Tss-In developed shoestring symptoms on tomato [15]. Cryoelectron microscopy of purified virus showed the presence of ~28 nm isometric particle size, its SDS-PAGE and western blotting showed CP of ~26 kDa and its dimeric form at ~52 kDa. Similar kind of pattern was previously observed in case of CMV-amaranthus strain [34].

Phylogenetic analysis with 21 different isolates of CMV (selected worldwide) at the nucleotide level clustered RNA1 and 2 with Japanese isolate and RNA3 with American and Australian isolates. At the amino acid level 1a and MP protein showed close resemblance to Japanese isolate, 2a and 2b to USA isolate, and coat protein to Australian isolate. Changes in the replicase protein and suppressor protein can affect the infectivity of the virus [26, 50, 54]. Reassortment is a natural mechanism which leads to genetic variation and new strain emergence in multipartite RNA viruses [6]. New CMV strains emerged due to reassortment among subgroup I and II strains [8].

From India, 15 complete RNA3 sequences (including present isolate) and 70 complete coat protein and 27 movement protein sequences have been submitted. Based on the comparison of 15 RNA3 sequence and their phylogenetic analysis it was found to be closely related to an isolate infecting carrot (EU642567; Aligarh isolate) an isolate closely related to Australian isolate, AF198103 [1]. Also an isolate (IA) from India showing 99 % identity to

Fig. 2 Phylogenetic relationships of complete RNA1 (a), RNA2 (b) and RNA3 (c) sequences of present CMV isolate with 21 different isolates of CMV, determined using the maximum-likelihood method based on the Jukes-Cantor model (implemented in MEGA5, with 1000 maximum-likelihood bootstrap replicates)



CP gene of USA isolate has been reported [12]. A subgroup II isolate from *Ocimum sanctum* was found closely related to European isolate, EU191025 [24].

The adaptation of the virus to the new environment and the host plants leads to the emergence of new isolates. In CMV there was report of specific adaptation to the host plant in case of soybean isolate from Indonesia [21]. So far from India complete genome of CMV from subgroup II has not been reported and resemblance of all reported isolates with Asian and European isolates is based on only RNA3 and CP phylogenetic analysis. In our study CMV isolate showed resemblance to Japanese and American isolate and

thus showing its mixed origin which might be making it more stable in this type of environment. The isolate is very competitive in its infection on different host plants taken during the study and thus affecting their growth. Previously from India reports of CMV subgroup-I incidence were prevalent, but now reports belonging to incidence of subgroup-II are increasing. Similar results were also reported from China [51]. These results indicate that sub group-II strains were evolving in such a manner that increases their successful infection rate in field. Thus the present study reports the first complete genome of CMV subgroup II from India, information related to complete genome of an

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