Transmission and properties of a new luteovirus associated with chickpea stunt disease in India

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Several luteoviruses are involved in the chickpea stunt disease (CpSD) etiology. Earlier surveys identified a new luteovirus, *Chickpea stunt disease-associated virus* (CpSDaV), widely associated with CpSD in India. This study investigated its properties and host range, and the aphid vector species involved in its transmission. Purified CpSDaV preparations have typical luteovirus properties: isometric particles of 28 nm in diameter, a single coat-protein of 24.2 kDa and one RNA species of c. 5 kb. Polyclonal antiserum produced to purify CpSDaV preparations was useful for virus detection. CpSDaV which is serologically related to *Beet western yellows virus* (BWYV), has a host range distinct from that of BWYV, and is vectored by *Aphis craccivora*. Based on these properties and sequence homology, CpSDaV is a new species in the genus *Polerovirus*, family Luteoviridae. CpSDaV inoculated chickpea plants developed typical stunt disease symptoms, confirming its role in CpSD etiology.

CHICKPEA stunt (CpSD), the most important virus disease of chickpea (*Cicer arietinum*), is endemic in India, and several other chickpea-growing countries of the world[1]. Diseased plants are stunted and discoloured due to leaf reddening or yellowing, and perform poorly. Early infected plants die prematurely. Several luteoviruses cause symptoms similar to stunt disease in different countries[2]: Pea leaf roll virus [synonymous with Bean leaf roll virus (BLRV)] in Iran[3]; Subterranean clover red leaf virus (SCRLV) [a strain of Soybean dwarf virus] and Beet western yellows virus (BWYV) in California[4,5]; and BLRV and BWYV in Spain[6]. In India, BLRV was thought to be involved in CpSD etiology, but the exact identity of the virus was not known[7]. Surveys conducted to identify viruses involved in CpSD in India revealed the occurrence of three different viruses: the leafhopper transmitted Chickpea chlorotic dwarf virus (CCDV; genus Mastrevirus, family Geminiviridae)[8]; a BLRV-like virus, detected in only a small proportion of CpSD plants; and a new luteovirus which was generically named as the chickpea luteovirus, was predominantly associated with CpSD[9]. To understand weather these two luteoviruses are distinct strains of BLRV or different luteoviruses, the coat-protein genes of these viruses were amplified using universal luteovirus primers by reverse transcription–polymerase chain reaction (RT–PCR) and sequenced[10]. This showed that the BLRV-like virus has 100% sequence homology to BWYV and was therefore regarded as BWYV. However, the chickpea luteovirus has 82% or less sequence homology to the coat-protein sequence of other characterized luteoviruses[11]. Therefore it was regarded as a new luteovirus and named as Chickpea stunt disease-associated virus (CpSDaV)[11]. However, properties of CpSDaV, the aphid vector involved in its transmission and its role in CpSD were not known[10]. In this study CpSDaV was purified and its properties, vector transmission, host range and role in CpSD etiology were determined.

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Stunt disease-affected chickpea plants were collected from Junagadh, Gujarat, India. Plants were assayed with CpSDaV polyclonal antiserum by double antibody sandwich (DAS)–ELISA[9] to select CpSDaV-infected plants. Virus (designated as A24 isolate) from a single stunt-affected chickpea plant was used as the source in this study.

To identify the aphid vector involved in CpSDaV transmission, *Aphis craccivora* and *Myzus persciae*, the two commonest aphid species involved in luteovirus transmission were used. Cultures of *A. craccivora* and *M. persicae* were collected from our institute, Hisar, Akola and Junagadh, and maintained on groundnut (*Arachis hypogea*) cv. JL24, and radish (*Raphanus sativus*) respectively. For virus transmission, aphids were fed on CpSDaV-infected chickpea plants for 24 h virus acquisition access. Five viruliferous aphids were transferred onto each healthy chickpea and groundnut seedlings and were given a 48 h virus inoculation access (IAP). Their feeding was terminated by spraying plants with 0.02% (v/v) Metasystox. Inoculated plants were monitored for symptoms and assayed by DAS–ELISA for CpSDaV, 3 weeks post inoculation (pi).

A. craccivora acquired CpSDaV from chickpea plants and transmitted it to chickpea, groundnut and other plants tested (Table 1). Nymphs and winged alates of *A. craccivora* efficiently transmitted the virus. Virus transmission was low when they were fed on the CpSDaV-infected chickpea for virus acquisition, but transmission was high when they were fed on CpSDaV-infected groundnut plants (Table 1). CpSDaV-infected groundnut did not show any overt symptoms, but these plants reacted strongly to CpSDaV antiserum in DAS–ELISA (Table 2). Although virus concentration was high in the infected chickpea plants, the reasons for low transmission were not clear. *Myzus persicae* failed to acquire virus from CpSDaV-infected chickpea plants, but when fed on the virus-infected groundnut, it transmitted virus to groundnut and chickpea but with only poor efficiency, indicating that it is a poor vector of CpSDaV. For routine virus transmission, *A. craccivora* was used as the vector and CpSDaV-infected groundnut plants as the virus source. CpSDaV cultures established on chickpea cv. WR315 were used for virus purification.

Table 1. Aphid transmission of CpSDaV

		Plants infected/plants inoculated (% infection)[a]	
Virus source plant	Test plant	*A. craccivora*[b]	*M. persicae*[b]
Groundnut	Groundnut	32/32 (100)	4/31 (12)
Groundnut	Chickpea	39/41 (95)	2/27 (7)
Chickpea	Chickpea	8/36 (22)	0/34 (0)
Chickpea	Groundnut	6/34 (17)	0/29 (0)

[a]Virus infection confirmed by DAS–ELISA.
[b]Aphids were allowed two days virus acquisition access period and two days virus inoculation access period.

Table 2. Host range of CpSDaV assessed using viruliferous *Aphis craccivora*[a]

Family, species	Plants infected/tested[b]	Symptoms	Virus concentration[c]
Amaranthaceae			
Gomphrena globosa	22/30	None	High
Leguminoceae			
Arachis hypogeae	34/34	None	High
Cassia obstrusifolia	6/15	None	Moderate
Cicer arietinum	8/36	Yes	High
Lens esculenta	24/28	None	High
Pisum sativum	4/32	None	Low
Trigonella foenum-gracum	25/27	None	High
Vicia faba	19/27	None	Moderate

[a]Aphids were fed on CpSDaV-infected groundnut plants.
[b]All plants were tested for virus by DAS–ELISA.
[c]$H = A_{405} > 1.0$ OD; $M = A_{405}$ 0.5–1.0 OD; $L = A_{405} < 0.5$ OD.

Luteoviruses are phloem-limited, occurs in low concentration and purification of such viruses is difficult. A new procedure was derived from the methods described by Horn *et al.*[8] and Van den Heuvel *et al*.[12] for CpSDaV purification from virus-infected chickpea plants. The plant tissue (100 g) was homogenized using four volumes of 0.1 M sodium citrate buffer, pH 6.0, containing 0.5% ethanol, 0.1% thioglycolic acid and 3% celluclast (Novo-Nordisc, Denmark) and stirred for 3 h. This was filtered through two layers of muslin cloth, and 1 : 1 chloroform and butanol mixture was added to 50% (v/v) final concentration and stirred for 10 min. The emulsion was separated by centrifugation at 13,680 *g* for 15 min, the aqueous phase was collected and NaCl and polyethylene glycol (mol. wt 8000) were added to a final concentration of 0.2 M and 8% (w/v) respectively, and stirred at room temperature for 2 h. The mixture was centrifuged for 20 min at 13,680 *g*, the pellets resuspended in 30 ml of 10 mM phosphate buffer (PB) pH 7.2, and stirred overnight at 4°C. This was clarified by centrifugation at 7100 *g* for 10 min, the supernatant layered on 15 ml of 30% sucrose in PB and centrifuged at 185,500 *g* for 4 h. Pellets were resuspended in 1 ml PB and layered on 10–40% linear sucrose density gradients prepared in PB and centrifuged for 3 h at 110,000 *g*. The light-scattering zone was not distinct in the gradients. Therefore, the gradients were fractionated into four 2.5 ml fractions and each fraction was diluted to 25 ml with PB and concentrated separately by centrifugation at 185,500 *g* for 4 h. Pellets were resuspended in 200 μl of PB and used for downstream applications.

For electron microscope (EM) studies, carbon film-coated 300 mesh copper grids were placed on a drop of purified virus preparation for 10 min. Grids were stained with 1% uranyl acetate and examined under a Phillip CM-20 EM. The highest concentration of isometric particles of 28 nm diameter was found in fraction 1 (Figure 1). Fraction 2 (35–50 mm depth from the top of the tube) contained few virus particles, and other two fractions from the bottom half of the tube contained negligible amounts of virus particles. Virus preparations from fraction 1 had UV-absorption characteristics typical of nucleoprotein with $A_{max} = 260$ nm, $A_{min} = 240$ nm, $A_{max/min} = 1.15$, A260/A280 = 1.66 (all values are the means of five experiments). Virus yields estimated assuming an extinction coefficient[13] of 8.6 was of 0.7–1.0 mg/kg chickpea tissue. The number and size of the coat protein and nucleic acid components of CpSDaV were determined by analysing purified virus preparations, as reported previously[14]. Preparations contained a single coat-protein of estimated size 24.2 kDa and a single RNA molecule of approximately 5 kb (Figure 2).

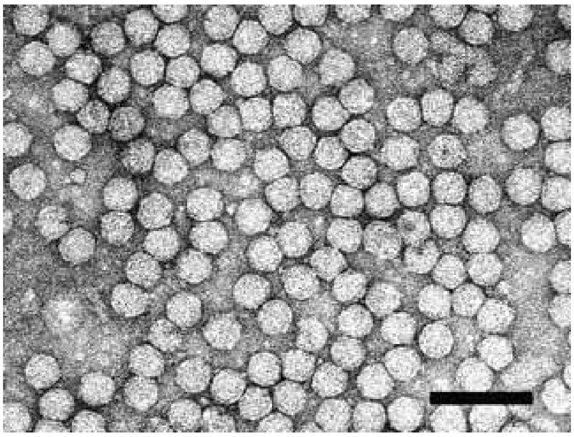

Figure 1. Electron micrograph of purified CpSDaV particles stained with 2% uranyl acetate. Bar = 80 nm.

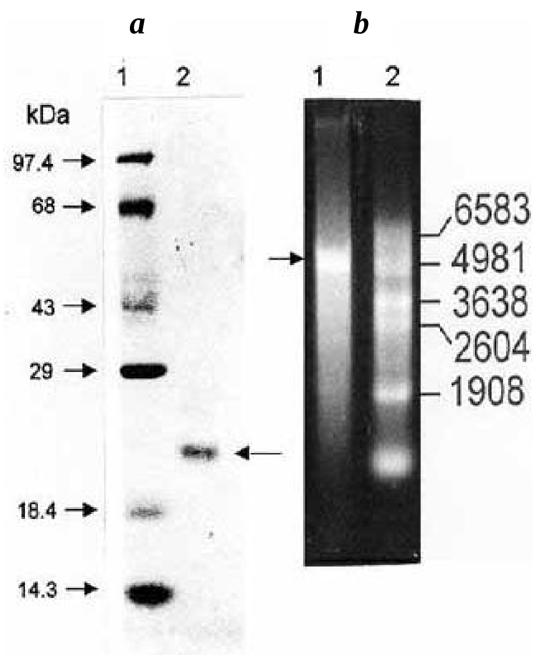

Figure 2. ***a***, Electropherogram of denatured coat-protein preparation of CpSDaV in 12% denaturing polyacrylamide gels. Gel was silver-stained to visualize proteins. Lane 1, Protein molecular weight markers; Lane 2, CpSDaV (arrow). ***b***, Electropherogram of CpSDaV genomic RNA separated in 1% agarose gel. Gel was stained with ethidium bromide. Lane 1, RNA extracted from purified CpSDaV preparation (arrow); Lane 2, RNA marker (size in bases).

Purified virus (70–100 μg) was used for producing polyclonal antibodies in a New Zealand white rabbit and gammaglobulins (IgGs) were extracted from polyclonal antiserum using the sodium sulphate method[15]. Immunosorbant electron microscopy (ISEM) was performed, as described by Roberts and Harrison[16]. Copper grids were coated with test antiserum diluted to 1 : 1000 in 70 mM phosphate buffer, pH 6.5, and placed on a 10 μl drop of purified CpSDaV preparation for 1 h. The grids were stained with 1% uranyl acetate and examined under the EM. Virus particles were counted under 50 viewing fields at 30,000 × magnification. The number of virus particles per 1000 μm^2 was calculated[17]. Grids coated with homologous polyclonal antiserum trapped approximately 2700 or more particles and BWYV polyclonal antiserum trapped around 1072 (average from three experiments) particles. Polyclonal antiserum to BLRV, Groundnut rosette assistor virus (GRAV), Potato leaf roll virus (PLRV) and SCRLV trapped few particles.

DAS–ELISA was performed according to Hobbs *et al*[15]. CpSDaV-infected leaf or stem tissues were extracted in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 and added into the wells of ELISA plates (Nunc, Denmark) pre-coated with 1 μg/ml concentration of homologous IgGs or IgGs of the following luteoviruses (sources in parenthesis): BLRV (L. Bos), PLRV (D. Z. Maat), BWYV (J. E. Duffus), GRAV (A. F. Murant) and SCRLV (G. R. Johnstone). The IgGs extracted from each polyclonal antiserum were conjugated to alkaline phosphatase by the glutaraldehyde method[18] and used as the detecting antibody, and *p*-nitrophenyl phosphate was used at 1 mg/ml as substrate. Test plates were incubated for 1–2 h at room temperature and OD measured at 405 nm in an ELISA plate reader. CpSDaV reacted strongly with homologous antiserum and BWYV antiserum, but did not react with BLRV, GRAV, PLRV and SCRLV antisera.

Thirty-three plant species belonging to leguminous and non-leguminous families were grown in growth chambers (Table 2). Nymphs of *A. craccivora* were fed on CpSDaV-infected groundnut plants for 48 h. Ten viruliferous aphids were transferred onto each test plant and allowed 48 h IAP. Plants were monitored for symptoms and tested for virus by DAS–ELISA[9] using CpSDaV antiserum, 4–6 weeks pi. Among the 33 species, eight tested positive for CpSDaV in DAS–ELISA (Table 2), but only chickpea plants showed symptoms (Table 2). Chickpea cv. WR315 seedlings inoculated with viruliferous *A. craccivora* or by cleft grafting[19] using scions from CpSDaV-infected chickpea plants, showed typical stunt disease symptoms (Figure 3), confirming that CpSDaV causes stunt disease in chickpea.

The following plant species were not infected by CpSDaV: *Brassica oleracea* var. *botrytis*, *B. oleracea* var. *capitata*, *Cajanus cajan*, *Capsicum annum*, *Chenopodium amaranticolor*, *C. quinoa*, *Coriandrum sativum*, *Datura stramonium*, *Glycine max*, *Helianthus annus*, *Lycopersicon esculentum*, *Medicago sativa*, *Nicotiana benthamiana*, *N. clevlandii*, *N. glutinosa*, *N. rustica*, *N. tabacum* (cvs. White Burley and Samsun N/N), *Phaseolus vulgaris* (cvs. French bean and Top crop), *Raphanus sativus*, *Solanum tuberosum*, *Trifolium alexandrinum*, *Vigna mungo*, *V. radiata* and *V. unguiculata*.

CpSDaV exhibited characteristic features of members in the family Luteoviridae, such as 28 nm diameter, a single coat-protein species of approximately 24 kDa, a

Table 3. Comparison of host range, coat-protein size and vector species of CpSDaV, BLRV, BWYV, GRAV, PLRV and SCRLV[5,22]

Host species	CpSDaV	BLRV	BWYV	GRAV	PLRV	SCRLV
Cicer arietinum	+	+	+	–	na	+
Arachis hypogeae	+	+	+	+	na	–
Datura stramonium	–	na	–	na	+	na
Gompherana globosa	+	–	+	+	+	+
Glycine max	–	+	+	+	na	+
Pisum sativum	+	+	–	+	–	+
Solanum tuberosum	–	na	+	na	+	na
Vicia faba	+	+	+	–	–	+
Vigna unguiculata	–	+	+	–	na	–
Viral protein	24.2 kDa	23 kDa	56 and 24 kDa	24 kDa	26 and 7 kDa	22.6 or 25 kDa
Insect vector	*A. craccivora*	*Acyrthosiphon pisum* *M. persicae*	*Ac. pisum* *M. persicae*	*A. craccivora* *A. gossyppi*	*M. persicae*	*Ac. solani* *Ac. pisum*

(+), Plants infected; (–), Plants uninfected; na, Data not available.

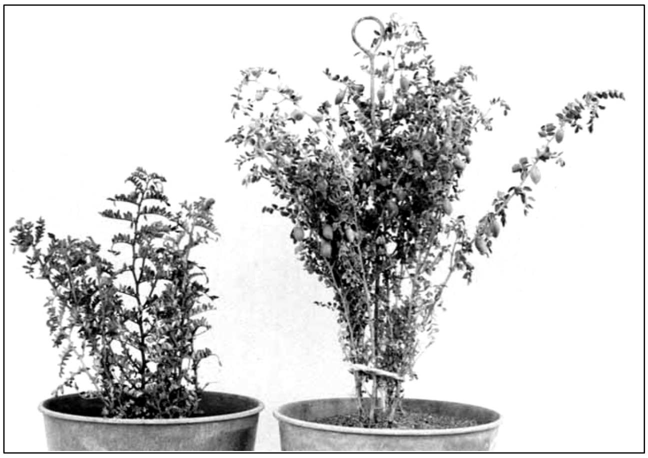

Figure 3. (Left) Chickpea inoculated with viruliferous *A. craccivora* showing typical stunt-disease symptoms. (Right) Uninoculated plant.

monopartite single-stranded RNA genome of approximately 5 kb, and transmission by aphids[11,20]. DAS–ELISA and ISEM tests showed that CpSDaV is distantly related serologically to BWYV, but unrelated to BLRV, GRAV, PLRV and SCRLV. The amino acid sequences of the coat protein of CpSDaV have similarities with BWYV (82%), GRAV (68%), PLRV (61%) and BLRV (55%)[10]. The CpSDaV host range is significantly different from BLRV, BWYV, GRAV, PLRV and SCRLV (Tables 2 and 3)[5,21]. Based on serological relativity and coat-protein sequence homology, CpSDaV is closer to BWYV than other luteoviruses. However, unlike BWYV, CpSDaV failed to infect *G. max* and *S. tuberosum*, but infected *Pisum sativum* and *Vicia faba*, and is transmitted by different species of aphids (Table 3). Hence CpSDaV is a distinct member in the genus *Polerovirus*, family Luteoviridae[22].

Chickpea plants either graft-inoculated with scions from CpSD plants or inoculated with *A. craccivora* fed on CpSDaV-infected groundnut developed typical stunt-disease symptoms, indicating the role of CpSDaV in stunt etiology. It is known that most of the luteoviruses cause leaf yellowing or reddening and stunting symptoms are confined to phloem tissues[23]. Leafhopper-transmitted geminiviruses (such as CCDV) mainly infect phloem and also produce stunt-disease symptoms[23]. This suggests that CpSD is caused by phloem-limited viruses and they can cause similar, if not identical symptoms, and cannot be distinguished by the symptoms they cause in chickpea. Therefore, efforts to control CpSD in the field should proceed first by identifying the virus involved to develop a specific control strategy. The abundance of different luteoviruses surviving in reservoir hosts and prevalence of different aphid vector species occurring in the proximity of chickpea crops, presumably determine which virus or what proportion of different viruses occurs in CpSD-affected chickpea each year. The identification of CpSDaV-infected groundnut as a good virus source for *A. craccivora*, warrants investigation to study its role in CpSDaV ecology, especially in Gujarat, where groundnut is a major crop and CpSD is endemic. The effect of synergistic interaction in the case of mixed infections among luteoviruses and between luteoviruses and CCDV on symptom severity and host-plant resistance is not known. It is evident from this and our earlier studies that CpSD in India is a caused by one (mainly CpSDaV or CCDV) or more (mixed infections) phloem-limited viruses, and disease-control strategy should be aimed at all the major viruses involved in its etiology.

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Evaluation of genotoxic potential of synthetic progestin ethynodiol diacetate in human lymphocytes *in vitro*

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The genotoxicity study of a synthetic progestin ethynodiol diacetate, used as oral contraceptives, was carried out on human lymphocyte chromosomes using sister chromatid exchanges (SCEs), replication index (RI) and chromosomal aberrations (CAs) as parameters. The study was carried out in the presence as well as in the absence of metabolic activation (S9 mix). The aim of the present study is to achieve a precise characterization of the genotoxic activity of ethynodiol diacetate and to establish the value of cytogenetic assays in order to determine the effect of the drugs, at therapeutic doses, to settle an improved risk assessment. Ethynodiol diacetate was studied at three different concentrations (50, 100 and 150 μg/ml of peripheral blood lymphocyte culture) and was found non-genotoxic in the absence of metabolic activation (S9 mix). But in the presence of S9 mix ethynodiol diacetate increases SCE ($P < 0.03$) and CA ($P < 0.005$) frequencies and inhibits lymphocyte proliferation ($P < 0.03$) at 150 μg/ml. The results suggest a genotoxic and cytotoxic effect of ethynodiol diacetate in human peripheral blood cultures *in vitro*.

SYNTHETIC progestins are widely used as oral contraceptives in addition to their use in the treatment of various menstrual disorders, various types of cancers, and in hormonal replacement therapy. For contraception, these are either used alone or in combination with estrogens. Progestins, like estrogens, diffuse easily across the cell membranes and bind to highly specific, soluble receptor proteins in the cytoplasm. These receptors are members of a large family of proteins that act as receptors for a wide range of hydrophobic molecules, including other steroid hormones, e.g. thyroid hormones and retinoids. The steroid receptor complex modifies the expression of specific genes by binding to control elements in DNA[1,2]. Ethynodiol diacetate is used either as single entity drug or in combination with estrogen, such as ethinyloestradiol or mestranol in oral contraceptives[3]. However, studies conducted on the mutagenic activity of various contraceptives and synthetic progestins are contradictory. A significant increase in the number of lymphocytes with DNA migration in alkaline comet assay and frequency of sister chromatid exchanges (SCEs) per metaphase were observed in oral contraceptive users as compared with their age-mat-

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