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# Production of polyclonal antibodies for Capsicum chlorosis virus (CaCV) infecting chilli in India through recombinant nucleocapsid protein expression and its application

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ABSTRACT

Bud necrosis and chlorotic spots causing virus affecting chilli crop in Tamil Nadu (India) was identified as *Capsicum chlorosis virus* (CaCV). Specific primers were used for amplification and sequencing of the nucleocapsid protein (NP) gene. Polyclonal antibody against the bacterially expressed NP from the CaCV-TN-CBE isolate was produced using recombinant DNA technology. NP gene was subcloned into the pET-28a (+) vector and expressed by transformation in BL21 (DE3) pLysS. The expressed protein was about ~34 kDa and was confirmed through western blot analysis using *Groundnut bud necrosis virus* (GBNV) polyclonal antiserum from ICRISAT, India. The purified recombinant protein was used to immunize rabbits to generate CaCV-specific polyclonal antiserum. The sensitivity levels of polyclonal antiserum thus raised was assayed through indirect ELISA or direct antigen coating (DAC)-ELISA using the recombinant protein as antigen. The recombinant antiserum produced in this study successfully detected the natural infection of CaCV on chilli plants collected from the field as well as on cowpea plants artificially inoculated with CaCV by using DAC-ELISA, DIBA and western blotting.

## 1. Introduction

Chilli (*Capsicum annuum* L.) is an important vegetable cum spice crop grown in almost all tropical and subtropical regions of the world. In India, chillies are cultivated in an area of around 1 million hectares with a total production of around 1.4 million tons of dry fruits and 2 million tons of fresh fruits (AGRISTAT, 2015). Though chilli pepper has many medicinal properties, it is vulnerable to a variety of diseases associated with viruses, fungi, bacteria, and phytoplasma (Singh, 2005). Among these, viruses pose a serious threat to chilli crop which is highly susceptible at all developmental stages. In India, more than 20 viruses are known to infect chilli and sweet peppers among which *Chilli leaf curl virus* (ChiLCV), a *begomovirus*, *Chilli veinal mottle virus* (ChiVMV), a potyvirus, *Cucumber mosaic virus* (CMV), a cucumovirus and *Groundnut bud necrosis virus* (GBNV), a tospovirus, are currently considered to be the most important (Reddy and Reddy, 2010).

*Capsicum chlorosis virus* (CaCV), belonging to the genus *Tospovirus*, is the causative agent responsible for an emerging viral disease in India affecting several economically important crops such as tomato (Kunkalikar et al., 2007), chilli pepper (Krishnareddy et al., 2008; Haokip et al., 2016), *Amaranthus* sp. (Sharma and Kulshrestha, 2014) and groundnut (Vijayalakshmi et al., 2016). CaCV has been reported to affect different crops in other countries *viz*., chilli pepper in Australia (McMichael et al., 2002), tomato in Thailand (Knierim et al., 2006; Chiemsombat et al., 2008), peanut in China (Chen et al., 2007), *Phalaenopsis* orchid and calla lily in Taiwan (Zheng et al., 2011; Chen et al., 2012) and wax flower (*Hoya calycina* Schlecter) in USA (Melzer et al., 2014). The virus particle is an enveloped, quasi-spherical structure of 80–120 nm in diameter and has a segmented RNA genome of three single-stranded RNA molecules (Small - S; Medium - M and Large - L). The RNAs of S and M segments are ambisense and L RNA is of negative sense. The L RNA encodes replicase-associated protein, whereas the M RNA encodes movement (NSm) protein and glycoprotein (Gn/Gc) and the S RNA encodes non-structural (NSs) and nucleocapsid protein (NP) (Holkar et al., 2016). Being an emerging threat to chilli cultivation, availability of commercial polyclonal antiserum for the detection of CaCV strain infecting chilli in India is urgently needed. Hence, this study describes the expression and purification of CaCV recombinant

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NP, using it for the production of a polyclonal antiserum for developing serology-based diagnostics for virus detection in the field.

## 2. Materials and methods

### 2.1. Detection of CaCV in chilli

The CaCV infected chilli plants exhibit concentric chlorotic and necrotic ring spots on the leaves and necrotic streaks or patches on the stem and buds which eventually leads to drying of the entire twigs. Five samples each from five different locations in Coimbatore, Tamil Nadu, India were collected and total RNA was extracted both from symptomatic and asymptomatic samples using total plant RNA extraction Kit (Sigma Aldrich, USA) and used to synthesize its respective complementary DNA (cDNA) employing the Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, USA). Polymerase chain reaction was performed using CaCV specific primer pair (GKCaCVCPF1: AACCAAT AGTTTGCCTCCG; GKCaCVCPR1: AGAGCAATCGAGG CACTA) and GBNV (GKPBNVCP: RTTACAATTCCAGCGAAGGAC; GKPBNVCPF: ATGTCT AACGTYAAGCAGCTC) corresponding to the nucleocapsid protein region (Haokip et al., 2016; Nagendran et al., 2018). CaCV-positive chilli samples were mechanically sap-inoculated to cowpea plants (cv.C-152) for propagation and maintenance of virus inoculum using chilled 0.1 M phosphate buffer (pH 7.0) containing 0.1 per cent β mercaptoethanol. Six days old cowpea plants (forty plants) were inoculated by gently rubbing the inoculum using the broad end of the pestle on the primary leaves, previously dusted with 600 mesh carborundum powder. The excess inoculum was washed away after a few minutes, with a jet of sterile distilled water. The inoculated plants were observed for symptomatic expression for about 3–7 days under glasshouse conditions in an insect proof cage

### 2.2. Cloning of complete nucleocapsid gene of CaCV

The total RNA extracted from the symptomatic mechanically sap-inoculated cowpea plants, was subjected to reverse transcription using Revert Aid First Strand cDNA synthesis kit (ThermoScientific, USA) as per the manufacturer's instructions. The complete NP gene (KC953852) was amplified by RT-PCR using the primer pair designed with the help of Primer3 software (CaCV-TN-CBE NP**F:**5′-TAC***GGATCC***ATGTCTAAC GTTAGGCAACTTACC-3′; CaCV-TN-CBE NP**R:**5′-TAA ***GGATCC***TTACA CCTCTATAGAAGTACTAGGC-3′- Bam HI restriction sites are in bold). The PCR conditions were optimized to: initial denaturation of 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplified product was then cloned into pGEMT Easy vector (Promega, Madison, WI, USA), transformed into *E.coli* DH5α and sequenced. The CaCV-NP gene, digested with BamHI restriction enzyme from the recombinant pGEMT plasmid, was subcloned into pET-28a (+) vector, digested using the same enzyme and transformed into *E. coli* -BL21 (DE3) pLysS and BL21 (DE3). Positive clones were identified by colony PCR, subjected to restriction digestion with site-specific enzymes and further confirmed through nucleotide sequencing of the inserted gene.

### 2.3. Purification of nucleocapsid protein in BL21

A starter culture of BL21 (DE3) pLysS and BL21 (DE3) harbouring pET recombinant clone was established. LB media (3 ml) containing chloramphenicol for BL21 (DE3) pLysS and plain LB media for BL21 (DE3) taken in a culture tube was inoculated with a sterile loop of cells taken from a culture plate and incubated overnight at 37 °C. This 3 ml culture grown overnight was sub cultured into 100 ml LB broth containing chloramphenicol and plain LB for BL21 (DE3) pLysS and BL21 (DE3) respectively. Broth culture was incubated with shaking at 37 °C until $OD_{600}$ reached 0.6. Two ml of the culture was removed for uninduced control. To the remainder, IPTG (isopropyl-b-D-thiogalactopyranoside) was added at five different concentrations *viz.*, 0.1, 0.5, 0.6, 0.8 and 1.0 mM from the 100 mM stock, incubated at different temperatures (26 °C, 32 °C, and 37 °C) and at various incubation times (2, 3, 5, 6 and 8 h). The flask was placed on ice for 5 min and the cells were harvested by centrifugation at 5000 × g for 5 min at 4 °C. The supernatant was saved for further analysis. The pellet was resuspended in lysis buffer and slowly dissolved by pipetting and incubated for 10–30 mins for the lysis reaction. The resuspended cells were sonicated for 6 min on an ice bath, for ten passes for about 30s. The soluble and insoluble fractions were separated by centrifugation at 13,000 rpm at 4 °C for 5 min. The cells were resuspended in lysis buffer and stored at −40 °C for further use. An aliquot was further analyzed by12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook and Russel, 2001). The expressed protein was purified according to the protocol described by Selvarajan et al. (2016). The insoluble sonicated fraction containing the expressed protein was dissolved in 1X SDS sample buffer and electrophoresed in 12% SDS-PAGE. The gel was stained with 0.1 M KCl solution and the bands were visualized after an incubation of 5–10 min. The desired protein band was excised and washed with distilled water. The protein band was crushed and then incubated overnight with elution buffer (50 mM TrisHCl, 150 mM NaCl and 0.1 mM EDTA) at 37 °C. The incubated protein sample was centrifuged at 12,000 rpm for one hour at 4 °C. The pellet containing gel pieces was discarded and the supernatant was dialyzed against elution buffer at 60 V for 1 *h* at a temperature of 4 °C. The dialyzed protein solution was collected and stored for further analysis. The purified protein was confirmed by SDS-PAGE and western blotting using GBNV specific polyclonal antiserum (ICRISAT, India).

### 2.4. Production of polyclonal antibody

The gel-eluted purified fusion proteins were further dialyzed to remove the salt impurities, lyophilized and quantified by a Bio-photometer (Eppendorf, Germany). To raise the polyclonal antiserum, 500 µl purified CaCV-NP fusion protein (300 µg/ml) emulsified with an equal volume of Freund's complete adjuvant was injected subcutaneously into New Zealand white rabbits. The rabbit was given 3 additional booster doses of 500 µl (200 µg/ml) each of the purified protein emulsified with Freund's incomplete adjuvant at weekly intervals. The first test bleeds were collected 35 days after initial immunization. One week after the final immunization, serum was collected three times, at 14-day intervals and the crude antiserum was mixed with 100% glycerol (1:1, v/v), amended with sodium azide and stored at −80 °C.

### 2.5. ELISA, Western blotting and DIBA assay

The antibody titer was estimated by Direct Antigen Coating-Enzyme Linked Immunosorbent Assay (DAC-ELISA) as per the procedure described by Hobbs et al. (1987) using the purified recombinant protein where the polystyrene plates were coated with 1 µg/well of purified antigen at dilutions of 1:32000, 1:16000, 1:8000, 1: 4000, 1:2000, 1:1000. Pre-immune sera were used as a control instead of the primary antibody and incubated at 37 °C for 3 *h*. The samples were replicated thrice. The plates were washed three times in PBS-Tween with an interval of 3 min for each washing. One per cent blocking solution at a rate of 200 µl was added to each well and incubated at 37 °C for 1 h. The plates were washed once with PBS-T. The crude antiserum of the first immune and second immune sera was added to each well @ 200 µl and incubated overnight at 4 °C. The plates were washed thrice with PBS-T allowing an interval of 3 min for each wash. The secondary antibody (IgG) conjugate linked with alkaline phosphatase (Genei, Bangalore) diluted in conjugate buffer at 1:5000 was added to each well @ 200 µl and incubated at 37 °C for 3 *h*, washed with PBS-T for three times with an interval of 3 min for each wash. The substrate, para-nitrophenyl phosphate (pNPP; 1 mg $ml^{-1}$ in substrate buffer) (Sigma Aldrich, USA)