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Immunocapture PCR Detection of ToLCNDV from Plant Extract by using Heterologous Virus Coat Protein Antisera

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ABSTRACT

Immunocapture-PCR (IC-PCR) is very versatile technique that has the potential to expedite the detection of plant viruses from plant species containing various forms of PCR amplification inhibitors. In current study, IC-PCR was used for the detection of Tomato leaf curl New Delhi *virus* (ToLCNDV) from plant extracts of *Nicotiana benthamiana* plants inoculated with ToLCNDV. To immunocapture the ToLCNDV, two antisera raised against Tomato yellow leaf curl virus-coat protein (TYLCV-CP) and African cassava mosaic virus-coat protein (ACMV-CP) were used. However, immunocapture of ToLCNDV could only be achieved by using the TYLCV-CP antisera followed by PCR detection by using specific and degenerate primers. ToLCNDV is geographically wide spread Begomovirus (Family Geminiviridae), considered as a close relative of TYLCV, and cause severe losses for many economical important crops in tropical and subtropical regions of the world. This is the first report of the use of heterologous polyclonal antibodies (PAbs), raised against a geographically distinct begomovirus, ToLCNDV. The described results have shown to exhibit a many fold increase detection sensitivity and reproducibility hence, can be adopted for routine detection of ToLCNDV from plant extracts.

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ZI performed the experiments. ZI and MK wrote the article.

Key words
Immunocapture PCR, ToLCNDV, Antisera, PAbs

INTRODUCTION

Tomato leaf curl New Delhi virus (ToLCNDV), a begomovirus (Family Geminiviridae), is geminate shaped particle of 18×22nm in size whose genome composed of two single-stranded DNA components (denoted as DNA A and DNA B). The outer shell of ToLCNDV is formed by coat protein (CP) encoded on DNA A. CP is involved in insect transmission, encapsidation, intra- and intercellular movement. Approximately 110 CP subunits structured as 22 pentameric capsomers forming the unique geminate shape particle (Rojas *et al*., 2001; Zhang *et al*., 2001). ToLCNDV is widely distributed in tropical and subtropical regions causing massive losses to agro-economical important crops in south and south-east Asia (Padidam *et al*., 1995). From last decade host range of ToLCNDV has increased enormously, now it has been reported from potato (Usharani *et al*., 2004), *Hibiscus cannabinus* (Raj *et al*., 2007; Srivastava *et al*., 2016), papaya (Raj *et al*., 2008), eggplant (Pratap *et al*., 2011) okra (Venkataravanappa *et al*., 2011), and several cucurbitaceous vegetables like ivy gourd, ridge gourd, cucumber, bitter gourd, pumpkin, long melon, bottle gourd, watermelon and chayote in India (Mandal *et al*., 2004; Sohrab *et al*., 2006, 2010; Tiwari *et al*., 2010). In Pakistan, besides tomato, ToLCNDV has also been reported from chilli (Hussain *et al*., 2004), bitter gourd (Tahir and Haider, 2005), and from weed *Eclipta prostrata* (Haider *et al*., 2006). While in Thailand, ToLCNDV has successfully been isolated from cucumber, bottle gourd and muskmelon (Ito *et al*., 2008). Very recently, ToLCNDV has been identified from Spain from vast range of plants including zucchini, cucumber, squash and melon (Juarez *et al*., 2014) and this virus has also been found in Tunisia (Mnari-Hattab *et al*., 2015).

Detection of plant viruses through immunocapturing followed by PCR is a versatile, simple, robust and delicate analytical technique. The hybrid method of virus detection is particularly advantageous in plants species or tissues partaking inhibitory substances. Additionally, antibody-mediated purification of viruses is quite simple as compared to other methods of isolation (Mulholland, 2009). Although, begomovirus infection can be characterized by typical symptoms, nonetheless, many precise, highly subtle, and reproducible diagnostic tests for begomoviruses detection have been established. Among available diagnostic methodologies, the widely adopted method is PCR (Rybicki and Hughes, 1990; Rojas *et al*., 1993; Deng *et al*., 1994; Wyatt and Brown, 1996). The conventional PCR procedures entail the provision of total genomic DNA of infected plants. Such genomic nucleic

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acids isolation and purification methods are not only strenuous but also may not be apposite to plant species; enriched with polysaccharides, phenolics and mucilage compounds (Wyatt and Brown, 1996; Biswas *et al*., 2014). To circumvent nucleic acid extractions, several alternate methods involving immunocapture of virus followed by PCR from crude plant extract have been described (Wetzel *et al*., 1991; Xie *et al*., 2013; Ghanim, 2014).

Undoubtedly, in IC-PCR prestigious selection of antibody will only allow the capture of viruses to be tailored to the assay requirements. Then selection of specific antibody at primary level will give rise to higher level of specificity at the PCR level. Much work on serological detection methods have been developed to detect begomoviruses, however, in all available studies specific monoclonal antibodies were used against respective viruses (Brown, 2000; Xie *et al*., 2013). In this study, purified antisera containing polyclonal antibodies (PAbs) against TYLCV-CP and African cassava mosaic virus-coat protein (ACMV-CP) were used in IC-PCR for ToLCNDV detection.

MATERIALS AND METHODS

Plant materials

Plant leaves used in this study were collected from *Nicotiana benthamiana* plants, inoculated with ToLCNDV, growing under glass house conditions at National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan.

DNA extraction and crude leaf extract preparation

In study presented here, 10 *N. benthamiana* plants infected with ToLCNDV were subjected for the IC-PCR detection of ToLCNDV. Whole genomic DNA isolation from *N. benthamiana* leaf tissues was achieved by CTAB method (Doyle and Doyle, 1990). While crude extracts were prepared by grinding one g of fresh and washed leaf in 20 mL of extraction buffer (2.4g Tris, 8g NaCl, 0.5mL Tween20, 0.2g KCl, 0.2g $NaNO_3$, pH=9.0). The extracts were subsequently centrifuged and supernatant was collected and used in assay.

IC-PCR

For IC-PCR, rabbit polyclonal antisera raised against TYLCV-CP and ACMV-CP (provided by Dr. Stephen Winter) were used for immunocapture of ToLCNDV. Antisera [diluted 2:1000 v:v in coating buffer (2.93g $NaHCO_3$,1.59g Na_2CO_3, 0.2g $NaNO_3$, pH=9.6/L)] were loaded to 0.5 mL sterile polypropylene microfuge tube, and incubated at 37°C for 2 h. After three consecutive washes with TBST (150mM NaCl, 10mM Tris-HCl, 0.05%, Tween20, pH=8.0), 150 µL ground plant extract were pipetted in and incubated at 37°C for 2 h or 4°C overnight. After incubation, the microfuge tubes were washed with TBST three times and dried; finally 10 µL ultrapure water was added, incubated at 96°C for 5 min followed by incubation at ice, finally, 5 µL was used in PCR reaction as a template.

Oligonucleotide primers and PCR amplification

PCR reactions consisted of 5 µL of template (immunocaptured ToLCNDV), 2.5µL 10X *Taq* polymerase buffer (Thermoscientific Fisher), 2.5µL (2 mM) dNTPs, 1µL (0.5µM) of each primer, 1.5µL (15mM) of $MgCl_2$ and 0.25µL (1.25 units) of *Taq* DNA polymerase (Thermoscientific Fischer). In a thermocycler (Eppendorf; Model, Master cycler gradient) tubes were preheated at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 1 min. Finally tubes were incubated for 10 min at 72°C. The degenerate primers CLCV1/CLCV2 and specific primers (PadCPPVXF/PadCPPVXR) were used for detection of ToLCNDV (Table I).

Examination of PCR products

The PCR amplified products were loaded and resolved in 1% agarose gels pre-stained with ethidium bromide. Photographs were taken by using UV Stratagene Gel Documentation System.

Prediction of models for CP

The amino acid sequences of CPs of ToLCNDV, ACMV and TYLCV were retrieved from the database with accession numbers AAA92807, CCH63378 and CAL64776, respectively, and used to unravel the structural aspects of these proteins. For the prediction of secondary

Table I.- Oligonucleotide primers used in the study.

Primer	Sequence (5' – 3')	Comments
CLCVI CLCV2	CCGTGCTGCTGCCCCCATTGTCCGCGTCAC CTGCCACAACCATGGATTCACGCACAGGG	Degenerate primers for detection of cotton viruses
PadCPPVXF PadCPPVXR	GCAAATCGATATGGCGAAGCGACCAG GGTCGACTATTAATTTGTGGCCGAATC	Specific primers designed for CP of ToLCNDV

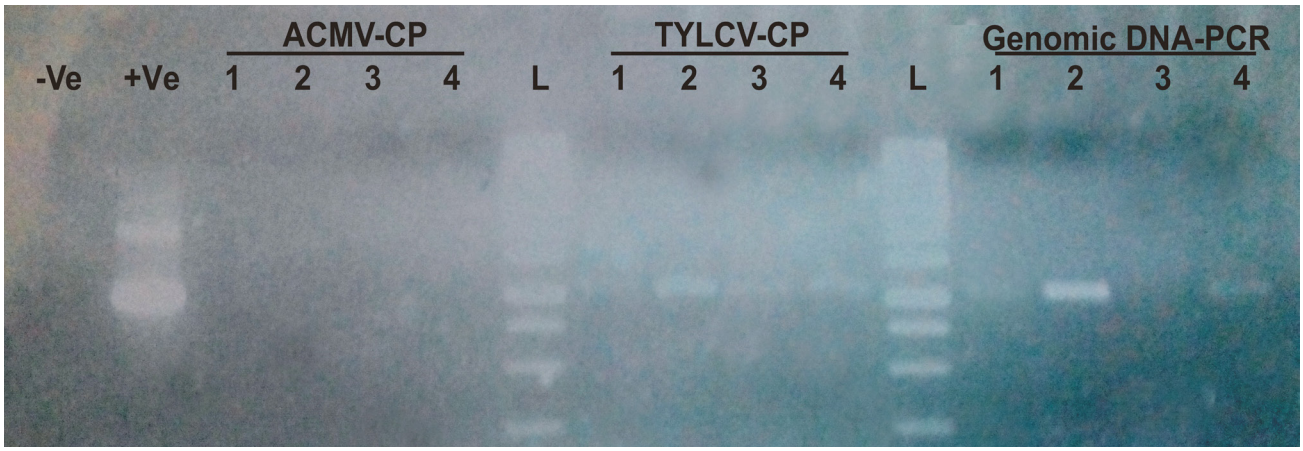

Fig. 1. PCR detection of immunocaptured ToLCNDV from the extracts of *N. Benthamiana* plants. The PCR products run on the gel were negative control (-ve), positive control (+ve) of PCR contain genomic DNA isolated from ToLCNDV inoculated plants as a template, ACMV-CP immunocaptured (1-4), TYLCV-CP immunocaptured (1-4) and PCR from genomic DNA of infected plants (1-4). 1kb DNA ladder (Thermoscientific fisher, SM#0313) was run on the gel to assess the size of required amplicon.

structures psipred was used while tertiary structures of all three CPs were constructed by using I-TASSER. Since no template was available in protein database showing sequence homology for modelling of CPs, therefore, I-TASSER server based threading technique was used to predict the 3D structures of the proteins (Zhang, 2008) that predicted five different best models for each protein.

RESULTS AND DISCUSSION

Detection of ToLCNDV by using TYLCV- and ACMV-CP antisera

The possibility of detection of ToLCNDV DNA-A from plant extract was examined by immunocapture PCR. In present study, two different available polyclonal antisera; CP of TYLCV and ACMV were used for the detection of ToLCNDV. Different working dilutions of antisera were tested and final results showed that minimal dilution at which ToLCNDV could easily be detected is 2:1000 (V:V). The result of three independent IC-PCRs showed expected amplification size for ToLCNDV DNA-A genome (1100bp, Fig. 1) by TYLCV-CP antiserum only. However, despite many concerted efforts, ToLCNDV DNA-A could not be immunocaptured by ACMV-CP antiserum demonstrating that TYLCV-CP antiserum was capable of trapping ToLCNDV DNA-A particles.

No PCR products could be amplified from extracts of healthy *N. benthamiana* plants. The results of IC-PCR detection with heterologous antisera are consistent with many studies, where antibodies generated against one type of virus were efficaciously used to detect serological related but distinct viruses, CLCuMuB was detected by using ACMV antibodies (Tabein *et al*., 2013), polyclonal ACMV-CP antibodies were used to capture Tomato leaf curl virus, different geminiviruses were detected by using monoclonal antibodies produced against ACMV (Thomas *et al*., 1986). Such interactions are also suggestive of sharing antigenic relationship between particle protein of ToLCNDV and TYLCV-CP. These types of interactions may be playing a pivotal role in synergism and virus evolution through recombination.

Comparison of conventional PCR with IC-PCR for ToLCNDV detection

A serological method for ToLCNDV detection based on immunocapturing with TYLCV-CP antiserum followed by PCR amplification was established. In the study, 100ng of purified plasmid containing ToLCNDV DNA-A clone yielded PCR amplification equivalent in intensity to 5µL of immunocaptured virion in IC-PCR (Fig. 1). The IC-PCR products of immunocaptured ToLCNDV (TYLCV-CP Lane 1-4) and ToLCNDV from genomic DNA (genomic DNA PCR lane 1-4) appeared as a 1.1 kb band in each virus (Fig. 1). These results are indicative of buoyant serological relationship between TYLCV and ToLCNDV. This assay is very subtle and detects ToLCNDV from plant leaf extract. The IC-PCR test method is comparatively inexpensive and free from genomic isolations. The comparison of the techniques, IC-PCR and PCR, revealed that detection of ToLCNDV through IC-PCR is more sensitive and reproducible than conventional PCR (Fig. 1). The reliability of IC-PCR was chiefly depends upon leaf sampling, sensitivity and specificity of the antisera used in serological methods and on specificity of primers used in PCR techniques. However, comparative studies showed

that IC-PCR is better test, because of its sensitivity towards the detection of DNA and RNA viruses, as compared to ELISA (Heinrich *et al*., 2001). When compared to ELISA tests or DNA extraction techniques and subsequent PCR detection, IC-PCR can be adopted as a valuable substitute for large scale testing/detection of begomoviruses. However, the main hindrance is availability of antisera against all known begomoviruses. Therefore, additional work is required to detect begomoviruses by using specific antibodies.

Sequence similarity in CPs of all three viruses

Amino acid pairwise alignment of CP of ToLCNDV, ACMV and TYLCV showed that ToLCNDV shared more sequence similarity with ACMV than TYLCV. However, homology model (constructed by using i-Tasser) showed that CPs of ToLCNDV and TYLCV shared close structural resemblance to each other (Fig. 2). The predicted secondary models for all three CPs showed that fourteen β-strands are present in ACMV-CP, while fifteen and

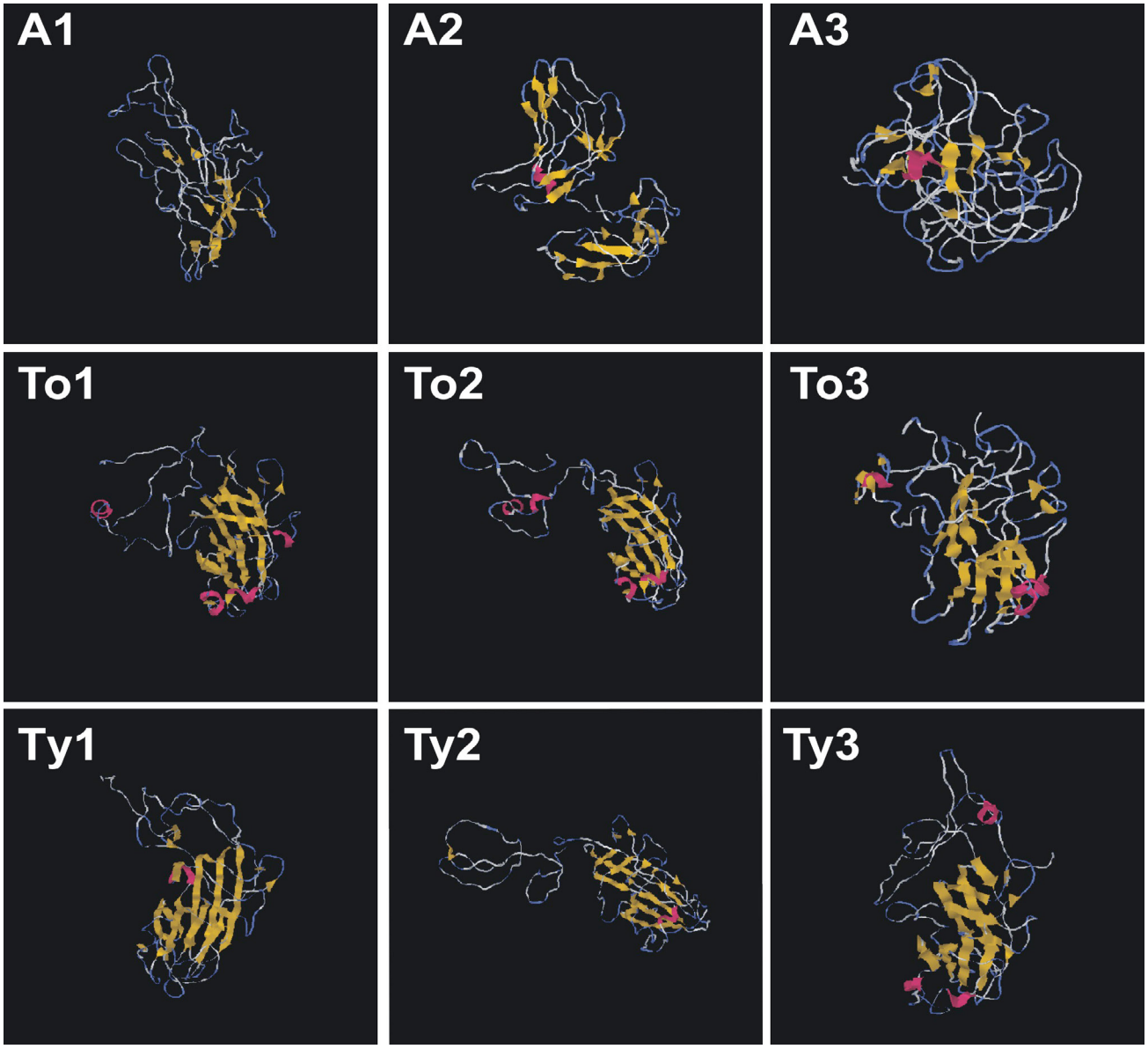

Fig. 2. The 3-D models of all three CPs predicted by thread based I-TASSER server. Different views of ACMV-CP models are designated as A1-A3, ToLCNDV-CP (To1-To3) and TYLCV-CP (Ty1-Ty3). In each model α-helices are represented by yellow color and β-sheats by purple color.

seventeen were found to be in ToLCNDV and TYLCV CPs, respectively. All these β-strands are present unevenly in the entire proteins, this distribution of β-strands in CPs of ACMV and ToLCNDV is same at N-terminus while CPs of TYLCV and ToLCNDV share more similarity at C-Terminus. The ACMV-CP different rest from others two in having three α-helices while remaining both, TYLCV-CP and ToLCNDV-CP, have single α-helix in their structure (data no shown). The predicted tertiary structures indicated that although less sequence identity present between ToLCNDV-CP and TYLCV-CP but both these proteins shared more structural resemblance to eacother. It is therefore, speculated that antisera raised against TYLCV-CP have the ability to interact immunogenically with ToLCNDV genome.

Many studies have revealed that different domains/motifs are present in CP of various begomoviruses through which CPs have the ability to bind nonspecifically with viral genomes (Kunik *et al*., 1998; Guerra-Peraza *et al*., 2005; Sharma and Ikegami, 2009). More comprehensive comparisons of the nucleotide homology of the CP genes of ACMV-JI (Stanley and Gay, 1983), TGMV (Hamilton *et al*., 1984) and BGMV (Howarth *et al*., 1985) indicated that the CP of begomoviruses have considerable amino acid sequence homology, thus the ability of some of the MAbs to react with distinct begomoviruses is not surprising. Such interactions showed that viruses have the ability to recombine each other under natural conditions, thus could lead to development of new strains as recombination plays an important role in the evolution of geminiviruses.

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Conflict of interest statement

The authors declare that there is no conflict of interests.

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