Comparison of direct binding polymerase chain reaction with recombinant coat protein antibody based dot-blot immunobinding assay and immunocapture–reverse transcription–polymerase chain reaction for the detection of sugarcane streak mosaic virus causing mosaic disease of sugarcane in India

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Direct-binding polymerase chain reaction (DB–PCR) test was developed for the detection of sugarcane streak mosaic virus (SCSMV–AP), a member of the new, undescribed genus in the family Potyviridae. Its sensitivity levels were compared to recombinant coat protein antibody-based tests like dot-blot immunobinding assay (DBIA) and immunocapture-reverse transcription–PCR (IC–RT–PCR) to detect virus in purified preparations and in sugarcane leaf extracts. In DBIA, DB–PCR and IC–RT–PCR, the virus was detected up to 5 ng, 0.01 ng and 0.001 ng in purified virus preparations and 10^{-2}, 10^{-3} and 10^{-4} dilutions in infected sugarcane leaf extracts, respectively. When compared to DBIA, IC–RT–PCR appears to be 5000-fold and 100-fold more sensitive with respect to purified virus and infected leaf samples respectively. DB–PCR is 10-fold less sensitive when compared to IC–RT–PCR. Further, shelf-life of recombinant antibody-coated tubes stored at 4°C was checked; it was found that they can be used for PCR up to one month. These PCR-based tests could be useful to screen sugarcane germplasm and in breeding and quarantine programmes.

SUGARCANE (*Saccharum officinarum* L.) is an important commercial cash crop and is the third largest crop in terms of value next to rice and wheat in India. The crop is grown extensively in Uttar Pradesh, Maharashtra, Punjab, Haryana, Gujarat, Bihar, Rajasthan, Tamil Nadu, Karnataka and Andhra Pradesh. Among the virus diseases that affect sugarcane, mosaic disease is important as its incidence is almost 100%; while considering the vast area under sugarcane cultivation, it results in significant yield losses[1,2]. Sugarcane streak mosaic virus – Andhra Pradesh isolate (SCSMV–AP), the pathogen causing mosaic disease in Andhra Pradesh, India was recently characterized and identified as a member of the new, undescribed genus of the family Potyviridae[3–6]. Further, the virus isolates causing mosaic disease of sugarcane in South Indian states were identified as pathotypes of SCSMV[7]. It is transmitted through vegetative propagules (setts) under natural conditions. So far, geographic distribution of SCSMV is confined to Pakistan and India[3,4]. It poses a considerable quarantine risk due to its spread through setts.

Identification of infected plants and development of virus-free planting material are the most successful methods of control, especially for viruses propagated through vegetative plant parts. For successful implementation of the control programme, it is necessary to have rapid, reliable and sensitive tests to screen sugarcane germplasm in quarantine and to determine the distribution of the virus in commercial fields[8]. Sometimes setts from symptomless sugarcane may carry the virus, and it may not be detected by serological methods. Detection of SCSMV–AP by nucleic-acid hybridization tests gives background with healthy samples due to pigment interference. Further, use of radioactive probes in less-developed laboratories is not feasible due to lack of facilities and other inherent limitations[8]. Attempts to detect SCSMV–AP by RT–PCR have been hampered by technical difficulties in obtaining suitable RNA preparations from sugarcane tissues. The quality of the polyclonal antiserum produced against virus depends on the purity of virus preparation used for immunization. Though ELISA was found to be useful for routine large-scale detection of SCSMV–AP, polyclonal antibodies raised against purified virions cross-react with host antigens and often give variable background reactions, limiting their use in ELISA to detect virus[8]. SCSMV–AP coat protein (CP) gene is over-expressed in *E. coli* and used as immunogen to produce high-quality antiserum (rCP antiserum). The rCP antiserum was used to develop an immunocapture–RT–PCR (IC–RT–PCR)-based assay for the detection and discrimination of SCSMV isolates in South India. Further, this test is found to be sensitive to detect the virus even from symptomless carriers, compared to other recombinant antibody-based direct antigen-coating ELISA and DBIA[9]. However, this approach to virus immobilization requires the prior purification of virus or recombinant CP for the production of antiserum. Preparation of antiserum may not be practical, especially for incompletely characterized viruses or viruses that are difficult to purify.

Here we describe a virus immobilization technique and DB–PCR that does not require the use of antiserum. Viruses from extracts of infected plants are non-specifically bound directly to a solid support and bound viruses can be detected directly by RT–PCR analysis. DBIA and IC–RT–PCR tests were done in parallel with the DB–PCR assay in order to compare the utility of each in the detection of the virus. Further, shelf-life of the antibody-coated PCR tubes was determined after storage for one month.

The virus was collected from commercial sugarcane fields and further maintained on *Sorghum bicolor* cv Rio

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in a wire-mesh house by mechanical inoculation[4,10]. It was also maintained on sugarcane through vegetative propagules periodically. The virus was purified from infected sorghum leaves using HEPES buffer by differential centrifugation followed by sucrose density-gradient centrifugation and quantified spectrophotometrically[4,10]. Polyclonal antiserum raised against recombinant CP was used in the present study[9].

For DBIA, DB–PCR and IC–RT–PCR, plant extracts were prepared from young, healthy and SCSMV–AP-infected sugarcane leaves by grinding in antigen extraction buffer (500 mM Tris-HCl; pH 8.3; containing 0.01 M Na_2SO_3; 2% polyvinyl pyrollidone, MW 40,000; 3 mM $NaNO_3$; 140 mM NaCl, and 0.05% Tween 20) at 1:10 dilution. The extracts were clarified by centrifugation (10,000 *g*, 10 min) and used for the assays. The clarified leaf extracts (10^{-1}–10^{-6}) and purified virus (100–0.0001 ng) were diluted with the antigen extraction buffer.

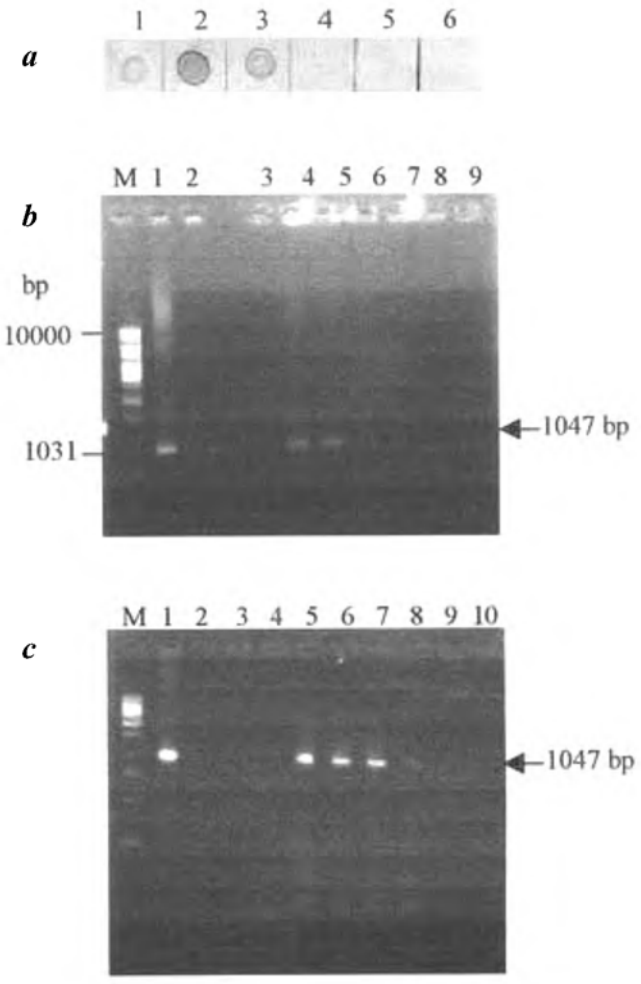

Figure 1. Comparison of three tests for detection of SCSMV–AP in sugarcane leaf extracts. ***a***, DBIA analysis: Lane 1, Healthy leaf extract at 10^{-1} dilution; lanes 2–6, 10^{-1}–10^{-5} dilutions of infected sugarcane leaf. ***b***, DB–PCR analysis: Lane M, 1 kb ladder; lane 1, Positive control; lane 2, Primers alone (-template); lane 3, Healthy leaf 10^{-1} dilution; lanes 4–9, 10^{-1}–10^{-6} dilutions of infected sugarcane leaf extract. ***c***, IC–RT–PCR analysis: Lane M, 1 kb ladder (Gibco-Brl); lane 1, Positive control; lane 2, Negative control; lane 3, Primers alone (-template); lane 4, Healthy leaf at 10^{-1} dilution; lanes 5–10, 10^{-1}–10^{-6} dilutions of infected sugarcane leaf extract.

DBIA was performed according to Berger *et al.*[11]. Nitrocellulose strips (Advanced Microdevices Pvt Ltd, Ambala Cantt.) were coated with leaf extract and purified virus (5 µl/strip). Antibodies produced against rCP (1:1000) were used as primary antibodies and HRP-labelled goat antirabbit antibodies (Genei, India) as secondary antibodies. Substrate was DAB/H_2O_2 (Sigma) for HRP system.

IC–RT–PCR was performed as described by Nalasco *et al.*[12]. Sterile, 0.2 ml polypropylene microfuge tubes (Tarsons, India) were coated with 50 µl of rCP antiserum (1:1000) in carbonate buffer (pH 9.6). To these tubes, 20 pmol of 3′ antisense primer corresponding to the 3′ terminal 30 nucleotides in SCSMV–AP sequence (5′TTT-TTTCCTCCTCACGGGGCAGGTTGATTG3′) was added. Immunocaptured virus was disrupted at 80°C for 10 min and chilled on ice. Reverse transcription reaction was done at 42°C for 60 min, followed by PCR with sense N-terminal eight amino acids of the SCSMV–AP sequence (5′GGA CAA GGA ACG CAG CCA CCT CAG 3′) and 3′ antisense primer. PCR was done using the following parameters: one cycle of 94°C for 2 min and 20 cycles of 94°C, 30 s; 55°C, 45 s; 72°C, 1 min and 15 s, followed by one cycle of 72°C for 10 min. The products were analysed by 1% agarose gel electrophoresis in TAE buffer[13].

DB–PCR was performed according to Rowhani *et al.*[14]. Sterile, 0.2 ml polypropylene microfuge tubes (Tarsons, India) were loaded separately with 50 µl of plant extracts and purified virus solutions, and incubated at 37°C for 60 min. After three washes with PBS-T followed by one wash with PBS, cDNA synthesis was done directly in the tubes and PCR was carried out, as previously described for IC–RT–PCR.

DB–PCR analysis using infected leaf and purified virus samples consistently produced DNA product of the predicted size (1047 bp) and such a specific product was not found in healthy controls (Figure 1 *b*, lane 3 and *c*, lane 4). Tenfold serial dilutions of crude homogenates and serial dilutions of purified virus samples were analysed to establish the detection limits of DBIA, DB–PCR and IC–RT–PCR assays for SCSMV–AP (Figures 1 and 2). The results are summarized in Table 1. The detection limits with respect to purified virus are 5, 0.01 and 0.001 ng and 10^{-2}, 10^{-3} and 10^{-4} with respect to infected leaf extract in DBIA, DB–PCR and IC–RT–PCR, respectively (Figures 1 and 2, and Table 1).

The results indicate that IC–RT–PCR is more sensitive than the other two tests employed. Compared with DB1A, DB–PCR is 500-fold and tenfold more sensitive in detecting the virus in purified preparations and in infected leaf extracts respectively. IC–RT–PCR is tenfold more sensitive than DB–PCR with both purified virus and leaf extract samples.

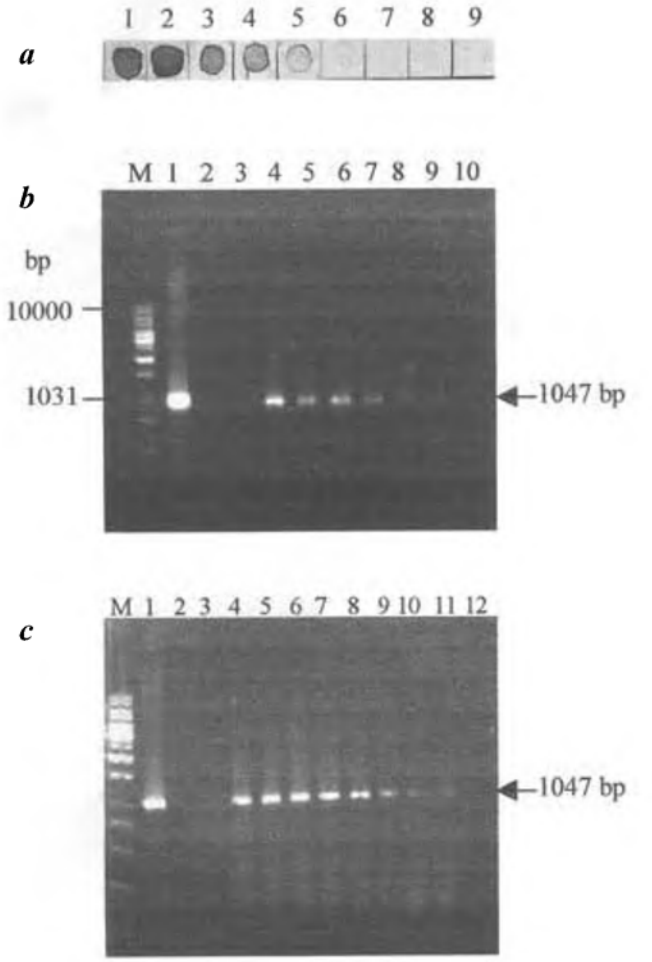

Figure 2. Comparison of detection sensitivity levels using purified virus (SCSMV–AP). ***a***, DBIA analysis: Lane 1, 100 ng; lane 2, 50 ng; lane 3, 25 ng; lane 4, 10 ng; lane 5, 5 ng; lane 6, 2.5 ng; lane 7, 1 ng; lane 8, 0.5 ng; lane 9, 0.1 ng. ***b***, DB–PCR analysis in 1% agarose gel: Lane M, 1 kb marker (Gibco-Brl); lane 1, PCR product obtained from pSCSMV–AP cDNA clone (positive control); lane 2, pUC 19 (negative control); lane 3, Primers alone (-template); lane 4, 100 ng; lane 5, 50 ng; lane 6, 10 ng; lane 7, 5 ng; lane 8, 1 ng; lane 9, 0.01 ng; lane 10, 0.001 ng. ***c***, IC–RT–PCR analysis in 1% agarose gel: Lane M, 1 kb ladder (Gibco-Brl); lane 1, Positive control; lane 2, Negative control; lane 3, Primers alone (-template); lane 4, 100 ng; lane 5, 50 ng; lane 6, 10 ng; lane 7, 1 ng; lane 8, 0.5 ng; lane 9, 0.1 ng; lane 10, 0.01 ng; lane 11, 0.001 ng; lane 12, 0.0001 ng.

Table 1. Comparison of sensitivity of DB–PCR with antibody-based DBIA and IC–RT–PCR for detection of SCSMV–AP

Nature of sample	DB–PCR	DBIA	IC–RT–PCR
Purified virus (ng)	0.01*	5	0.001
Infected sugarcane leaf extract	10^{-3}	10^{-2}	10^{-4}

*Values represent detection limits expressed as virus concentration per reaction (5 µl for DBIA, 50 µl for DB–PCR and IC–RT–PCR).

Total nucleic-acid extraction from sugarcane is time consuming and not amenable for processing of a large number of samples. We failed to detect SCSMV–AP by RT–PCR, despite many trials in getting good total nucleic acid preparations following various methods. DBIA does allow the detection of virus directly from extracts of sugarcane plants, but it lacks the sensitivity required for the detection in symptomless carriers, and pigment interference is the major limitation.

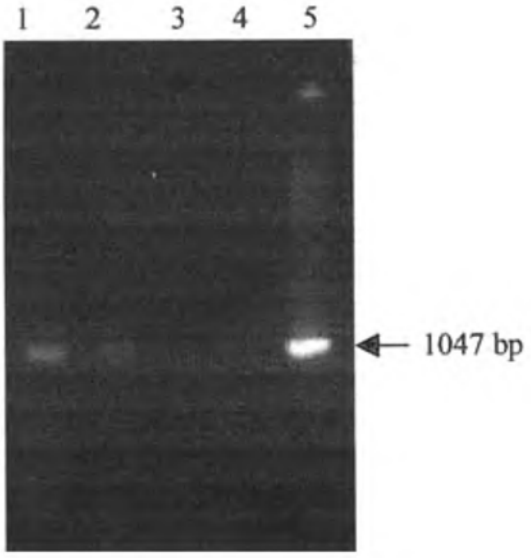

Figure 3. Agarose gel (1%) electrophoresis of IC–RT–PCR-amplified 1047 bp-product in antibody-coated tubes stored for one month at 4°C. Lanes 1 and 2, Amplified DNA products in coated tubes stored for one month; lane 3, Negative control; lane 4, Primers alone (-template); lane 5, Positive control.

Recombinant antibody-based IC–RT–PCR is found to be sensitive enough to detect the virus. But to avoid the risk of raising polyclonal antiserum, a non-specific virus immobilization system, i.e. DB–PCR was developed. However, the detection levels achieved by DB–PCR were tenfold lower than those of IC–PCR analysis. The reduction in sensitivity could be due to fewer virions directly bound to the PCR tubes compared to antibody-coated tubes.

The PCR-based detection techniques described here simplify the methods previously reported for the detection of SCSMV and its pathotypes[7–9], while dramatically increasing the level of sensitivity over that at which viruses are currently detected.

In order to determine the shelf-life of the antibody-coated tubes, the tubes were stored at 4°C for one month and the purified virus at 50 ng/50 µl (1 µg/ml) was subjected to IC–RT–PCR in coated tubes. Amplified DNA band was seen even after one month from the stored immunocapture tubes (Figure 3; lanes 1 and 2). This shows that several PCR tubes can be coated with antibodies at one time and that the stored tubes are suitable for screening purpose.

Finally, we recommend DBIA for routine large-scale testing of SCSMV and PCR-based tests for its detection in germplasm and breeding programmes, plant quarantine

and in approaches leading to production of virus-free sugarcane planting material by tissue-culture technology.

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ACKNOWLEDGEMENTS. M.H. is a recipient of Research Associateship from the Council of Scientific and Industrial Research, New Delhi. We thank Prof. V. Muniyappa, University of Agricultural Sciences, Bangalore for providing healthy sugarcane.

Received 25 July 2003; revised accepted 18 September 2003