

tacts with an interatomic distance of 4 Å or less in human oxyhaemoglobin. No such contacts exist in met and R2 structures.

The results presented above show that the three crystallographically independent molecules in human methaemoglobin crystals have structures intermediate between the R and R2 structures, with different levels of closeness with one or the other of the latter. During the period when this communication was being prepared, three crystal structures of bovine carbonmonoxyhaemoglobin were reported¹⁰. The molecule in one of the three is very similar to human haemoglobin R2 structure, while those in the other two have subunit arrangements intermediate between those in the R and R2 structures. The three crystals were, however, grown under different conditions. In the present work, haemoglobin molecules with different degrees of closeness to R and R2 states are observed in the same crystal. Thus, haemoglobin appears to have several relaxed states with varying degrees of similarity among them.

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Antibody and nucleic acid probe-based techniques for detection of sugarcane streak mosaic virus causing mosaic disease of sugarcane in India

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Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) and direct antigen coating (DAC)-ELISA tests were evaluated for detection of sugarcane streak mosaic virus (SCSMV-AP), a new member of *Tritimovirus* genus in the family Potyviridae, in leaf extracts, sugarcane juice and purified virus. The virus was detected up to 1/3125 and 1/625 dilutions in infected sugarcane leaf, 5 µl and 10 µl/well in sugarcane juice, 1/3125 and 1/3125 dilutions in infected sorghum leaf and 10 ng and 50 ng/ml for purified virus in DAS-ELISA and DAC-ELISA tests, respectively. A cDNA clone pSCSMV-AP (495 bp) specific to SCSMV-AP was selected as diagnostic ³²P and DIG (digoxigenin) probe. In slot-blot hybridization analysis, ³²P and DIG probes reacted with total nucleic acid extracts from infected sugarcane leaf (10⁻⁵ and 10⁻⁴), infected sugarcane juice (10⁻³ and 10⁻⁴) and infected sorghum leaf (10⁻⁵ and 10⁻⁵). With both the probes the virus was detected in purified virus up to 50 pg and viral RNA up to 1 pg level. DAS-ELISA appears to be sensitive and ideal for routine large-scale detection of virus in small leaf tissue and cane juice samples of sugarcane. Nucleic acid-based tests could be useful in screening of sugarcane germplasm.

SUGARCANE (*Saccharum officinarum* L.) is one of the most important commercial crops in the world. The viruses known to naturally infect the crop are sugarcane

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mosaic potyvirus (SCMV), sorghum mosaic potyvirus (SrMV), sugarcane badnavirus (SCBV), sugarcane streak geminivirus (SSV), sugarcane Fiji disease furovirus (FDV), sugarcane mild mottle closterovirus (SCMMV), sugarcane yellow leaf luteovirus (ScYLV) and peanut clump furovirus (sugarcane isolate) (SCRLMV) from different parts of the world¹⁻⁶. All these viruses readily disseminated through vegetative propagating material (setts).

Sugarcane streak mosaic virus (SCSMV-AP), a new member of *Tritimovirus* genus of the family Potyviridae, is a recently recognized pathogen of mosaic disease of sugarcane in Andhra Pradesh, India⁷. Further, the virus isolates causing mosaic disease of sugarcane in South Indian states were identified as pathotypes of SCSMV-AP, but not the strains of SCMV subgroup as has been claimed previously⁸. In India, the mosaic disease of sugarcane (almost 100% incidence) was reported to decrease the yield of sugar significantly⁹.

Literature survey reveals the detection of sugarcane viruses like SCBV, FDV and SCMV based on immunological and molecular approaches¹⁰⁻¹⁹. Immunofluorescent and dot-immunobinding assays were developed to detect the wheat streak mosaic tritimovirus in a single wheat curl mite²⁰. In India, no sensitive techniques have been developed to detect the viruses infecting sugarcane in planting material. Meristem tip culture and thermotherapy are inefficient for management of mosaic disease^{9,21}. Identification of infected plants and development of virus-free clones is the most successful method of control. For successful implementation of these programmes, it is necessary to have specific, rapid and sensitive virus detection tests for indexing of sugarcane on a large scale. In this paper, we report the development and evaluation of antibody (ELISA) and nucleic acid probe (³²P and DIG labelled)-based techniques (slot-blot hybridization) for detection of SCSMV-AP isolate in sugarcane and sorghum.

The virus was collected from commercial sugarcane fields and further maintained on *Sorghum bicolor* cv. Rio in a wire-mesh house by mechanical inoculation^{7,22}. It was also maintained on sugarcane through vegetative propagules (setts) periodically. Virus was purified from infected sorghum leaves, using HEPES buffer by differential centrifugation followed by sucrose density gradient centrifugation and quantified spectrophotometrically^{7,23}.

Polyclonal antiserum raised against purified virus was used in the present study²³. For DAS-ELISA, IgG was separated from crude SCSMV-AP antiserum by ammonium sulphate precipitation followed by DEAE cellulose column chromatography^{24,25}. Alkaline phosphatase (Sigma) was conjugated to SCSMV-AP IgG by one-step glutaraldehyde method²⁶.

ELISA plates with flat-bottom wells (Polysorb, Nunc) were used. The composition of buffers and plate-

washing procedures were followed as described by Clark and Bar-Joseph²⁶. The volumes of reactants added at each step were 200 μ l/well. Plates were incubated in each step for 90 min at 37°C prior to substrate addition in a humid box. The antigen samples used were infected sugarcane leaf sap (1/5–1/15625 dilution), cane juice from infected sugarcane (200, 100, 50, 10, 5, 1 μ l/well), healthy and infected sorghum leaf sap (1/5–1/15625 dilution) and purified virus (2 μ g–10 ng/ml). For DAC-ELISA, the antigens were extracted (1 g/4.5 ml) and subsequent dilutions were made in 0.05 M carbonate buffer, pH 9.6 containing 0.01 M DIECA, whereas for DAS-ELISA, they were extracted and diluted in PBS-TPO containing 0.01 M DIECA.

For DAC-ELISA, the plates were directly coated with antigen samples prepared in carbonate buffer, pH 9.6 (ref. 27). Crude SCSMV-AP polyclonal antiserum at 1:500 dilution in PBS-TPO was added after washing. ALP-labelled goat antirabbit antibodies (Genei, Bangalore) at 1:1000 dilution in PBS-TPO were added and incubated. Then the plate was incubated with p-nitrophenyl phosphate (Sigma) (5 mg/10 ml) for 90 min at room temperature. The absorbance readings (A_{405}) were recorded in Bio-Tek Ceres 900 ELISA reader over buffer controls. Readings twice those of healthy controls were considered as positive. DAS-ELISA described by Clark and Bar Joseph²⁶ was followed. SCSMV-IgG at 1:500 dilution in carbonate buffer, pH 9.6 was added to the plates as trapping antibodies. Antigen samples prepared in PBS-TPO were added after washing the plate followed by ALP-labelled SCSMV-AP IgG at 1:500 dilution. After washing, the substrate was added and incubated at room temperature for 90 min. The absorbance values were recorded as described under DAC-ELISA.

Total nucleic acids were extracted from 200 mg each of healthy and virus-infected sorghum, sugarcane and cane juice (2 ml) samples according to procedure of Smith *et al.*¹². The isolated virus RNA (100 ng–0.01 pg), disrupted purified virus (100 ng–0.1 pg), total nucleic acid extracts of infected sugarcane, sorghum leaves (10^{-1} – 10^{-6} dilution), infected cane juice and healthy sorghum leaves (10^{-1} – 10^{-5} dilution) were prepared and made up to 200 μ l with 10X SSC. Nylon membranes (Boehringer Mannheim) were equilibrated in 10X SSC for 10 min and inserted into slot-blot filtration manifold (Hybrislot manifold, GIBCO-BRL). The test samples along with healthy controls were denatured in boiling water for 5 min and chilled quickly in ice bath and loaded onto the membrane with gentle vacuum. The membranes prepared in duplicates were air-dried and baked at 80°C for 2 h.

The *Bam*HI/*Hind*III fragment released from cDNA clone pSCSMV-AP (ref. 7) was labelled with DIG (Boehringer Manniheim kit manual) and α -³²P dATP (Amersham International) by following random primer

labelling method as described by Feinberg and Vogelstein²⁸ and purified through Sephadex G-50 spun column²⁹. Hybridization and further processing of nylon membranes which are probed with ³²P and DIG labels were carried according to Sambrook *et al.*²⁹ and Boehringer Mannheim kit manual, respectively.

Several serological and nucleic acid-based techniques have been developed from time to time to detect sugarcane viruses. Detection of these viruses in infected sugarcane plants is limited by the type of tissue sample taken from the test plant¹¹. The choice of these techniques is based on the available laboratory facilities and expertise, sensitivity, cost and time factor. ELISA-based tests are preferred in several laboratories, especially in large-scale indexing of planting materials^{30,31}. In the present study, SCSMV-AP was detectable at 1/3125 and 1/625 dilution of infected sugarcane leaf, 5 µl and 10 µl/well of sugarcane juice, 1/3125 and 1/3125 dilution of infected sorghum leaf and 10 ng and 50 ng/ml of purified virus in DAS-ELISA and DAC-ELISA tests, respectively (Table 1). Healthy sugarcane

Table 1. Evaluation of ELISA tests for detection of SCSMV-AP

Nature of sample	Sample concentration	ELISA	
		DAS	DAC
Infected sugarcane leaf	1/5	2.95 ^a	1.12
	1/25	2.20	0.54
	1/125	1.20	0.39
	1/625	0.98	0.23
	1/3125	0.45	0.16
	1/15625	0.20	0.04
Infected sugarcane juice (µl/well)	200	0.99	0.64
	100	1.37	0.81
	50	1.26	0.69
	10	0.68	0.36
	5	0.39	0.14
	1	0.20	0.05
Infected sorghum leaf	1/5	0.99	2.01
	1/25	1.37	1.68
	1/125	1.26	1.18
	1/625	0.68	0.84
	1/3125	0.49	0.31
	1/15625	0.20	0.10
Healthy sorghum leaf	1/5	0.21	0.09
	1/25	0.10	0.04
Purified sugarcane virus (µg/ml)	2.0	xxx	2.71
	1.0	2.97	1.62
	0.5	2.14	0.94
	0.1	1.56	0.34
	0.05	1.42	0.27
	0.02	1.23	0.17
	0.01	0.56	0.12
0.001	0.23	0.10	

a, Values are an average absorbance of A₄₀₅ of three wells recorded after 90 min of adding substrate, xxx, not readable.

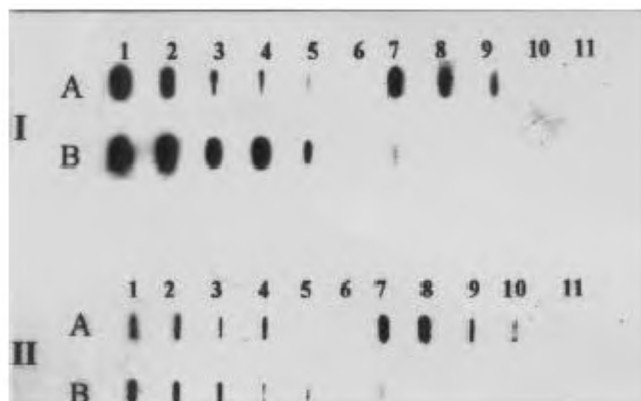


Figure 1. Slot-blot hybridization analysis of SCSMV-AP in total nucleic acid extracts of sugarcane leaf, juice and sorghum leaves. *I*, ³²P label, *II*, DIG label. *A*, 1–6 : 10⁻¹–10⁻⁶ dilutions of infected sugarcane leaf; 7–11 : 10⁻¹–10⁻⁵ dilutions of infected sugarcane juice; *B*, 1–6 : 10⁻¹–10⁻⁶ dilutions of infected sorghum leaf; 7–11 : 10⁻¹–10⁻⁵ dilutions of healthy sorghum leaf.

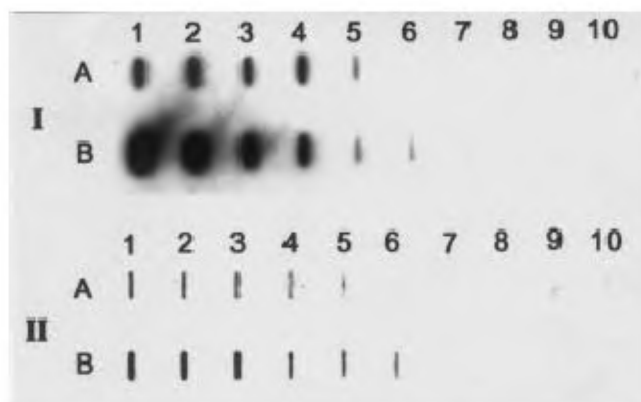


Figure 2. Detection of SCSMV-AP by slot-blot hybridization analysis. *I*, ³²P label, *II*, DIG label; *A*, Purified virus. 1, 100 ng; 2, 10 ng; 3, 1 ng; 4, 500 pg; 5, 50 pg; 6, 25 pg; 7, 10 pg; 8, 1 pg; 9, 0.5 pg; 10, 0.1 pg; *B*, Isolated virus RNA. 1, 100 ng; 2, 10 ng; 3, 1 ng; 4, 500 pg; 5, 50 pg; 6, 1 pg; 7, 0.5 pg; 8, 0.1 pg; 9, 0.01 pg; 10, Buffer control.

was not included in the tests as the virus-free sugarcane was not available, in spite of sincere efforts to procure it from national research institutes. No reaction was observed with healthy sorghum leaf antigens in both the tests. There is significant difference in the sensitivity levels of virus detection with both tests in sugarcane leaf, sugarcane juice and purified virus. Though DAC-ELISA is economic and requires less time, DAS-ELISA appears to be specific, sensitive and ideal for large-scale detection.

Nucleic acid-based techniques appear to be more sensitive for detection of viruses even in very small quantities of test samples. Labeled nucleic acid probes have been used in various laboratories in developed and developing countries for detection of several plant viruses^{31,32}. The main advantages of using cloned cDNA

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probes are that they can be prepared in large amounts when needed and provide absolute purity and specificity of the probe.

In the present study, cDNA insert from clone pSCSMV-AP (495 bp) specific to SCSMV-AP RNA (ref. 7) which covers the 3'-untranslated region (UTR) and C-terminal part of the coat protein region of viral RNA was used as probe in slot-blot hybridization studies. With ^{32}P and DIG probes, total nucleic acid extracts reacted up to 10^{-5} and 10^{-4} of sugarcane leaf, 10^{-3} and 10^{-4} of infected cane juice and 10^{-5} and 10^{-5} dilutions of infected sorghum leaf, respectively (Figure 1). Purified virus and viral RNA reacted up to 50 pg and 1 pg levels, respectively with both the probes (Figure 2). A slight reaction at 10^{-1} dilution of healthy sorghum extract could be due to the pigment interference in colour development with respect to DIG probe (Figure 1). There is a difference in sensitivity levels with both ^{32}P and DIG probes in the detection of virus in sugarcane leaf and cane juice, but no significant difference with respect to purified virus and RNA samples.

Application of nucleic acid hybridization-based tests using radioactive probes for virus detection in less-developed laboratories is not feasible due to lack of facilities for handling radioisotopes and other inherent limitations³². During the last 5–6 years, many researchers have shown that nonradioactive probes can be used successfully for virus detection and can be substituted for ^{32}P without loss of sensitivity³¹. The advantage of DIG probe is that it can be reused 3–4 times without significant loss of sensitivity of the probe. However, the main obstacle in using digoxigenin system for field samples is the binding of substances in crude plant tissue homogenates (in addition to nucleic acids) to the hybridization membranes. Pigmentation on the blots confuses the interpretation of colour development and bound substances can block or interfere with colorimetric detection, causing false positives. Among the tests evaluated in the present study, nucleic acid based tests appear to be sensitive and could be especially useful in screening sugarcane germplasm in breeding programmes.

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