INCREASED RISK OF NEW VIRUS INFECTIONS IN THE SOUTH AFRICAN SUGARCANE INDUSTRY: PREPARING FOR THE FUTURE

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Abstract

Sugarcane is subject to many viral infections that can cause serious yield losses. The development of diagnostic tools and the study of emerging viruses in sugarcane are essential for viral disease control. Pathogen detection and identification are important, especially in quarantine procedures to prevent the introduction of exotic diseases into the South African sugar industry. The *Potyviridae* (transmitted by aphids and which include *Sugarcane mosaic virus*, the causal agent of sugarcane mosaic) and the *Geminiviridae* (which include *Sugarcane streak virus* (SSV) and *Maize streak virus* (MSV)) are important pathogenic viral families. The *Caulimoviridae*, that include badnaviruses such as *Sugarcane bacilliform virus* and *Banana streak virus* (causing streak in bananas) can also become a threat to the sugar industry. The *Closteroviridae* cause diseases such as beet yellows (caused by *Beet yellows virus*) and wheat yellow leaf (caused by *Wheat yellow leaf virus*) that may also be a potential threat to sugarcane.

The aim of this project was to design generic primers for the detection of all viral families that can possibly infect sugarcane. New molecular tests were introduced in the SASRI quarantine glasshouse as a result of this project. These tests include the detection of *Maize streak virus* with the polymerase chain reaction (PCR), *Sugarcane bacilliform virus* (PCR), *Peanut clump virus* with reverse transcriptase (RT-PCR), a wider range of species in the *Potyviridae* family (RT-PCR) as well as a protocol to amplify Tospovirus species vectored by thrips. Using primers from the current study, *Maize streak virus* was recently identified on cultivar N44, a first report of MSV infecting sugarcane in South Africa.

Keywords: sugarcane, diseases, viruses, quarantine, molecular detection

Introduction

The international movement of plant germplasm is necessary because of increasing demands for food, access to germplasm with desirable traits, and access to newly developed or identified varieties for evaluation or inclusion in commercial breeding operations (Harris *et al*., 2000). Plant quarantine policies and regulations exist in many countries to prevent the movement of undesirable pests and/or the introduction of exotic pests when plant germplasm is moved from one location to another (Martin *et al*., 2000). Clonal grasses (including sugarcane) are classified as 'high risk' quarantine material because they have the potential to carry a number of serious exotic diseases (Harris *et al*., 2000). Pathogens such as viruses, phytoplasmas, spiroplasmas and virus-like pathogens are known to infect *Poaceae* species. Plants infected by these pathogens generally show stunting, streaks, stripes, lines or bands, mosaic, mottling or flecking, chlorosis or yellowing, reddening or red stripes, deformation, galls or enations. Sometimes these pathogens cause symptomless infections (Harris *et al*., 2000). Viruses can cause considerable losses in the sugarcane crop yield and quality,

especially when a latent or symptomless infection occurs. Latent infections usually result in plants that grow more slowly, attain a smaller size and react in various other ways that go unnoticed (Waterworth and Hadidi, 1998).

According to Rybicki and Pietersen (1999), there are three major groups of viruses that seem to be most important among new disease-causing agents worldwide. These could be called 'emerging' in terms of apparently new viruses causing new and serious diseases. The most important of these are in the taxonomic families *Potyviridae*, *Geminiviridae*, *Bunyaviridae* of the genus Tospoviruses, and the pararetroviruses such as *Banana streak virus* (BSV). Weeds and grasses may serve as reservoirs for many plant viruses and their vectors (Rybicki and Pietersen, 1999).

Objectives
A first objective of this project was to identify families of viruses that are pathogenic to sugarcane and could be a quarantine risk, and therefore could become of economic importance in the future to the South African sugar industry. The aim was to develop a PCR system using broad spectrum or universal primers for the detection of families of plant viruses for quarantine purposes. Since primer design is improving and sequences are constantly added to the databases, the first step was to check for published primers for the virus groups of interest. The second part of the project was to test generic primers designed in the first objective against a number of viruses and hosts to establish their ability to reliably amplify specified viral sequences. In the following section the families of viruses that were investigated will be discussed.

General presentation

Potyviridae
Potyviridae is an important pathogenic viral family that includes the Potyviruses (Table 1). Potyviruses are one of the most successful groups of plant pathogens in the world (Rybicki and Pietersen, 1999).

New Potyviruses seem to appear with new crop introductions wherever these occur (Rybicki and Pietersen, 1999), and are a serious quarantine threat for sugarcane because of latent infections. The Potyviruses are the causal agent of sugarcane mosaic in sugarcane. The SCMV complex has been shown to consist of four distinct Potyviruses and includes strains of *Johnsongrass mosaic virus* (JGMV), *Maize dwarf mosaic virus* (MDMV), *Sorghum mosaic virus* (SrMV) and SCMV (Yang and Mirkov, 1997). Additionally the SCMV complex consists of different strains, namely SCMV-A, SCMV-B, SCMV-BC, SCMV-Brisbane, SCMV-Bundaberg, SCMV-D, SCMV-E, SCMV-Isis, SCMV-MDB, SCMV-Sabi, SCMV-SC (Gough and Shukla, 1981; Oertel *et al.*, 1997; Shukla *et al.,* 1987) and MDMV-B, which are close to SCMV-A (Handley *et al.,* 1998). *Sorghum concentric ringspot virus, Sorghum red stripe virus, Grass mosaic virus, European maize dwarf virus, Maize mosaic virus* and possibly also *Abaca mosaic potyvirus* (the only SCMV strain to have a non-grass as its natural host), may also be strains of SCMV *(*McKern *et al.,* 1991; Shukla, 1994*;* Alegria *et al.,* 2003; Chen *et al.*, 2002*).*

Natural hosts for MDMV are *Sorghum bicolor*, *S. halepense* and *Zea mays*. SrMV was originally grouped with *Sugarcane mosaic potyvirus*, and so its described strains are SCMV-I, SCMV-M, SCMV-H and SCMV-H SI (Giorda *et al.*, 1986). RT-PCR-based RFLP analysis was used to distinguish strains of SCMV and SrMV (Yang and Mirkov, 1997). Sequencing of the sugarcane striate mosaic virus indicated that the pathogen causing striate mosaic of

sugarcane is related to a Carlavirus (Choi and Randles, 1997). The virus is spread between plants by aphids (*Melanaphis sacchari*) and transmitted in cuttings as well as by mechanical inoculation. Mite-transmitted species of the family *Potyviridae* include *Ryegrass mosaic virus* (RGMV), *Wheat streak mosaic virus* from the genus *Tritimovirus*, *Brome streak mosaic virus* (BrSMV) and *Agropyron mosaic virus* (Stenger *et al.*, 1998). Published sequences of most of the abovementioned species were used in the process of finding universal primers that would detect the *Potyviridae* family as a whole.

Table 1. Summary of the genus, species and strains of the *Potyviridae* family that might be infectious to sugarcane.

Family	Genus	Species	Strain
Potyviridae	*Potyvirus*	Sugarcane mosaic virus complex: *Johnsongrass mosaic virus* (JGMV), *Sugarcane mosaic virus* (SCMV)	*Sugarcane mosaic virus* (SCMV) strains: SCMV-A, SCMV-B, SCMV-BC, SCMV-Brisbane, SCMV-Bundaberg, SCMV-D, SCMV-E, SCMV-Isis, SCMV-MDB, SCMV-Sabi, SCMV-SC, MDMV-B *SCMV-I, SCMV-M, SCMV-H and SCMV-H SI
		Sorghum mosaic virus (SrMV)	***Sorghum concentric ringspot virus, Sorghum red stripe virus, Grass mosaic virus, European maize dwarf virus*
		Maize dwarf mosaic virus (MDMV)	
	Tritimovirus	*Sugarcane streak mosaic virus, Wheat streak mosaic virus*	

*Possible strains of SCMV
**Possible strains of SrMV

Geminiviridae
Mastreviruses, which fall into the family *Geminiviridae*, are responsible for streak diseases on graminaceous plants in Africa and Asia, and include *Maize streak virus* (MSV) and *Sugarcane streak virus* (SSV). The virus is transmitted by leafhoppers such as *Cicadulina mbila* and *C. bipunctatus* (syn. *bipunctella*). Earlier it was presumed that MSV overwinters in grasses and/or crops such as wheat, that overlap maize crops. However, gene sequence studies have shown that a different strain of MSV is common in grasses. In Bigarre *et al.* (1999) the Natal (type) strain, Egyptian strain and Mauritius strain are considered as distinct virus species (*Sugarcane streak virus* or SSV, *Sugarcane streak Egypt virus* or SSEV and *Sugarcane streak Mauritius virus* or SSMV). Until recently SSV was considered to be a strain of *Maize streak mastrevirus;* however, sequencing has shown that three species, SSV, MSV and *Panicum streak virus* comprise the African cereal streak viruses, and they are close to *Digitaria streak mastrevirus*. Susceptible plant species includes *Saccharum officinarum* and *Zea mays*. SSV affects leaves, shoots and tillers of sugarcane. The virus is transmitted in cuttings, not transmitted by seed and not transmitted by mechanical inoculation.

Members of the *Geminiviridae* family are given in Table 2.

Table 2: Members of the *Geminiviridae*.

Family	Genus	Species	Strains
Geminiviridae	*Mastrevirus*	*Sugarcane streak virus, Maize streak virus, Wheat dwarf virus*	SCS (Natal) SCS Mauritius (SCSMV) SCS Egypt (SCSEV) SCS Senso stricto (SA)
	Begomovirus	*Tomato yellow leaf curl virus* (transmitted by white flies)	
	Curtovirus	Beet curly top virus	

Closteroviridae

The taxonomy of the family *Closteroviridae* was revised by Martelli *et al*. (2002) and three genera, *Ampelovirus*, *Closterovirus*, and *Crinivirus,* were identified (Table 3).

Table 3. Summary of the *Closteroviridae* family of viruses.

Family	Genus	Species
Closteroviridae	Closterovirus	*Beet yellows virus* *Wheat yellow leaf virus* *Citrus tristeza virus* *Mint vein binding virus*
	Crinivirus	*Lettuce infectious yellows virus* *Tomato chlorosis virus* *Sweet potato chlorotic stunt virus*
	Ampelovirus	*Grapevine leafroll - ass virus 3* *Sugarcane mild mottle (mosaic) virus*

Under laboratory trial conditions susceptibility to infection by the *Closteroviridae* family of viruses was found in several plant families as well as in the *Graminae* (in which sugarcane falls). These viruses, including *Sugarcane mild mottle (mosaic)* (SCMMV), (Ampelovirus group), are spread by mealybugs and soft scale insects. *Sugarcane mild mosaic closterovirus* can cause mild mosaic and chlorotic stripes in sugarcane. The virus is transmitted by *Saccharicoccus sacchari,* the pink sugarcane mealybug. SMMV has only been found in mixed infections with *Sugarcane bacilliform badnavirus*, although it is not known whether the association is obligatory (Lockhart *et al*., 1992).

Luteoviridae

The natural host of *Sugarcane yellow leaf virus* (SCYLV), a member of the *Luteoviridae* family, is sugarcane, and this virus can cause severe leaf yellowing in the crop. The *Luteoviridae* are transmitted by insects of the *Aphididae* family (aphids). SCYLV can be transmitted by *Melanaphis sacchari* and *Rhopalosiphum maydis* (Scagliusi and Lockhart, 1997). Yellow leaf syndrome in Brazil significantly decreases the sugar yield of variety SP71-6163 (Lockhart *et al.,* 1996). Effects on yields have also been reported in other countries such as Colombia, the USA (Florida), Guadelupe in the West Indies and Reunion Island.

Cereal yellow dwarf polerovirus and *Barley yellow dwarf luteovirus* also belongs to the family *Luteoviridae* (Table 4). Natural hosts for *Cereal yellow dwarf polerovirus* are *Avena sativa* (oats), *Cynodon dactylon*, *Dactylis glomerata* (cocksfoot), *Eragrostis curvula*,

Hordeum vulgare (barley), *L. perenne* (perennial ryegrass), *Sorghum bicolor*, *Triticum aestivum* (wheat), *Triticum durum* and *Zea mays* (maize). It causes yellowing, stunting and reddening in these crops (Henry *et al*., 1992; Kendall *et al.,* 1996; McKirdy and Jones, 1993). Sequences from all three genera were used to design primers.

Table 4. Summary of putative *Luteoviridae* viruses that could become a threat to sugarcane.

Family	Genus	Species
Luteoviridae	*Polerovirus*	*Sugarcane yellow leaf virus* *Potato leafroll virus* *Cereal yellow dwarf virus*
	Luteovirus	*Barley yellow dwarf virus*
	Enamovirus	*Pea enation virus-1*

Bunyaviridae

There is concern in the South African industry that *Fulmekiola serrata* (sugarcane thrips) could become a virus vector. *Thrips tabaci*, for example, is responsible for the transmission of at least 12 virus diseases in southern Africa including *Tomato spotted wilt virus* (TSWV) (Table 5) (Hartwig, 1985). Worldwide, the tospoviruses are an important emerging viral group infecting species in 34 dicotyledon and seven monocotyledon families including the *Poaceae*. For example, *Cynodon dactylon*, a common grass weed in sugarcane fields, has been found to host TSWV following natural infection (Jorda *et al*., 1995). *Rice stripe tenuivirus* affects leaves, shoots and tillers of rice. The virus is spread between plants by *Delphacidae* (planthoppers). The virus is not seed-transmitted but it can be transmitted by mechanical inoculation (but only with great difficulty).

Table 5. Summary of the *Bunyaviridae*.

Family	Genus	Species
Bunyaviridae	*Tospovirus*	*Tomato spotted wilt virus*
	Tenuivirus	*Rice stripe virus*

Reoviridae

Fiji disease fiji virus was first reported in *Saccharum* species in Australia and the Phillipines in 1883. The symptoms include leaf deformation and leaf galls. Susceptible host species include *Saccharum officinarum*, *Zea mays* and *Sorghum bicolor*. The virus is transmitted by a the insect *Perkinsiella saccharicida. Fiji virus disease* is a serious risk for the South African sugarcane industry, as it does not occur in the country at present. *Saccharum* spp. can act as diagnostically insusceptible host species of *Maize rough dwarf fiji virus* (Table 6).

Table 6. Summary of family *Reoviridae* infecting *Graminae*.

Family	Genus	Species
Reoviridae	*Fijivirus*	*Fiji disease fiji virus* *Maize rough dwarf fiji virus*

Caulimoviridae

Sugarcane bacilliform virus (SCBV) is a pathogen of sugarcane and can cause yield losses in some varieties (Braithwaite *et al.*, 1995). The symptoms of infection are variable, some varieties display flecks or freckles on the leaves while other varieties stay symptomless. The major vector is the pink sugarcane mealybug, *Saccharicoccus sacchari.* SCBV can be transmitted by *S. sacchari* from infected sugarcane to banana and *Banana streak virus* (BSV) can be mechanically transmitted to sugarcane, although no foliar symptoms are observed. Because of this it was suggested that BSV and SBV are strains of the same virus (Braithwaite *et al.*, 1995) and these sequences were used to design primers for the *Caulimoviridae* family (Table 7).

Table 7. Members of the family *Caulimoviridae.*

Family	Genus	Species
Caulimoviridae	*Badnaviruses*	*Sugarcane Bacilliform virus* *Banana bacilliform badnavirus* *Banana streak badnavirus*

Sequiviridae

The *Sequiviridae* consists of two genera, namely Waikavirus and Sequivirus. The waikavirus, *Maize chlorotic dwarf virus* (MCDV), is carried by the black-faced leafhopper, *Graminella nigrifrons* Forbes. The leafhopper overwinters in the perennial weed Johnson grass (*Sorhum halepense*) that can be infected with other sugarcane pathogens such as *Leifsonia xyli* subsp *xyli*. MCDV infects maize and some other graminaceous hosts, but not rice. *Rice tungro spherical virus* (RTSV), a member of the waikaviruses, infects rice and some other graminaceous hosts.

Viruses not assigned to a family

African peanut clump pecluvirus (also known as African peanut clump furovirus, and *Sugarcane red leaf mottle virus*) falls in the *Pecluvirus* genus, and has not been assigned to a Family (Table 8). In sugarcane it causes chlorotic stripes that extend along the leaf blade, and that become white, rusty or wine-red, depending on the variety. This virus has not been detected in South Africa and is a serious quarantine risk. *Indian peanut clump pecluvirus* is also part of the genus *Pecluviruses*, has also not been detected in South Africa. Natural hosts for *Indian peanut clump virus* are *Sorghum arundinaceum, Sorghum bicolor* and *Setaria italica.*

Table 8: Viruses unassigned to a family.

Family	Genus	Species
Unassigned	*Pecluvirus*	*Peanut clump pecluvirus* *Indian peanut clump virus*

Virus-like families

Sugarcane chlorotic streak virus-like pathogen infects *Saccharum officinarum*, *Brachiaria mutica*, *Imperata cylindrica* and *Pennisetum purpureum*. The pathogen causes chlorotic streaking of leaves. It is speculated that the virus is spread between plants by a vector insect, a mite or possibly a leafhopper. There is no specific assay for chlorotic streak because the causal agent has not been identified (Magarey and Egan, 2000).

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Materials and Methods

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Virus identification: A literature search was done to identify families of viruses that are pathogenic to sugarcane or grass species, or which could become important pathogens in the South African sugar industry in the future.

Primer design: In the first phase, the literature was scanned to find published primer sequences for the detection of viral families that could affect sugarcane. Additionally, published sequences of viruses were obtained from the Genbank database and these sequences were aligned to design generic primers. After possible primers were identified by sequence comparisons, the designed primers were tested for their self-annealing or primer dimer formations, as well as the determination of their melting points by using the OligoCalc software (http://www.basic.northwestern.edu/biotools/oligocalc.html). Primer sequences were then compared to published sequences on the Genbank database to confirm their ability to detect the targeted sequences. In the case where no primers could be designed for a family or a viral genus, specific published species primers were used to put diagnostic tests into place to be used in general pathology and quarantine diagnosis.

Sequencing of amplified fragments: PCR-based sequencing reactions were carried out in a thermal cycler (Eppendorff Gradient) with ABI PRISM TM dye terminator cycle sequencing using the Perkin Elmer ready reaction kit with AmpliTaq R DNA Polymerase, FS. DNA was amplified in a 20 µl reaction mixture containing 4 µl terminator ready reaction mix, 4 µl 2.5X sequencing buffer, 0.2 µM primer and 100 ng. The following cycling parameters were used: an initial denaturation cycle of 1 min at 96ºC, 30 cycles of 10 sec at 96ºC, 5 sec at 50ºC and 4 min at 60ºC. Extension products were precipitated in 80 µl of 75% isopropanol to obtain a working concentration of 5% (v/v) for 15 minutes at room temperature. The pellet was dried on a heating block and resuspended in 20 µl of Hi-Bi Formamide, heated for 5 min at 100ºC, cooled on ice and loaded in the ABI Prism Genetic Analyser for sequence analysis.

Alignment of sequences and identification: Sequences obtained were compared with sequences on the Genbank database using the BLASTN search tool.

Potyviridae
Total RNA extractions were done from sugarcane leaves infected with SCMV (from various sugarcane varieties from South Africa), by using the Qiagen RNeasy Mini kit (Cat. no. 74904) according to the manufacturer's instructions. Total RNA was eluted in 30 µl DEPC-treated water and stored at -80ºC.

Primers for the *Potyviridae* were designed by aligning known sequences obtained from the Genbank (BLASTn) database (Primer pair P10 and P11). The following sequences were used in the alignment: *Sorghum mosaic virus, Maize dwarf mosaic virus, Sugarcane mosaic virus* (all strains) and *Johnsongrass mosaic virus*. Primer sequences were compared with published sequences on the Genbank database to confirm their ability to detect the targeted sequences. Other primers used in this study include primer pairs SCMVF4 and SCMVR3 (Alegria *et al.*, 2003) and Oligo1 and Oligo2 (Grisoni *et al.,* 2004).

Geminiviridae
DNA extraction: DNA was extracted from sugarcane leaves infected with *Sugarcane streak virus* (Variety Uba), *Maize streak virus* (South African variety N44) by using the Qiagen DNeasy Plant Mini Kit (Cat. no 69104) according to the manufacturer's instructions.

Primers to detect streak were designed by aligning known streak viral sequences obtained from the Genbank (BLAST) database. These include the *Sugarcane streak virus* strains Natal, Reunion and Egypt as well as the Maize streak virus sequence. Primer sequences were compared with published sequences on the Genbank database to confirm their ability to detect the targeted sequences. Published primers (Rybicki and Hughes, 1990) were also used in this project.

PCR conditions and amplification (Mastreviruses): PCR was prepared in a 25 µl volume, containing 10X buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), $MgCl_2$ (75 mM), dNTP mix (4 mM), 0.275 µM of each primer, 1 unit of Taq DNA polymerase and 2 µl of the DNA template. The amplification profile consisted of initial denaturation of 2 min at 94ºC, followed by 30 cycles of denaturing at 94ºC for 1 min, primer annealing at 53ºC for 30 sec and primer extension at 72ºC for 1 min and a final extension cycle at 72ºC for 5 minutes. PCR products were visualized in agarose gels (2%) stained with ethidium bromide and viewed under UV light.

Closteroviridae
Primer design: Known sequences of the *Closteroviridae* obtained from the Genbank (BLAST) database were aligned and we attempted to design degenerate primers for this family. Degenerate primer pair HSP1 and HSP2 (Tian *et al.,* 1996) was used on various sugarcane varieties without any symptoms. These primers can be used to amplify extracted RNA from *Citrus tristeza virus* as well as *Grapevine leafroll associated virus 3*. Specific primers for *Citrus tristeza virus* (PEX 1 and PEX 2, PIN 1 and PIN 2) as well as *Gapevine leafroll associated virus 3* (C547 and H229, Acheche *et al*., 1999) are available but were not used in this study.

RT-PCR was carried out using the Titan One-Step RT-PCR kit (Roche, Cat. no. 1 855 476). The 25 µl RT-PCR reaction mix consisted of 2 µl eluted RNA, 2.5 µl of the 10X buffer, 200 µM (0.2 mM), 0.2 mM each of dNTP mix, 5 mM DTT solution, 1 µl of the Titan Enzyme mix, 2.5 mM $MgCl_2$, 10 mM each of the primer as well as DEPC-treated water. The RT-PCR programme was 42ºC for 45 min, 94ºC for 15 min, 30 cycles at 94ºC for 30 sec, 50ºC for 30 sec and 72ºC for 40 sec, with a final 72ºC extension for 5 min. The amplified product was analysed by electrophoreses through a 1% agarose gel.

Luteoviridae
Isolation of total RNA: RNA extractions were done from sugarcane leaves infected with SCYLV (from South Africa), by using the Qiagen RNeasy Mini kit (Cat. no. 74904) according to the manufacturer's instructions. Total RNA was eluted in 30 µl DEPC-treated water and stored at -80ºC.

Primer design: Primers were designed by comparing several published *Luteoviridae* sequences. Primer pair L1/L4 was designed by comparing yellow leaf virus sequences published on the Genbank database from Brazil, China, Peru, Cuba, Reunion and a sequence from variety CP66-357 to each other by using the ClustalW software on the Internet. Primer sequences (YLS 462 and YLS 111) were kindly provided by Dr M Irey, US Sugar Corporation, FL, USA. These primers amplify part of the gene encoding the coat protein of SCYLV and were used in detection of the virus.

RT-PCR was carried out using the Titan One-Step RT-PCR kit (Roche, Cat. no. 1 855 476). The 25 µl RT-PCR reaction mix consisted of 2 µl eluted RNA, 2.5 µl of the 10X buffer, 200 µM (0.2 mM), 0.2 mM each of dNTP mix, 5 mM DTT solution, 1 µl of the Titan Enzyme

mix, 2.5 mM MgCl$_2$, 10 mM each of the primer and DEPC-treated water. The RT-PCR programme was 45ºC for 45 min, 94ºC for 2 min, 35 cycles at 94ºC for 1 min, 56ºC for 1 min and 72ºC for 1 min, with a final 72ºC extension for 10 min. The amplified product was analysed by electrophoreses through a 1% agarose gel.

Bunyaviridae

Isolation of total RNA: RNA extractions were done from sugarcane leaves infected with SCMV and SCYLV (from South Africa), by using the Qiagen RNeasy Mini kit (Cat. no. 74904) according to the manufacturer's instructions. Total RNA was eluted in 30 µl DEPC-treated water and stored at -80ºC.

RT-PCR was carried out using the Roche One-Step RT-PCR kit (Cat. no. 1 855 476) according to the manufacturer's instructions. Primer pair BR60 and BR65 (Eiras *et al.,* 2001) was used to amplify extracted RNA. The 25 µl RT-PCR reaction mix consisted of 2 µl eluted RNA, 2.5 µl of the 10X buffer, 200 µM (0.2 mM), 0.2 mM each of dNTP mix, 5 mM DTT solution, 1 µl of the Titan Enzyme mix, 2.5 mM MgCl$_2$, 10 mM each of the primer, and DEPC-treated water. The RT-PCR programme was 50ºC for 30 min, 95ºC for 15 min, 35 cycles at 94ºC for 1 min, 48ºC for 48 sec and 72ºC for 1 min with a final 68ºC extension for 7 min. The amplified product was analyzed by electrophoreses through a 1% agarose gel.

Reoviridae

RNA was kindly provided by Grant Smith from BSES Ltd, Australia.

Primer pair FDV7F and FDV7R (Smith and van der Velde, 1994) was used for the amplification of *Fiji disease virus*.

RT-PCR was carried out using the Roche One-Step RT-PCR kit (Cat. no. 1 855 476) according to the manufacturer's instructions.

Caulimoviridae

DNA extraction: DNA was extracted from sugarcane leaves infected with *Sugarcane Bacilliform virus* (obtained from Salem Saumtally, MSRI, Mauritius) by using the Qiagen DNeasy Plant Mini Kit (Cat. no. 69104) according to the manufacturer's instructions.

PCR amplification was standardised for *Sugarcane bacilliform virus* (SCBV) from infected sugarcane clones with two sets of primers namely the *Badnavirus* group primers published by Baranwal *et al.* (2005) and SCBV specific primers by Braithwaite *et al.* (1995).

Viruses not assigned to a family

Peanut clump viral RNA was kindly provided by Jean-Claude Girard (CIRAD, France) and Claude Bragard and Benjamin Dieryck from the Phytopathology Unit at UCL, Belgium. A primer pair designed by Bragard *et al*. (2000) was used for the amplification of *Pecluviruses* (*Peanut clump virus*).

RT-PCR was carried out using the Titan One-Step RT-PCR kit (Roche, Cat. no. 1 855 476). The 25 µl RT-PCR reaction mix consisted of 2 µl eluted RNA, 2.5 µl of the 10X buffer, 200 µM (0.2 mM), 0.2 mM each of dNTP mix, 5 mM DTT solution, 1 µl of the Titan Enzyme mix, 2.5 mM MgCl$_2$, 10 mM each of the primer and DEPC-treated water. The RT-PCR programme was 42ºC for 60 min, 94ºC for 2 min, 35 cycles at 94ºC for 10 sec, 55ºC for 30 sec and 72C for 1 min, with a final 72ºC extension for 8 min. The amplified product was analysed by electrophoreses through a 1% agarose gel.

Results

No universal primers could be found in the literature that would amplify all members of a viral family. Attempts to design primers for viral families using sequences from the Genbank database also proved to be very difficult and no primers could be designed. Eventually published primers used for the diagnosis of specific viruses were used and the tests were refined to suit the SASRI laboratories.

Potyviridae
The most suitable primers designed for detection of *Potyviridae* were P11 and P10 (Table 9).

Table 9. Primers designed to amplify most members of the *Potyviridae*.

P11 FORWARD 5'GC(T/A)CC(A/G)TAT(A/C/G)(G/T)C(A/C/T)GA(A/G)AC(A/T)GC(/C/A)CT3'
P10 REVERSE 5'TTTAGTGATGC(C/A)GC(T/A)GAAGCGTA3'

Although no amplification could be obtained by using designed primers P10 and P11, future work will include optimisation of the PCR. Primer pair SCMVF4 and SCMVR3 (Alegria *et al.*, 2003) amplified SCMV-infected plants successfully (Figure 1a). Primer pair Oligo1 and Oligo 2 (Grisoni *et al.,* 2004) amplified a 300 bp fragment from all sugarcane RNA (SCMV-infected as well as non-infected sugarcane) tested (Figure 1b). This fragment will be sequenced to determine whether it originates from viral or plant genes. No positive controls for *Johnson grass mosaic virus*, *Maize dwarf mosaic virus*, *Sorghum mosaic virus*, *Sugarcane streak mosaic virus* or *Sugarcane striate mosaic virus* could be obtained for testing primers by RT-PCR.

No amplification of *Sugarcane striate mosaic virus*, *Sorghum mosaic virus* and *Sugarcane streak mosaic virus* could be achieved when the available sugarcane RNA samples (varieties N44, N36, N45 and N42 from SASRI) were used as template in these reactions. No positive controls could be obtained for these viruses and thus the RT-PCR parameters could not be optimised for these PCRs.
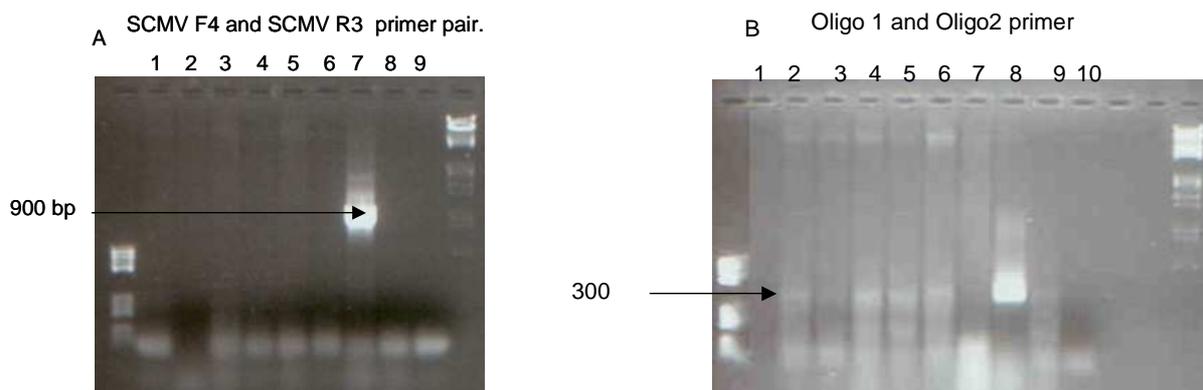

Figure 1. Amplification of sugarcane RNA with primer pair SCMVF4 and SCMVR3 (A) and primer pair Oligo1 and Oligo2 (B). First lane: molecular weight marker 5, Lane 1: variety N44, Lane 2: variety N44, Lane 3: variety N42, Lane 4: variety N36, Lane 5: variety N45, Lane 6: sugarcane damaged by thrips, Lane 7: SCMV-infected cane, Lane 8: disease-free sugarcane, Lane 9: negative control, Lane 10: water control, Last lane: molecular weight marker 3.

Geminiviridae (*Mastreviruses*)
The sequence of the designed primers is listed in Table 10.

Table 10. Sequences of primer pair used in this study for the detection of streak viruses.

| G4 F: 5'AG(T/G/C)(G/T)(T/G)(G/T)(T/G)(G/C/T)CATCG(G/C)TTCGT3' |
| G6 R: 5'CTGTACATCCTCGGGCCAACAAGAAC3' |

Streak virus sequences (G4/G6) were designed by aligning known streak viral sequences obtained from the Genbank (BLAST) database. These include the *Sugarcane streak virus* strains Natal, Reunion and Egypt, as well as the *Maize Streak virus* sequence. A fragment of 1200 bp was amplified from DNA extracted from variety N44 (collected on the lower south coast near Umzimkulu in the KwaZulu-Natal province of South Africa) with apparent *Maize streak virus* symptoms as well as from a grass that grew on the edge of the field where the symptoms were found when using primer pair V2/C1 (Rybicki and Hughes, 1990). A fragment of 900 bp was amplified from the DNA when primer pair G4/G6 was used on the same samples (Figure 2). Both the fragments were purified with the Qiagen Gel extraction kit and sequenced. The sequences from using primer pair G4/G6 failed, except for one sequence using primer G4. The result for this sequence when it was compared to the published sequences for the different streak viruses also showed the highest homology to *Maize streak virus*. Sequences obtained with using primers V2 and C1 were compared to published sequences on the Genbank database (BLASTn) and the results indicated that the strain of virus found in N44 was *Maize streak virus*.
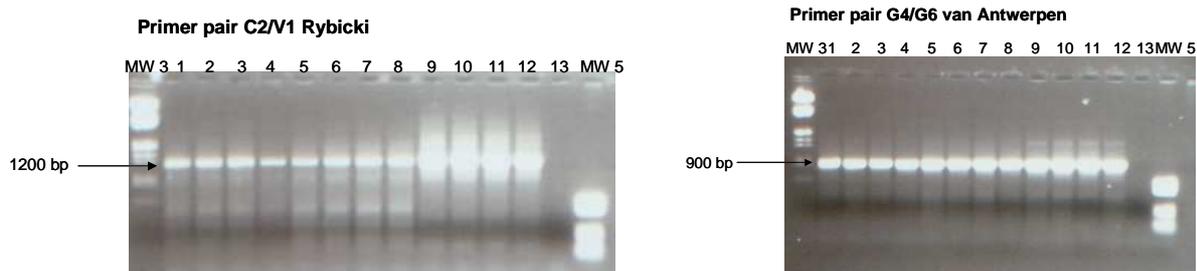

Figure 2. Amplification products for primer pairs V2/C1 (left) and G4/G6 (right). First lane: molecular weight marker 3, Lanes 1-4: variety N44 (collected 24 May), Lanes 5-8: variety N44 (collected 30 May), Lanes 9-12: grass from field next to Variety N44, Lane 13: water control, Last lane: molecular weight marker 5.

Reoviridae
A full set of primers to amplify the family Reoviridae could not be designed, so specific primer pair FDV7F and FDV7R (Smith *et al.,* 1994) were used and successfully amplified a 450 bp fragment from Fiji disease virus. None of the South African samples tested positive for Fiji disease virus.

Closteroviridae
No primers could be designed that could amplify the *Closteroviridae* in this study. The generic primers published by Tian *et al*. (1996) were used for amplification in this study but did not amplify *Closteroviruses* in any of the sugarcane RNA (varieties N42, N44, N45 and N46 from SASRI) used in this study. For further studies with these primers, a positive control for the *Closteroviridae* will have to be obtained.

Luteoviridae

Primer pair L1 and L4 did not amplify the SCYLV from sugarcane RNA and it was thus decided to carry on testing for SCYLV with the primers provided by M Irey. Amplification parameters for primer pair L1/L4 will be tested further in order to amplify more members of the *Luteoviridae* that can be of significance in the sugarcane industry. None of the other genuses in the *Luteoviridae* family (poleroviruses and enamoviruses) could be amplified in this project due to the lack of positive controls. This will also be investigated further.

Bunyaviridae

Two amplification products were detected under UV light, with sizes of 300 and 200 bp respectively (Figure 3). However, these fragment sizes were smaller than the expected 514 bp for primer pair BR60/BR65. The amplified product occurred in all the sugarcane RNA samples used in this study, so it might be a non-specific amplification of sugarcane RNA. These fragments will be sequenced to determine their identity.
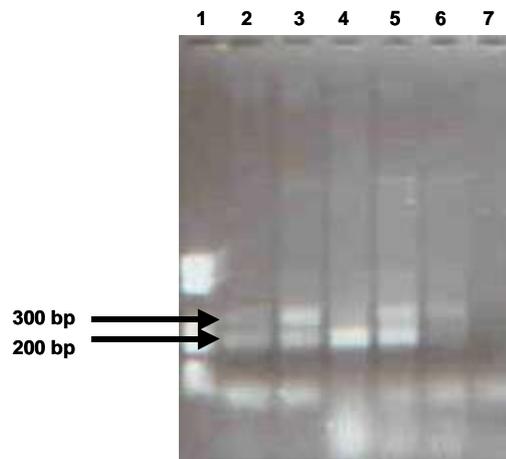

Figure 3. RT-PCR amplification of sugarcane RNA using primer pair BR60/BR65 (Eiras *et al.*, 2001). Lane 1: molecular weight marker 5, Lane 2: variety N44 from Umzimkulu (collected 24 May 2007), Lane 3: variety N44 from Umzimkulu (collected 24 May 2007), Lane 4: sugarcane damaged by thrips, Lane 5: sugarcane infected with SCMV, Lane 6: disease-free sugarcane RNA Lane 7: water control.

Caulimoviridae

No amplification of DNA was achieved when the *Badnavirus* group primers were used in PCR. However, SCBV-specific primers (SCBV F and SCBV R) amplified a 221 bp DNA fragment in *Sugarcane bacilliform virus* infected leaves received from Mauritius (Figure 4).
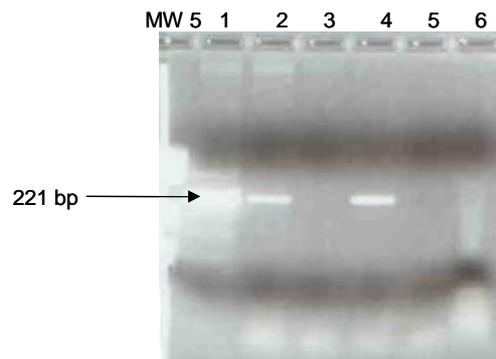
Figure 4. Amplification products of SCBV DNA by using primer pair SCBV F and SCBV R. First lane: molecular weight marker 5, Lane 1: 5 μl of SCBV-infected sugarcane leaf DNA, Lane 2: 4 μl of SCBV-infected sugarcane leaf DNA, Lane 3: 3 μl of SCBV-infected sugarcane leaf DNA, Lane 4: 2 μl of SCBV-infected sugarcane leaf DNA, Lane 5: 1 μl of SCBV-infected sugarcane leaf DNA, Lane 6: negative control.

Viruses from an unassigned family

The primers designed by Bragard *et al.* (2006) amplified a 210 bp fragment from the peanut clump viral RNA provided by Claude Bragard and Benjamin Dieryck. However, fragments of about 190 and 300 bp were amplified from sugarcane variety N44, from grass found next to a field with streak symptoms, and from varieties N39 and N40 with the primers specific for peanut clump virus. This fragment will be sequenced and compared with other published sequences on the NCBI Blast programme.

Conclusion

Effective quarantine requires an adaptable approach to diagnosis (Harris *et al*., 2000). Viruses are intracellular parasites. They often infect plants such as sugarcane systemically and are usually transmitted through sugarcane cuttings. Although in some grass hosts they cause no symptoms, it is as important, from a quarantine standpoint, to recognise and exclude such plants because the pathogens may cause disease in other host plants such as sugarcane. Traditionally, viruses have been detected using one of the following approaches or as a combination, (i) visual inspection for symptoms, (ii) transmission by mechanical inoculation and/or vectors to a range of indicator plants, (iii) electron microscopy to search for characteristic particles, cells or other cytological features in infected tissue, and (iv) serological testing (immunoassays) to detect the presence of antigens of the pathogen.

However, in the past 10 years, various versions of the polymerase chain reaction (PCR) to detect specific gene sequences of the pathogen have proven to be superior to these traditional diagnostic methods for many purposes. To refine the process more, PCR can be performed with universal primers that would be able to detect most members of a viral family (Alegria *et. al*., 2003). In this study most of the universal primers designed did not amplify the targeted RNA or DNA and thus more attention will be given to the optimisation of PCR parameters in future work.

Tospoviruses replicate in their thrips vectors, thus the insects not only spread the virus but also serve as a virus host (Meena *et al*., 2005). The two fragments (200 bp and 300 bp, respectively) that were amplified with the BR60/BR65 degenerative primers could be detected in all sugarcane RNA samples used. Future work will include the sequencing of these two fragments, as well as the use of another primer pair designed by Chen *et al*. (2005) for the detection of tospoviruses. Positive controls for this reaction should be obtained from other sources. A survey where all grasses and sugarcane will be sampled all over the sugar industry, will commence shortly to detect any tospoviral infection in other parts of the industry.

Amplification of *Sugarcane streak virus* DNA has not previously been done at SASRI because of the absence of a positive control. However, when the outbreak of streak symptoms occurred in variety N44 in the sugar industry, *Maize streak virus* could be amplified from these samples by using the primers published by Rybicki and Hughes (1990) as well as the primers designed in this current project (G4/G6). This places SASRI in a position to now use this molecular tool to detect streak virus in quarantine and add the test to the battery of tests that are done in quarantine to screen imported clones of sugarcane for diseases. Further work will include the optimisation of sequencing with primer pair G4/G6 and to also sequence the sugarcane streak virus present in the variety Uba at SASRI.

Sugarcane bacilliform badnavirus was detected in nucleic acid from diseased sugarcane leaves by PCR amplification of a 221 bp fragment with primer pair SCBVF5 and SCBVR5. Amplification of SCBV sequences from three different sugarcane varieties revealed considerable variability in the viral populations, both within single infected plants and between infected plants, suggesting that the SCBV isolates sequenced to date may not be representative of the range of virus variability. In this study the primers used for the amplification of the Australian strains of SCBV also amplified the Mauritian strain of the group, which make the primers safe to use for most SCBV strains worldwide. This method can be applied to screening of germplasm in quarantine and in the field.

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