High throughput multiplex real time PCR assay for the simultaneous quantification of DNA and RNA viruses infecting cassava plants

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Running head: Detection of cassava viruses by multiplex real-time PCR

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ABSTRACT

Aims: To develop a multiplex TaqMan-based real-time PCR assay (qPCR) for the simultaneous detection and quantification of both RNA and DNA viruses affecting cassava (*Manihot esculenta*) in eastern Africa.

Methods and Results: The diagnostic assay was developed for two RNA viruses; *Cassava brown streak virus* (CBSV) and *Uganda cassava brown streak virus* (UCBSV) and two predominant DNA viruses; *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV), which cause the economically important cassava brown streak disease (CBSD) and cassava mosaic disease (CMD), respectively. Our method, developed by analysing PCR products of viruses, was highly sensitive to detect target viruses from very low quantities of 4 to 10 femtograms. Multiplexing did not diminish the sensitivity or accuracy compared to uniplex alternatives. The assay reliably detected and quantified four cassava viruses in field samples where CBSV and UCBSV synergy was observed in majority of mixed-infected varieties.

Conclusions: We have developed a high-throughput qPCR diagnostic assay capable of specific and sensitive quantification of predominant DNA and RNA viruses of cassava in eastern Africa.

Significance and Impact of Study: The qPCR methods are a great improvement on the existing methods and can be used for monitoring virus spread as well as for accurate evaluation of the cassava varieties for virus resistance.

INTRODUCTION

Cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) are two major constraints to cassava production in Africa. CMD is caused by nine DNA virus species in Africa (Legg *et al.* 2015), which are commonly referred to as cassava mosaic begomoviruses (CMBs) (genus *Begomovirus*, family *Geminiviridae*). Among them, *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) and EACMV-like species such as the Uganda variant (EACMV-Ug) are widespread in the eastern parts of sub-Saharan Africa (SSA) (Legg *et al.* 2015). EACMV-Ug caused a major outbreak of CMD in Uganda in the 1990s (Zhou *et al.* 1997), which has then spread across neighbouring countries in the Great Lakes region of eastern Africa causing annual losses of 1.2-2.3 billion US$ to African cassava farmers (Thresh *et al.* 1997). These losses are highly likely to be an underestimate as the disease has now expanded into new countries in Angola (Kumar *et al.* 2009) and Cameroon (Akinbade *et al.* 2010) in central Africa.

CBSD is caused by two RNA viruses; *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) (genus *Ipomovirus*; family *Potyviridae*), which are collectively referred to as cassava brown streak viruses (CBSVs). CBSD was largely confined to the lowland areas of eastern Africa for over 70 years since its first description in the 1930s (Hillocks and Jennings, 2003). In recent years, however, severe outbreaks were reported in inland regions of Uganda (Alicai *et al.* 2007) and areas around Lake Victoria including north western Tanzania, south western Kenya (Legg *et al.* 2011) and it has now been on a west-ward movement having been reported in Burundi, Rwanda and DR Congo (Bigirimana *et al.* 2011;

Mulimbi *et al.* 2012). A baseline survey conducted in Kenya, Tanzania, Malawi and Uganda on the economic impact of CBSD indicated a yield loss of 3.2-7.0% in the eastern African region (Hillocks *et al.* 2015), which is equivalent to an annual loss of 1.16-2.53 million tons of fresh cassava roots with a market value of approximately US$290-632 million.

Two strategies were proposed for minimizing these losses and to control the spread of CBSD and CMD in the region. A prevention strategy involving assurance of the health of planting material and cultivation of resistant cassava, and a detection and monitoring strategy involving accurate and affordable laboratory-based diagnosis and large-scale surveillance (Legg *et al.* 2015). A range of PCR-based diagnostic and quantitative methods are currently used for diagnosis and survey of field infections (Alabi *et al.* 2008; Abarshi *et al.* 2010; Moreno *et al.* 2011; Abarshi *et al.* 2012; Adams *et al.* 2013), virus indexing in tissue culture-derived planting material (Maruthi *et al.* 2014a) and screening for new sources of resistance (Kaweesi *et al.* 2014; Maruthi *et al.* 2014b). More effective and holistic implementation of the monitoring and virus management strategies would require a sensitive, high-throughput and cost-effective method which can simultaneously quantify both CBSVs and CMBs for virus indexing and resistance screening. The real time quantitative (qPCR) can fulfil these requirements, considering its high sensitivity combined with the characteristic specificity of the TaqMan probe chemistry. In this study, we report the development of multiplex TaqMan-based qPCR assay for the detection and quantification of the two CBSVs and ACMV and EACMV in a single tube reaction. Standard quality assurance tests such as sensitivity, specificity, intra- and inter-assay variability were performed for the new method. The impact of multiplexing on sensitivity was assessed and the

method was subsequently applied to determining virus quantities in field infections of CMD and CBSD in local east African cassava germplasm.

MATERIALS AND METHODS

Plant and virus material

Two CBSD viruses, CBSV-[Mz:Nam:07] and UCBSV-[UG:Kab:07], originally collected from Mozambique and Uganda, respectively (Abarshi *et al.* 2012) were maintained in the cassava var. Albert. The two CMD viruses ACMV-[UG:Nam:97] and EACMV-[UG:Nam:97] were maintained in var. Ebwanateraka (Maruthi *et al.* 2002). All plants were kept in the quarantine glasshouse facility of the Natural Resources Institute (NRI) in the UK. Leaf samples from upper, middle and lower parts of the 12 to 24 month-old plants with viral symptoms were collected for subsequent nucleic acid extraction. Leaf samples from 81 field-grown six month old cassava plants, representing sixteen cassava varieties, were collected in 2014 from Naliendele in southern Tanzania and preserved dried at room temperature until nucleic acid extraction. Only the fifth leaf from top, which was considered to be adequate for virus detection in cassava (Abarshi *et al*., 2012), was analysed for field samples.

Sample preparation and end-point PCR protocols

Total nucleic acid was extracted from fresh (100 mg) or dried (33 mg) cassava leaf tissue using the cetyl trimethyl ammonium bromide CTAB extraction method (Maruthi *et al.* 2002; Abarshi *et al.* 2010). Extracts were eluted in 100 µl sterile molecular grade water, quantified using a Nanodrop spectrophotometer, and stored at -20^{o}C for subsequent analysis.

End-point PCR amplification of ACMV, EACMV, CBSV and UCBSV was performed on field-collected samples. ACMV and EACMV were tested using the common forward primer CMBRep/F and the reverse primers – ACMVRep/R and EACMVRep/R respectively (Alabi *et al.* 2008), and CBSV and UCBSV using the common forward primer CBSV F2 and the reverse primers – CBSV R7 and CBSV R8, respectively (Abarshi *et al.* 2012).

Primer and probe design for real-time PCR

Primers and probes specific to CBSV, UCBSV, ACMV and EACMV were designed in addition to the existing ones (Table 1). Selection of primer and probe sequences for each virus was based on the multiple alignments of full-length virus sequences found in GenBank database. For designing primers and probes for ACMV and EACMV, 342 DNA-A sequences representing all nine CMBs found in Africa were aligned. Primer and probe sequences for ACMV and EACMV were selected from regions of DNA-A conserved in and unique to the virus species of interest. CBSV and UCBSV probe sequences were selected from conserved regions of the coat protein gene, between existing common forward and reverse primers, after aligning five and eight full-length virus sequences, respectively. All probes were labelled with specific fluorophores at the 5' end and a dark quencher at the 3' end (Table 1). A secondary ZEN™ quencher (IDT, Coralville, IA) was introduced between the fluorophore and primary quencher for CBSV- and EACMV-specific probes for reducing background noise and improving fluorescent signal. Melting temperature, % GC content and potential for self- and hetero-dimer formation were checked for selected primer and probe sequences using the online primer design web tools such as OligoAnalyzer and Thermoscientific's multiple primer analysers.

Multiplex real time PCR

A two-step multiplex qPCR protocol exploiting the TaqMan chemistry was used for quantitative assay of CBSV, UCBSV, ACMV, EACMV as well as its variant EACMV-Ug. Complementary DNA (cDNA) was synthesized from RNA extracts using Improm II^{TM} Reverse transcription kit (Promega, Southampton, UK) following manufacturer's instructions. A nucleic acid template consisting for each sample of 2 µl of cDNA mixed with 1.5 µl of 10X dilution of nucleic acid extraction was prepared prior to the PCR. Reactions were carried out in a mix made from 2X Express qPCR supermix universal (Life Technologies, Paisely UK), and EACMV, ACMV, CBSV and UCBSV-specific primers and probes, at quantities indicated in Table 1. Targets were amplified using the Eppendorf's Mastercycler ep realplex (Eppendorf, Cambridge, UK) in a cycle consisting of 2 minutes at 50 ^{o}C, 2 minutes at 94 ^{o}C followed by 40 PCR cycles of 15 seconds at 94 ^{o}C, 20 seconds at 54 ^{o}C and 30 seconds at 60^{o}C. In a single run, samples were analysed in triplicate wells for cassava viruses. The cassava gene – serine threonine phosphate (PP2A), which has been established as the best reference endogenous gene for quantification (Moreno *et al.* 2011), was amplified as an internal control in separate triplicate wells. Relative quantities for each of the four cassava viruses – ACMV, EACMV, CBSV and UCBSV in field cassava samples were calculated, using the relative quantification $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001) with PP2A as a reference gene and previously known infected plants as reference samples.

The multiplex qPCR was tested for sensitivity and specificity of detection for each intended target. Sensitivity experiment was carried out using 10X serial dilution standards generated from purified amplicons of each target obtained from conventional PCR. The PCR products were purified using reSource PCR purification kit (Source BioScience LifeSciences, Nottingham UK).

Sensitivity of detection of each virus was determined from lowest quantity of the purified virus amplicon detectable at maximum Cq of 35. Same Cq limit was used for all samples tested in this study. A standard curve was generated for each target and used to determine their reaction efficiency and correlation coefficient. Specificity of the multiplex qPCR was assessed by testing the primers and probes in the presence of target and non-target viruses and on samples negative for any one of the four viruses but positive for the remaining three. Intra- and inter-assay variation of Cq values was determined in single and mixed virus template samples. Variation was expressed as standard deviation from mean Cq values. The virus quantities detected were normalized to the quantities of CBSV and UCBSV detected in glasshouse propagated cassava var. Albert.

Statistical analysis

Cq values from uniplex and multiplex quantitative assays of single and combinations of virus targets were compared to determine the potential effect of multiplexing on test accuracy. The Wilcoxon signed rank test was applied to test for significant changes in Cq arising from multiplexing under the assumption of no significant difference between Cq values from both assay methods. The test was implemented using the 'coin' package (Hothorn *et al.* 2008) of the R-statistical analysis software version 3.1.0 (R Development Core Team, 2014). For each virus, variation in Cq values across three runs was determined by calculation of standard deviation. Statistical significance of this variation was verified by analysis of variation (ANOVA) using the R-software. The Mann-Whitney test was deployed to test for difference in CBSV or UCBSV quantities between cases of single and mixed infections with both viruses. Possible interaction between mixed infection effect and effect of variety on virus quantity was tested using two-way

ANOVA also implemented on the R statistical analysis platform (R Core Development Team, 2014).

RESULTS

Specificity, sensitivity, intra- and inter-assay Cq variation of multiplex qPCR

Four sets of primers and probes successfully detected the four viruses CBSV, UCBSV, ACMV and EACMV when all were present in a single tube (Figure 1a). The specificity of primers and probes was confirmed when removing one target virus at a time did not result in its amplification from primers and probes specific for other viruses (Figures 1b and 1c). Specificity was further confirmed when only intended virus target was amplified using primers and probe specific to a single virus in assays including templates of all four viruses (data not shown). For each virus, Cq < 35 was obtained for positive samples whereas Cq values in negative samples were undetermined or ≥ 35.

Limits of virus detection were assessed by using serial dilutions of purified PCR amplicons of known quantities and testing it either in the presence of one (uniplex) or four (multiplex) sets of primers and probes. The lowest quantity detected (Cq <35) ranged from 4.0 femtograms (fg) of purified EACMV sequence fragments to 12.5 fg of ACMV amplicons, using the multiplex method (Table 2) (1 fg = 10^{-6} ng). Limits of detection for CBSV and UCBSV were 9.88 fg and 9.53 fg, respectively. The detection of viruses in either uniplex or multiplex conditions did not affect the lower limit of detection. Reaction efficiencies for analysis of each virus species were all above 90% both in multiplex or uniplex conditions, except for 83% for EACMV in uniplex.

Variations in Cq values of the same samples across triplicates of a single plate (intra assay) or across three separate runs (inter assay) were determined (Table 3). Intra-assay variability was lowest for CBSV for which standard deviation from mean Cq values ranged from 0 to 0.57 and highest for UCBV at range 0.47 to 1.39 (Table 3). Higher values of variations were obtained between assays with standard deviation values ranging from 0.47 for ACMV to 2.80 for EACMV. However, Cq values measured for each virus did not vary significantly ($P > 0.05$, ANOVA) between runs across samples of single and mixed virus templates (Table 4).

Comparison of uniplex and multiplex qPCR assays

In a single run, quantitative Cq values obtained from multiplex and uniplex assays of each virus target were not significantly different (Figure 2). Differences in Cq values ranged from ±0.12 to ±2.32 but majority of these differences were an increase in Cq arising from multiplexing. In all four virus targets, the Wilcoxon signed rank test showed that multiplexing did not result in significant change in Cq value at threshold of $p = 0.05$, when Cq values obtained using the multiplex or uniplex assays were compared (Figure 2).

Detection and quantification of cassava viruses in field-cultivated cassava

The TaqMan multiplex assay and end-point PCR methods when used simultaneously for detecting CBSV and UCBSV in 16 varieties of field grown cassava plants showed that the former detected higher virus prevalence in field samples (Table 5). Using end-point PCR, CBSV was detected in 30 out of the 81 samples tested but the qPCR detected one additional positive sample. Likewise, additional 18 UCBSV infections were identified by multiplex qPCR compared to end-point PCR. A total of 55 plants were detected to be positive for either CBSV or UCBSV or both by qPCR. Among these, over 48% were infected by both viruses while 51.4% were

single infections of CBSV or UCBSV. The mean quantity of CBSV in doubly infected samples was on average 6-times higher than singly infected samples across varieties. This difference is significant (Mann and Whitney, Z= -2.796, $p<0.05$) and there is no evidence ($P > 0.05$, ANOVA) of it being associated with variety genotype. The samples found positive only in qPCR were found to have higher Cq values reflecting a lower titre of virus (data not shown). The incidence of CMBs was much lower, with only one sample found positive in end point PCR for EACMV, and an additional two samples found infected with ACMV and EACMV by qPCR.

Five of the varieties tested were completely free of all four viruses (Table 5). Among the varieties which have at least one of the five plants infected, NDL 2003/031, Mkumba, Pwani, KBH 2002/494 and the known tolerant variety Kiroba have much lower CBSV quantities compared to Mahiza, Albert and Naliendele, which also has the highest CBSV incidence (Figure 3a). UCBSV distribution in the assayed varieties is very similar to the above observation for CBSV. The varieties Mahiza, Naliendele, Albert in addition to KBH 2002/477, which was infected only by UCBSV, have the highest UCBSV quantities. On the other hand, Mkumba, Kiroba, and KBH 2002/494 have the lowest UCBSV quantities (Figure 3b).

Discussion

The causal viruses of CMD and CBSD are the most destructive pathogens of cassava in Africa and their specific detection in the field is crucial for epidemiology, quarantine, resistance identification and breeding. A multiplex diagnostic tool reduces the time, cost and effort associated with the detection of pathogens and is therefore an important asset in managing diseases. Several tools, using conventional PCR have already been designed for both CMBs and

CBSVs (Alabi *et al.* 2008; Abarshi *et al.* 2010, 2012; Mbanzibwa *et al.* 2011), however, the absence of quantitative data limits the discrimination between tolerant varieties that sustain low quantities of viruses, and susceptible varieties with high level of virus in the plant (Maruthi *et al.* 2014b). When available, such studies were developed for single virus (e.g. for CBSV by Moreno *et al.* 2011) or for the viruses of the same group (e.g. for CBSV and UCBSV by Adams *et al.* 2013), but not for CBSVs and CMBs together, while infection by both groups of viruses is a common feature in farmer fields.

In this study we developed a real time qPCR method which allows accurate and sensitive quantitative detection of four major viruses of cassava – ACMV, EACMV, CBSV and UCBSV, simultaneously in a single tube reaction for the first time. Evaluation of specificity showed that the method can uniquely detect each of the four viruses. Each virus was detected in samples containing the virus alone or in combination with other viruses hence showing that the assay is specific and not hampered by the presence or absence of other virus templates. Quantification was demonstrated to be reliable due to the high reaction efficiency (>90%) of each primer and probes combinations, low limits of detection for each target varying between 12.5 and 4 fg and reproducibility between assays as demonstrated by $P > 0.05$ from ANOVA test of inter-assay quantitative Cq values. The method was also reproducible enough to be useful for assaying samples with multiple infections – a situation which is to be expected in survey of field virus infections and screening for resistance to multiple cassava viruses.

The multiplex qPCR method was successfully applied to screen 81 samples of 16 varieties of field-grown cassava plants. The method showed superior results to the end point PCR and was able to detect the four viruses in additional samples that failed to amplify in the latter. Apart from

the var. Mahiza, CBSV and UCBSV quantities in these additional samples were among the lowest of the 81 samples tested, suggesting that the improved sensitivity more than broader specificity is the reason for the enhanced detection. The qPCR method demonstrated to be more sensitive than conventional PCR and thus a more precise tool for quarantine and resistance screening purposes.

Across virus species assayed in the field samples, CBSV and UCBSV were by far the most prevalent with 37 out of the 81 field samples being tested positive for at least one or both viruses. Out of these, 19 samples (51.4%) were infected with both viruses. These data are similar to earlier reports of CBSV and UCBSV mixed infections in different areas of Tanzania hence re-affirming the observation that both viruses are not always geographically separate and can co-infect a single plant (Mbanzibwa *et al.* 2011; Abarshi *et al.* 2012). For varieties infected with both viruses, mean CBSV quantities were generally higher for mixed infection cases than in singly-infected samples suggesting the possible existence of synergism between CBSV and UCBSV in field-infected cassava. The absence of any significant ($P > 0.05$) interaction or association between higher virus quantities in mixed infection samples and the genotype effect suggests that both factors exert independent effects on virus quantity of samples. Synergism between viruses infecting cassava has already been reported. ACMV and EACMV synergised to produce increased symptom severity and higher accumulation of virus DNA in field-grown cassava (Fondong *et al.* 2000). Evidence of synergism by CBSVs, however, has been debated. Kaweesi *et al*. (2014) has recently suggested the existence of competition between CBSV and UCBSV as possible explanation for the higher CBSV than UCBSV titres in co-infected cassava. However, UCBSV is known to accumulate at lower levels compared to CBSV and show milder

symptoms even in single infections (Winter *et al.* 2010; Mohammed *et al.* 2012). Ogwok *et al.* (2015) reported higher accumulation of total viral RNA (i.e. CBSV and UCBSV) in field-collected samples diagnosed for both viruses compared to those diagnosed for CBSV alone. The current study has presented evidence of an overall substantially higher CBSV quantities in mixed infected plants compared to single infections and shown that this difference is not influenced by genotype determinant of virus accumulation levels. It is important to understand the interaction between CBSV and UCBSV in cassava varieties and their implications on durability of CBSD resistance.

The multiplex qPCR method provides an appropriate tool for the cost effective study of CBSV-UCBSV interaction and ultimately CBSVs-CMBs interaction in cassava. The ability to detect four cassava viruses in a single tube will reduce time, reagents and consumable costs approximately four-fold compared to corresponding uniplex alternatives, hence is more suitable for high-throughput applications. Prior to the current study, the SYBR green chemistry has been utilized for uniplex quantification of CBSV (Moreno *et al.* 2011) and *South African cassava mosaic virus* (SACMV) (Allie *et al.* 2014). Real time qPCR methods, based on the TaqMan chemistry, have also been developed for quantification of ACMV and EACMV in separate uniplex assays (Otti *et al.* 2013) and CBSV and UCBSV in duplex reactions (Adams *et al.* 2013) although target masking effect limits application of the latter for quantification purposes (Adams *et al.* 2013). The evidence of high sensitivity, specificity and reproducibility presented for the method here represents the first demonstration of the potential to effectively quantify all the four most important viruses plaguing cassava production in eastern and central Africa.

Supporting Information:

Figure_4_SuppInfo.gif: Figure S1. Amplification plot from multiplex qPCR assay of 10 times serial dilutions of a. EACMV b. ACMV c. CBSV and d. UCBSV templates.

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Conflict of interest

The authors declare no conflict of interest on this work.

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1 Table 1: Primers and probes used for the detection of ACMV, EACMV, CBSV and UCBSV

Virus target	**Primer/probe**	**Sequence (5′ - 3′)**	**Quantity ($\mu molL^{-1}$)**	**Reference**
ACMV	ACMVAV2F1	RCAGGCGAAGTTGTKGCTA	0.3	This study
	CMBAV2R	TAWGGGCTGTCGAAGTTCAG	0.3	This study
	Probe	ROX-TGTCGAAGCGWCCAGGMGATATCATCATTTCCAC-BHQ2	0.4	This study
EACMV/	CMBRep/F	CRTCAATGACGTTGTACCA	0.12	Alabi *et al*. (2008)
EACMV-Ug	Neweac-alt/R*	CATGGAGACCGATCAGTATTGTTC	0.12	This study
	Probe*	FAM-TCTTKGGAG/ZEN/ACAGATCCAGGTGTCCACAT-IABkFQ	0.06	Otti *et al*. 2013
CBSV	CBSV F3	GGARCCRATGTAYAAATTTGC	1.0	Abarshi *et al*. (2012)
	CBSV R4	GCWGCTTTTATYACAAAMGC	1.0	This study
	Probe	JOE-TTCCAGCCA/ZEN/AGCAATWYTGATGTATCAGAATAGTGTGA-IABkFQ	0.2	This study
UCBSV	CBSV F3	GGARCCRATGTAYAAATTTGC	1.0	Abarshi *et al*. (2012)
	CBSV R4	GCWGCTTTTATYACAAAMGC	1.0	This study
	Probe	TAMRA-ACTATGAGGAAGGTTATGAGAAACTTCTCTAGCCAAGC-BHQ2	0.2	This study
PP2A	PP2AF	TGCAAGGCTCACACTTTCATC	0.5	Moreno *et al*. (2011)
	PP2AR	CTGAGCGTAAAGCAGGGAAG	0.5	Moreno *et al*. (2011)
	Probe	JOEN-CTTTCTGTT/ZEN/GCCCCCACCATGC-IABkFQ	0.2	This study

2 *Developed in Dr Lava Kumar's laboratory at IITA-Ibadan, Nigeria.

Table 2. Limits of detection, reaction efficiency for multiplex and uniplex assays of four cassava virus species

	Multiplex qPCR assay			Uniplex qPCR assay		
Virus species	Lower detection limit (femtogram)	Reaction efficiency (%)	R^2 (%)	Lower detection limit (femtogram)	Reaction efficiency	R^2 (%)
CBSV	9.9	95.0	99.7	9.9	97.0	99.7
UCBSV	9.5	92.0	97.7	9.5	90.0	97.3
ACMV	12.5	90.0	99.2	12.5	96.0	99.9
EACMV	4.0	93.0	98.8	4.0	83.0	99.2

R^2 = coefficient of determination

Table 3. Standard deviation of intra- and inter-run Cq values of single and combined virus template samples generated using the multiplex qPCR method.

		Intra-assay	Inter-assay
Virus detected	In the presence of virus templates	Mean Cq ± SD (n=3)	Mean Cq ± SD (n=3)
ACMV	ACMV+	19.06 ± 0.47	21.71 ± 0.76
	ACMV+CBSV+UCBSV+	19.66 ± 0.05	20.87 ± 1.68
	ACMV+EACMV+CBSV+	20.04 ± 0.78	20.83 ± 0.63
	ACMV+EACMV+UCBSV+	21.02 ± 0.75	20.48 ± 0.47
	ACMV+EACMV+CBSV+UCBSV+	21.36 ± 0.54	21.04 ± 0.63
EACMV	EACMV+	24.00 ± 0.34	24.26 ± 0.65
	EACMV+CBSV+UCBSV+	23.75 ± 0.22	24.85 ± 0.99
	EACMV+ACMV+UCBSV+	24.12 ± 0.26	26.47 ± 2.80
	EACMV+ACMV+CBSV+	24.80 ± 0.51	25.24 ± 1.69
	EACMV+ACMV+CBSV+UCBSV+	24.51 ± 0.12	25.46 ± 1.76
CBSV	CBSV+	27.15 ± 0.00	27.17 ± 1.34
	CBSV+UCBSV+ACMV+	28.28 ± 0.57	26.92 ± 1.24
	CBSV+UCBSV+EACMV+	28.08 ± 0.24	26.70 ± 1.22
	CBSV+ACMV+EACMV+	28.24 ± 0.17	26.72 ± 1.58
	CBSV+UCBSV+ACMV+EACMV+	30.81 ± 0.12	27.76 ± 1.07
UCBSV	UCBSV+	27.09 ± 0.70	28.70 ± 1.20
	UCBSV+CBSV+EACMV+	23.71 ± 0.17	26.90 ± 1.14
	UCBSV+ACMV+EACMV+	23.18 ± 1.89	29.46 ± 1.63
	UCBSV+CBSV+ACMV+	23.28 ± 1.39	27.73 ± 0.83
	UCBSV+CBSV+ACMV+EACMV+	25.33 ± 0.46	26.23 ± 0.92

+ indicates the presence of the virus template in the PCR reaction.

11

12 Table 4: ANOVA test results for variation of inter-run Cq values across single and mixed
13 template samples of each virus species.

Virus species	Mean Cq of virus samples*	*df*	*F*	*P*
ACMV	21.3	4	0.35	0.79
EACMV	25.6	4	0.49	0.74
CBSV	27.1	4	0.43	0.78
UCBSV	27.8	4	0.95	0.48

14 *Mean Cq of single and mixed virus template samples measured over three separate runs.
15

16 Table 5: Incidence of ACMV, EACMV, CBSV and UCBSV in 16 field-grown cassava varieties
17 measured by end-point PCR and multiplex qPCR assays.

Cassava variety	Number infected/ Number tested by end-point PCR				Number infected/ Number tested by multiplex qPCR			
	ACMV	EACMV	CBSV	UCBSV	ACMV	EACMV	CBSV	UCBSV
Albert	0/5	0/5	4/5	0/5	0/5	0/5	5/5	2/5
Naliendele	0/5	0/5	2/5	3/5	0/5	1/5	2/5	4/5
NDL 2003/031	0/5	0/5	0/5	0/5	1/5	0/5	1/5	0/5
KBH 2002/66	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Kiroba	0/6	0/6	4/6	0/6	0/6	0/6	4/6	5/6
KBH 2002/477	0/5	0/5	0/5	3/5	0/5	0/5	0/5	3/5
NDL 2005/1472	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
KBH 2002/26	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
Pwani	0/5	0/5	4/5	0/5	0/5	0/5	5/5	2/5
NDL 2003/111	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Mkumba	0/5	0/5	5/5	0/5	0/5	0/5	5/5	2/5
KBH 96/1056	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
KBH 2005/1471	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
KBH 2002/494	0/5	0/5	4/5	0/5	0/5	0/5	3/5	1/5
Mahiza	0/5	0/5	5/5	0/5	0/5	0/5	5/5	5/5
KBH 2002/482	0/5	1/5	1/5	0/5	0/5	1/5	1/5	0/5
Total number infected/ tested	0/81	1/81	30/81	6/81	1/81	2/81	31/81	24/81

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