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Full Length Research Paper

Occurrence of *Cucumber mosaic virus* subgroup IA and IB isolates in pepper in Nigeria

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This study characterized *Cucumber mosaic virus* (CMV) infecting pepper in Southwestern Nigeria with the aim of providing up-to-date information on the taxonomic subgroup(s) of the parading strains in the study area. Fifty pepper leaf samples with one or more symptoms of mottling, mosaic, necrosis, leaf distortion and stunting were collected from eight commercial farms across Southwestern Nigeria and screened for CMV using antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA). From the seropositive samples, four representative samples were selected, labelled PSWN1-4 before subjected to reverse transcription polymerase chain reaction (RT-PCR) and nucleic acid sequencing using a pair of primers specific for the amplification of RNA 3 genomic fragment of CMV. These were followed by multiple nucleotide sequence alignment and phylogenetic estimations of the isolates alongside selected CMV strains that were reported in previous studies using the molecular evolutionary genetics analysis (MEGA) software. The result of the ACP-ELISA test revealed that 14 (28%) of the samples collected were positive for CMV infection. The resulting nucleotide sequences from RT-PCR revealed 98% nucleotide homologies with several corresponding sequences of CMV strains from the Genbank. Further analysis of the PSWN nucleotide sequences through multiple nucleotide sequence alignment revealed the absence of the *Eco*R1 restriction site found only within the examined genomic portion of RNA 3 of subgroup II strains. These features defined the detected isolates as subgroup I strains. Phylogenetic analysis and estimations of genetic distances, conclusively distinguished the pepper-infecting CMV isolates from Southwestern Nigeria as members of subgroups IA and IB, which are the most virulent subgroups.

Key words: *Cucumber mosaic virus* (CMV), pepper, Southwestern Nigeria.

INTRODUCTION

Cucumber mosaic virus (CMV) infects more than one thousand two hundred plant species worldwide causing

viral epidemics and economic loss in several economically important crops including tomato (Palukaitis and Garcia-

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Arenal, 2003a). The virus causes mosaic symptoms in cucumber and other cucurbits; mosaic, systemic necrosis and shoestring-shaped leaf in tomato; mosaic and ringspot in pepper; withering in spinach; mosaic and stunting in clover, lupins and lucerne. They also cause stunting in soybean; mosaic and infectious chlorosis in banana; and mosaic and dwarfing in many other species of dicotyledonous and monocotyledonous plants (Palukaitis, 2003).

CMV has a worldwide distribution (both in tropical and temperate climates) and noted to have the widest host range for any plant virus (Palukaitis and García-Arenal, 2003b), including more than one thousand two hundred species in over a hundred families of angiosperm (Douine et al., 1979; Edwardson and Christie, 1991).

Most CMV strains have a restricted geographic distribution and symptom variants, making identification of CMV often difficult (Palukaitis and García-Arenal, 2003b). Strains of CMV have been classified on the basis of serological typing (Devergne and Cardin, 1973), peptide mapping of the coat protein (Edwards and Gonsalves, 1983) and sequence similarity of the genomic RNAs (Gonda and Symons, 1978; Piazzolla et al., 1979; Owen and Palukaitis, 1988). These classifications divided CMV strains into either Subgroup I or Subgroup II (Owen and Palukaitis, 1988).

Phylogeny based on analysis of RNA 3 sequences indicates that subgroup I strains can be further subdivided into subgroups IA and IB, in which subgroup IA strains are more closely clustered together than subgroup IB strains. Subgroups IA and II appear to be monophyletic (Roossinck et al., 1999).

The presence of the characteristic *Eco*R1 restriction site, 5' GAATTC 3', located at a particular region within RNA 3 gene fragment of the subgroup II strains, has also been used by researchers to differentiate members of the subgroup II strain from Subgroup I (Eni et al., 2013). The *Eco*R1 restriction site is found around nucleotide 1866-1871 of the RNA3 of subgroup II (Kayode, 2014). Subgroup I strains have been shown to be more virulent in crops than the subgroup II strains (Wahyuni et al., 1992; Zhang et al., 1994). Cross protection occurs between strains from all subgroups.

In view of the potential devastating impact of the different CMV subgroups on pepper production, this study was designed to molecularly identify and characterize the CMV isolates infecting pepper on selected commercial farms in major pepper producing areas of Southwest Nigeria. This research work reported the incidence and classification of the common strains of CMV in pepper within Southwestern Nigeria into subgroups. The knowledge of the taxonomic subgroup(s) of the isolated pepper-infecting CMV strains in farms provided an insight into the degree of virulence of the parading strains of CMV in the study area, since virulence activity and devastating impact of CMV strains has been reportedly linked to strain's subgroup membership.

MATERIALS AND METHODS

Sample collection

Fresh, young expanded leaves were collected from pepper plants showing symptoms of CMV diseases. The samples were preserved in air-tight McCartney bottles pre-loaded with silica gel (Sigma Aldrich) and covered with cotton wool. Fifty (50) samples were collected from eight commercial farms (Table 1) within the study area.

Serological indexing for virus detection in collected samples

Antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA) method was used for the detection of CMV in collected leaf samples as described by Hughes and Tarawali (1999). From each of the leaf sample, 100 mg were ground in 1 ml of antigen extraction/coating buffer (1:10 w/v). One hundred microlitres of the antigen ground was dispensed into each well of the ELISA plate. The plate was covered and incubated overnight at 4°C, then washed three times with PBS-Tween by flooding for 3 min each time. The plate was drained, tap dried and blocked with 200 µl per well of 3% (w/v) dried non-fat skimmed milk in PBS-Tween. The plate was covered, incubated at 37°C for 30 min, emptied and tap dried. 100 µl of polyclonal antibody was added to each well and diluted with conjugate buffer in the ratio 1:2000. The plate was covered and incubated at 37°C for 1 h, then washed three times with PBS-Tween by flooding for 3 min each time before tap drying. 100 µl of goat anti-rabbit alkaline phosphatase conjugate diluted in conjugate buffer was added into the each well. The plate was covered and incubated at 37°C for 1 h. The plate was finally washed three times with PBS-Tween by flooding for 3 min each time before tap dried. 100 µl of 0.5 mg/ml of p-nitrophenyl phosphate substrate in substrate buffer was added per well. The plate was placed in the multiscan ELISA plate reader provided with 405 nm filter and the reading was taken after 1 h and overnight. Samples with values exceeding twice the reading of the healthy control were considered CMV positive.

Nucleic acid extraction

Four seropositive samples with the highest ELISA readings were selected for molecular characterization of the detected CMV. The samples were labeled PSWN1, PSWN2, PSWN3 and PSWN4. The cetyltrimethylammonium bromide (CTAB) method was used for nucleic acid extraction as described by Abarshi et al. (2010) and Dellaporta et al. (1983). CTAB RNA extraction buffer pH 8.0 was made by mixing 2% molecular biology grade cetyltrimethyl ammonium bromide powder (Sigma H6269) (w/v), 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, and 0.2% β-mercaptoethanol (v/v) (was added just before use).

Procedure for RNA extraction

One hundred milligrammes of tomato leaf were ground in 1000 µl of nucleic acid extraction buffer in a sterile mortar and pestle. The sap was poured into new sterile tube and vortex briefly before incubating in water bath at 60°C for 10 min. The plant sap was brought to room temperature and equal volume of the mixture containing phenol, chloroform and iso-amyl alcohol in the ratio 25:24:1 was added. Sap was vortexed, centrifuged at 12000 rpm for 10 min and 450 µl of the supernatant was pipetted into new

Table 1. Sample sources and GPS coordinates.

Sample source	Ifo, Ogun State	Orile Ilugun, Ogun State	Olodo Village, Ogun State	Aramoko, Ekiti State
GPS	N 6° 48′ 11.662"	N 7° 14′ 37.175"	N 7° 17′ 15.568"	N 7° 42′ 34.181"
	E 3° 12′ 29.471"	E 3° 31′ 21.413"	E 3° 39′ 32.959"	E 5° 03′ 01.952"

Sample source	Iyana-Offa, Oyo State	Eruwa Road, Oyo State	NIHORT Ibadan, Oyo State	Ido LGA, Oyo State
GPS	N 7° 29′ 57.473"	N 7° 28′ 11.295"	N 7° 24′ 46.624"	N 7° 03′ 27.738"
	E 4° 04′ 40.155"	E 3° 45′ 11.738"	E 3° 51′ 51.179"	E 3° 43′ 09.816"

sterile tube. One microlitre of 1 U/µl DNase enzyme (Promega USA) was added to degrade the DNA portion of the nucleic acid. Cold isopropanol 300 µl was added, mixed and sap incubated for 1 h at -20°C. The mixture was centrifuged at 12000 rpm for 10 min to sediment the nucleic acids. The supernatant was gently decanted to ensure the pellets were not disturbed. 500 µl of 70% ethanol was added to the pellets and centrifuge at 12000 rpm for 5 min. The ethanol was decanted and the RNA air dried at room temperature. RNA pellets were suspended in 50 µl TE buffer for further use and storage at -80°C.

Reverse transcription polymerase chain reaction (RT-PCR)

With the aim of amplifying the 3' end of the coat protein (CP) gene and C-terminal noncoding region of RNA3 of CMV, RT-PCR was carried out using the CMV specific primers, 5' GCC GTA AGC TGG ATG GAC AA 3' and 5' TAT GAT AAG AAG CTT GTT TCG CG 3' as described by Wylie et al. (1993). Primers were synthesized by Inqaba Biotechnological Industries Limited, Walker Street 525, Muckleneuk 0002, Pretoria, South Africa. The PCR mix used for cDNA synthesis and amplification in a one step reaction contains 2 µl of the template RNA, 1 µl of 10 pm reverse primer, 1 µl of 10 pm forward primer, 3 µl of 25 mM MgCl$_2$, 10 µl of PCR buffer, 1 µl of 10 mM dNTP, 0.24 µl of reverse transcriptase (1 U/µl) and 0.24 µl of Taq polymerase (1 U/µl) (Promega USA). RT-PCR was accomplished with the amplification programmed for one cycle cDNA synthesis of 44°C for 30 min and 95°C for 5 min and 35 cycles of amplification with 45 s of denaturation at 95°C, 45 s of annealing at 54°C and 45 s of extension at 72°C followed by one cycle of final extension for 7 min at 72°C. The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis.

Sequence analysis

The PCR amplicons were purified with the addition of 70% ethanol and centrifugation at 9000 rpm for 45 s before carrying out sequencing. ABI 3130xL Genetic Analyzer (Applied Biosystems, California, USA), available at the Bioscience Center of IITA (International Institute of Tropical Agriculture) Ibadan was used for the nucleotide sequencing. Sequence similarity search of the GenBank database was done using the NCBI basic alignment search tool (BLAST) program.

Phylogenetic and molecular evolutionary analysis

A multiple nucleotide sequence alignment was conducted alongside corresponding CMV sequences from the Genbank sequence database, which have already been used in other studies. With the aim of determining the CMV taxonomy subgroup(s) of the PSWN isolates, the nucleotide sequences obtained were aligned with selected sequences of CMV subgroups IA, IB and II strains using the CLUSTALW program (Larkin et al., 2007). *Tomato aspermy virus* (TAV) and *Peanut stunt virus* (PSV) were used as out-group reference members of the genus *Cucumovirus* for rooting the phylogenetic tree. Corresponding sequences of CMV from neighbouring countries of Benin and Cameroun, available on the GenBank database were also selected for evolutionary comparison with the Nigerian strains. The analysis involved a total of 14 selected corresponding sequences from Genbank and 4 sequences obtained from this study. Phylogenetic and evolutionary relationship analysis was done on Molecular Evolutionary Genetics Analysis (MEGA) version 7 software package (Tamura et al., 2011) and a tree was created using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. The optimal tree with the sum of branch length = 1.10493691 is shown. All positions containing gaps and missing data were eliminated. There were a total of 427 nucleotide positions in the final dataset.

RESULTS

ELISA test result and reverse transcription polymerase chain reaction (RT-PCR)

Fourteen (28%) of 50 samples showed the presence of CMV infection. All four representative samples selected for PCR and the disease control sample presented the expected amplicon bands of approximately 500 base pairs on the agarose gel (Plate 1). The positive control also revealed an amplicon of the same size as observed in the test samples. No amplicon was seen in the healthy control sample. This confirms the presence of CMV infecting pepper in Southwest Nigeria.

Sequence analysis and multiple nucleotide sequence alignment result

The obtained nucleotide (Appendix) sequences revealed high level similarities with corresponding CMV sequences available on the Genbank database. All four sequences recorded sequence identity of 98% with several CMV sequences in the Genbank. The percentage identities of the PSWN isolates with eight selected corresponding CMV sequences from the Genbank are shown in Table 2.
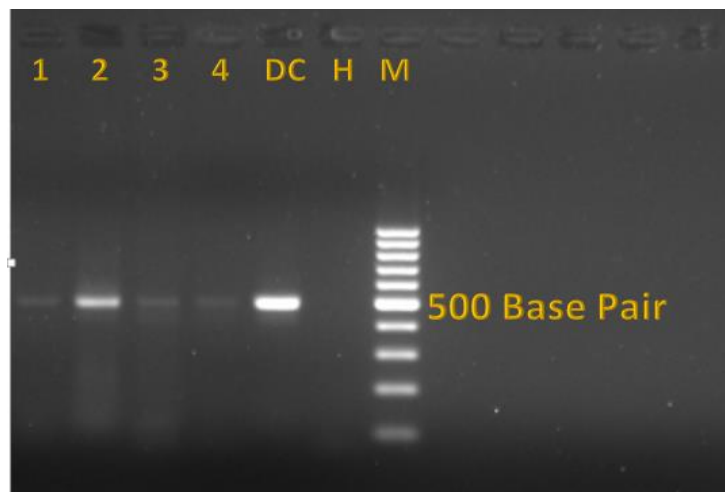
Plate 1. Agarose gel revealing amplicons of approximately 500 Base pair (bp). Wells 1-4 contain amplicons for CMV seropositive samples (PSWN 1-4), DC contains disease control, H is the healthy control (negative) and M is 100-1000 bp ladder.

Table 2. Percentage nucleotide sequence identities of PSWN isolates with corresponding sequences of eight selected strains of CMV from the Genbank database.

Accession	PSWN1							
	EU274471	LC066509	LC066500	EU428827	U22821	LC066515	LC066518	AM114273
% Identity	98	98	98	98	98	98	98	98
	PSWN2							
	KX014666	AJ810259	JND54635	KM272276	EF178298	KU947031	JN692495	AY861415
% Identity	98	98	98	97	97	95	94	95
	PSWN3							
	KU947031	KM272276	KM272275	AJ810259	JN054635	KX014666	EF593025	KC527768
% Identity	98	98	98	97	96	96	95	95
	PSWN4							
	AY861412	AY861415	KX014666	AY861413	AY861410	JN054635	AJ810259	KU947031
% Identity	98	98	98	98	98	98	98	97

Multiple nucleotide sequence alignment revealed a very high sequence homology among the PSWN isolates. The alignment also revealed the existence of a higher degree of sequence homology between the PSWN isolates and subgroup I strains than exist between PSWN isolates and the subgroup II strains. The characteristic *Eco*R1 restriction site, 5' GAATTC 3', which was found only within nucleotide 338-343 of the subgroup II strains but absent in the subgroup I strains and PSWN isolates (as enclosed in the box in Figure 1), distinguished the PSWN isolates as members of the subgroup I. *Tomato aspermy virus* (TAV) and *Peanut stunted virus* (PSV) were defined as out-group members as both revealed a relatively low degree of homology with all the CMV strains.

Phylogenetic and molecular evolutionary analysis

The phylogenetic tree further confirmed that the CMV strains characterized in this study belong to subgroups IA and IB as the isolates clustered together with members of CMV subgroup I with >70% bootstrap support (Figure 2). Isolates PSWN 2 - 4 clustered into subgroup IA while PSWN 1 clustered into subgroup IB.

Previously reported CMV strains from Cameroon (accession EU 428827), Benin (accession EU274471) and Nigeria (accession KM091954) also clustered into subgroup IA. Hence, the detected PSWN isolates and reported CMV strains from the named African countries all belong to Subgroup I. *Tomato aspermy virus* and PSV
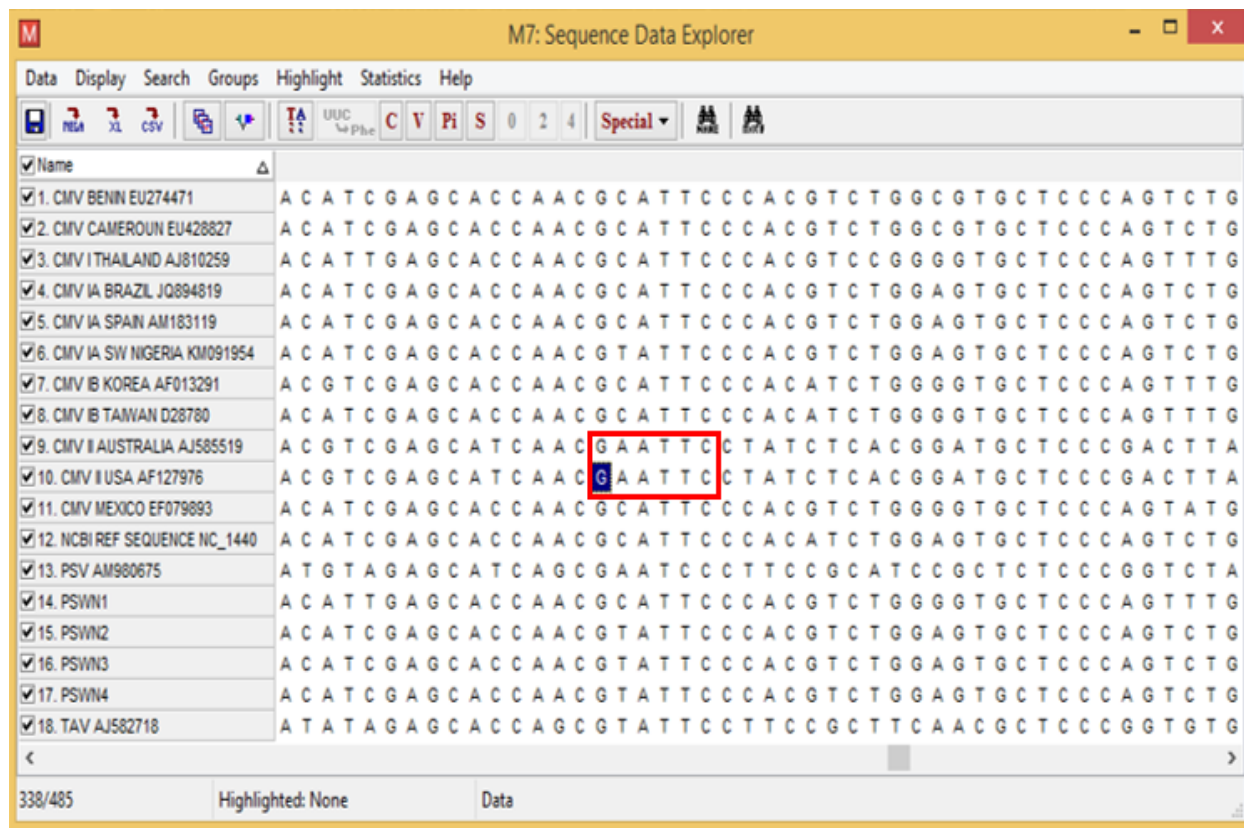
Figure 1. A section of the multiple nucleotide sequence alignment of the 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 of the four PSWN isolates of CMV and selected corresponding sequences of CMV strains of subgroups IA, IB and II, *Tomato aspermy virus* (TAV) and *Peanut stunted virus* (PSV). The characteristic *Eco*R1 restriction site, 5' GAATTC 3', which was found only within nucleotide 338-343 of the subgroup II strains as indicated by the box.

strains remain the out-group members as they formed a distinct branch separated away from all the CMV strains.

DISCUSSION

The outcome of the ACP-ELISA diagnostic test adopted for detection of CMV in pepper leaf samples was effective. This implies that this method is still valid for surveillance study of CMV. The 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 of the PSWN isolates that were amplified in this study produced amplicons of approximately 500 nucleotides as expected (Plate 1) (Wylie et al., 1993). Multiple nucleotide sequence alignment (Figure 1) of the obtained PSWN sequences (Appendix) alongside the corresponding sequences of selected CMV subgroups IA, IB and II strains using the CLUSTALW2 program (Larkin et al., 2007) revealed the presence of the *Eco*R1 site located at nucleotide 338-343 of the RNA 3 fragment of CMV subgroup II strains and its absence in CMV subgroups IA, IB and the PSWN isolates described by Eni et al. (2013). This clearly distinguished all the PSWN isolates as

members of subgroup I.

In addition, CMV strains from Benin (accession EU274471), the strain from Cameroun (accession EU428827) and strain from Nigeria (accession KM 091954) isolated previously from yam, banana and tomatoes, respectively were placed into subgroup I alongside the PSWN isolates in the phylogenetic tree (Figure 2). This is an indication that subgroup I stains of the virus is the most predominant subgroup in this region of Africa. These findings are supported by the report of Palukaitis and García-Arenal (2003b) that most CMV strains have a restricted geographic distribution.

The phylogenetic analysis and estimations of genetic distances in this study yielded results that correlated with of Roossinck et al. (1999) thus classifying the CMV strains into three distinct subgroups; IA, IB and II. The result of distinguished all the pepper-infecting CMV isolates from Southwestern Nigeria as members of subgroup I with three isolates (PSWN2, 3 and 4) classified as subgroup IA strains and PSWN1 as subgroup IB strains. The phylogenetic tree also revealed that CMV strains within each subgroup radiate from a single point of origin, indicating the evolution of each
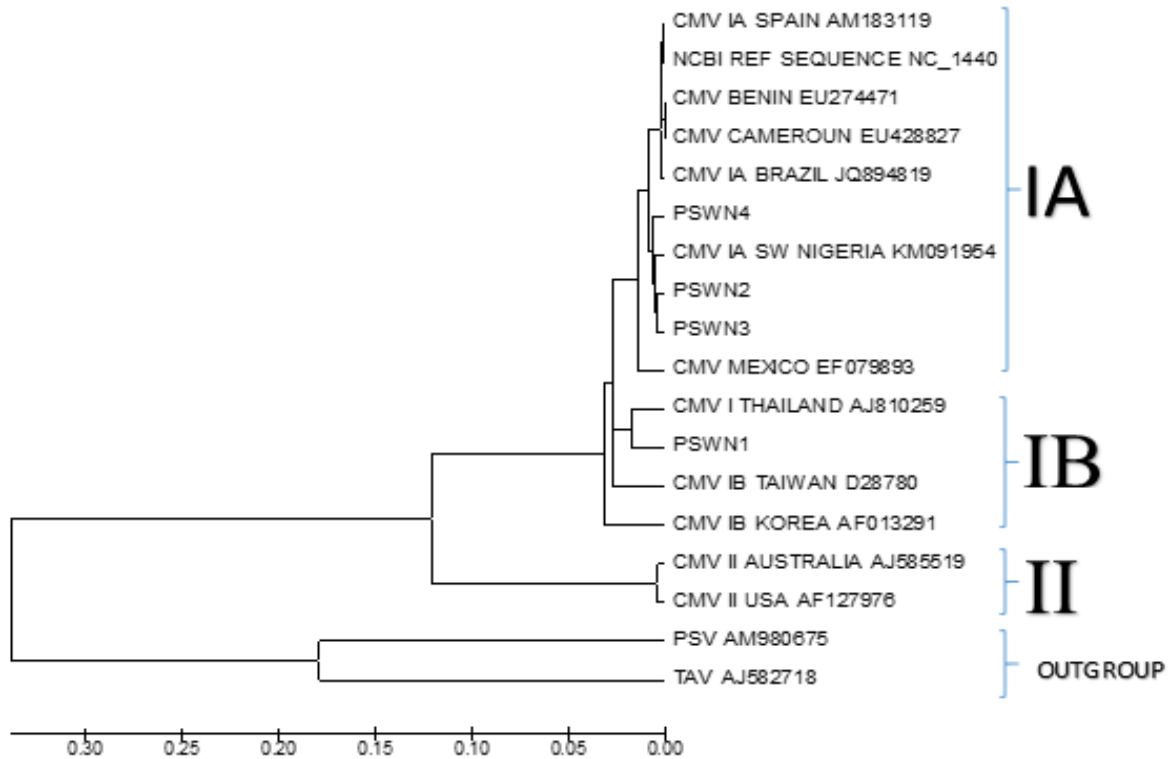
**Figure 2.** Neighbour-joining phylogenetic tree. Tree was constructed based on the nucleotide sequences of the 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 of the Southwest Nigerian isolates (PSWN1-4) and 12 selected CMV strains of subgroups IA, IB and II. *Tomato aspermy* virus and *Peanut stunted* virus defined as out-group.

subgroup from a single common ancestry. Strains belonging to subgroups IA and II were observed to be more closely clustered within their respective group when compared with strains within subgroup IB. This indicates that a monophyletic relationship exist within the two subgroups while a polyphyletic relationship exist in subgroup IB as reported by Roossinck et al. (1999). These features validate the phylogenetic tree obtained, hence the veracity of the PSWN strains as subgroup I members is established.

The fact that CMV strains reported in this study and other crops in southwestern Nigeria and the neighbouring countries belong to subgroup I (Eni et al., 2013; Kayode et al., 2014), which is the virulent group, may be due to the fact that these areas are all located within the tropical region of Africa possessing identical climatic and vegetative features which are specifically conducive for the proliferation of the subgroups IA and IB strains (Kayode, 2018).

CMV has been detected in several plant seeds and its numerous host range includes cultivated crops and weed species. It is therefore possible that contaminated seed lots and alternative weed and crop host plants serve as sources of CMV inoculum to cultivated pepper in affected farms. High aphids population observed on many farmlands within the study area (Kayode, 2014, 2018) have been attributed to be responsible for the rapid spread of CMV from infected crops and weeds to cultivated pepper plants.

Although the occurrence of CMV has been reported in several crops including pepper in several countries worldwide, to our knowledge, this is the first molecular identification of CMV subgroups IA and IB in cultivated pepper in Southwestern Nigeria.

This information on the genetic diversity of the common CMV strains in the study area is of economic importance in the development of control methods. The awareness of the fact that Subgroup IA and IB strains of CMV which are the most virulent strains (Wahyuni et al., 1992; Zhang et al., 1994), isolated from pepper as reported in this study is an eye-opener to the need for urgent control and effective management measures to minimize crop losses within the study area.

Considering the fact that the presence of alternative host plants and availability of transmitting aphid populations are required for CMV outbreaks and epidemic in pepper, farmers are therefore advised to get rid of all perennial weeds and alternative host plants reservoir in and around proposed farm lands; leave a distance of at least 5 m between pepper farms and

surrounding weeds; plant pepper earlier to avoid high aphid populations that usually occur later in the planting season; plant late settings as far as possible from fields used to produce early pepper and other vegetables; lookout for the first symptoms of any virus disease (if detected, farmers should pull out and burn the infected plants after spraying with insecticide to kill any insect vectors); early monitoring of aphid populations within the season and application of appropriate insecticide when necessary. Safety standards and regulations must be adopted during application of insecticides to prevent food poisoning and health hazards.

CMV as observed has been shown to present many symptom variants, making the identification often difficult from symptoms alone, the production of affordable, rapid, easy-to-use, on-the-spot ELISA diagnostic kit should be encouraged and facilitated to enhance surveillance studies, early detection and control of outbreaks and epidemics in the study area.

Other management strategies for the control of viral diseases of pepper include: planting of virus-free seeds obtained from credible sources rather than collection of seeds from previously harvested tomato fruits that might have been infected and raising of pepper seedlings in screen houses or nets using sterilized soil during the nursery stage.

Abbreviations

BLAST, Basic alignment search tool; **CMV,** cucumber mosaic virus; **EDTA,** ethylenediaminetetraacetic acid; **ELISA,** enzyme linked immunosorbent assay; **PSV,** peanut stunted virus; **RT-PCR,** reverse transcription polymerase chain reaction; **TAV,** tomato aspermy virus.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDIX

Nucleotide sequences of the 3' end of the coat protein gene and C-terminal noncoding region of RNA 3 of the four Southwest Nigerian CMV isolates (PSWN1-4) infecting pepper reported in this study.

Genomic sequence 1: Isolate PSWN1

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        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
             10            20            30            40            50
SWNcmv1   ATACGCTGAC TTTGCCGATT TGATTCTACC GTGTGGGTGA CAGTCCGTAA
|

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
             60            70            80            90           100
SWNcmv1   AGTTCCTGCC TCCTCGGACT TATCCGTTGC CGCCATCTCT GCTATGTTCG

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
            110           120           130           140           150
SWNcmv1   CGGACGGAGC CTCACCGGTA CTGGTTTATC AGTATGCCGC ATCTGGAGTC

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
            160           170           180           190           200
SWNcmv1   CAAGCCAACA ACAAACTGTT GTATGATCTT TCGGCGATGC GCGCTGATAT

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
            210           220           230           240           250
SWNcmv1   AGGTGACATG AGAAAGTACG CCGTCCTCGT GTATTCAAAA GACGATGCGC

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
            260           270           280           290           300
SWNcmv1   TCGAAACGGA CGAGCTAGTA CTTCATGTTG ACATCGAGCA CCAACGTATT

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
            310           320           330           340           350
SWNcmv1   CCCACGTCTG GAGTGCTCCC AGTCTGATTC CGTGTTCCAG AACCCTCCCT

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
            360           370           380           390           400
SWNcmv1   CCGATCTCTG TGGCGGGAGC TGAGTTGGCA GTTCTGCTAT AAACTGTCTG

        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
            410           420           430           440           450
SWNcmv1   AAGTCACTAA ACGTTTTTAC GGTGAACGGG TTGTCCATCC GCTTTAACGG
```

Genomic Sequence 2: Isolate PSWN2

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              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   10           20           30           40           50
SWNcm√2    TTTTTGTTTA GCTTCGGCAG TTGTTCTTCG GTGTGTGAAG GTGCTGTCCG

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   60           70           80           90           100
SWNcm√2    TCCCGCTTAC GGCGGACCTG TCCGTTTCCG CCATCTCTGC TATGTTCGCG

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   110          120          130          140          150
SWNcm√2    GACGGAGCCT CACCGGTACT GGTATATCAG TATGCTGCAT CTGGTGTTCA

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   160          170          180          190          200
SWNcm√2    AGCCAACAAC AAATTGTTGT ACGATCTTTC AGTGATCGCG CTGATATTG

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   210          220          230          240          250
SWNcm√2    GTGACATGAG AAAGTACGCC GTGCCCGGGA TTCAAAAAAA AGGATGCGCT

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   260          270          280          290          300
SWNcm√2    CGAAGACGGA TGAACTAGTA CTTCATGTTG ACATTGAGCA CCAACGCATT

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   310          320          330          340          350
SWNcm√2    CCCACATCTG GAGTGCTCCC AGTTTGAACT CGTGTTTTCC AGAACCCTCC

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   360          370          380          390          400
SWNcm√2    CTCCGTTTCC TGTGGCGGGA GCTGAGTTGG TAGTGTTGCT ATAAACTACC

              . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
                   410          420          430          440          450
SWNcm√2    TGAAGTCACT AAACGCTTTG GGGTGAACGG GTTGTCCATC CAGCTTTAAA
```

Genomic Sequence 3: Isolate PSWN3

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          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
               10             20             30             40             50
SWNcnv3   TTGCCATGAC ATCTGTATTC AGCTGTTTGA TTCTACCGTG TGGGTGACAG
```

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          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
               60             70             80             90            100
SWNcnv3   TCCGTAAGGT TCCTGCCTCC TCGGACTTGT CCGTTTCCGC CATCTCTGCT
```

```
          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
              110            120            130            140            150
SWNcnv3   ATGTTTGCGG ACGGAGCCTC ACCGGTACTG GTTTATCAGT ATGCTGCATC
```

```
          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
              160            170            180            190            200
SWNcnv3   TGGTGTTCAA GCCAACAACA AGTTGTTGTA TGATCTTTCA GTGATGCGCG
```

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          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
              210            220            230            240            250
SWNcnv3   CTGATATTGG TGATATGAGA AAGTACGCCG TGCTCGTGTA TTCAAAAGAC
```

```
          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
              260            270            280            290            300
SWNcnv3   GATGCGCTCG AGACGGACGA ACTAGTACTT CATGTCGACA TTGAGCACCA
```

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          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
              310            320            330            340            350
SWNcnv3   ACGCATTCCC ACGTCTGGGG TGCTCCCAGT TTGAACTCGT GTTTTCCAGA
```

```
          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
              360            370            380            390            400
SWNcnv3   ACCCTCCCTC CGTTTTCTGT GGCGGGAGCT GAGTTGGTAG TGCTGCTATA
```

```
          . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |
              410            420            430            440            450
SWNcnv3   AACTATCTGA AGTCACTAAA CGCTTTGCGG TGAACGGGTT GTCCATCCAG
```

Genomic Sequence 4: Isolate PSWN4

```
         ....|....| ....|....| ....|....| ....|....| ....|....|
             10         20         30         40         50
SWNcm4   TGGGTATGTT TTCGAAAACA TCAGTATCCT TTGCCGAATT TGATTCTACC


         ....|....| ....|....| ....|....| ....|....| ....|....|
             60         70         80         90        100
SWNcm4   GTGTGGGTGA CGGTCCGTAA AGTTCCTGCC TCCTCGGACC TGTCCGTTTC


         ....|....| ....|....| ....|....| ....|....| ....|....|
            110        120        130        140        150
SWNcm4   CGCCATCTCT GCTATGTTCG CGGACGGAGC CTCACCGGTA CTGGTTTATC


         ....|....| ....|....| ....|....| ....|....| ....|....|
            160        170        180        190        200
SWNcm4   AGTATGCTGC ATCTGGCGTT CAAGCCAACA ACAAATTGTT GTATGATCTT


         ....|....| ....|....| ....|....| ....|....| ....|....|
            210        220        230        240        250
SWNcm4   TCAGTGATGC GCGCTGATAT TGGTGACATG AGAAAGTACG CCGTGCTCGT


         ....|....| ....|....| ....|....| ....|....| ....|....|
            260        270        280        290        300
SWNcm4   GTATTCAAAA GACGATGCGC TCGAGACGGA TGAACTAGTA CTTCATGTTG


         ....|....| ....|....| ....|....| ....|....| ....|....|
            310        320        330        340        350
SWNcm4   ACATTGAGCA CCAACGCATT CCCACGTCCG GAGTGCTCCC AGTTTGAACT


         ....|....| ....|....| ....|....| ....|....| ....|....|
            360        370        380        390        400
SWNcm4   CGTGTTTTCC AGGATCCTCC CTCCGTTTTC TGTGGCGGGA GCTGAGTTGG


         ....|....| ....|....| ....|....| ....|....| ....|....|
            410        420        430        440        450
SWNcm4   TAGTGTTGCT ACAAACTGCC TAAAGTCACT AAACGCTTTT GCGGTGAACG
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Full Length Research Paper

Evaluation of commercial rapid test kits to determine the effective diagnostic method for dengue in a low resource setting

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Conventional commercially available rapid immuno-chromatographic tests (ICTs) or diagnostic kits were evaluated for their sensitivity, specificity, cost and turnaround time (TAT) results with Dengue IgM/IgG capture enzyme linked immunosorbent assays (ELISA) as the standard test, in blood samples from a cross-section of individuals with clinical features suggestive of dengue fever attending health care facilities in the country. Blood samples taken from over 100 consented participants were analyzed using the two rapid ICTs (SD Bioline Dengue Duo NS1/IgM/IgG and Panbio Dengue Duo Cassette) and compared with the Dengue IgM/IgG capture ELISAs. Standardized questionnaire was used to obtain bio and epidemiological data of the participants. The laboratory evaluation also assessed the TAT to complete the tests as well as the cost for each test method. The laboratory analysis on a given number (n=93) revealed that the SD Bioline was more sensitive (39.9%) than the Panbio (22.1%; p=0.005), and specificities for both were 100%. The SD Bioline includes an extra biomarker test with the same TAT and differs in cost by USD$ 1.14 as opposed to the Panbio. The ELISA has a cost of USD$ 8.07 and despite its longer TAT, it has the advantage of running more samples (1 vs 96) at a given time. While SD Bioline may be the better choice with a higher sensitivity, dengue ELISAs should also be favourably considered as an option for diagnostic purposes. In a resource strapped setting like the laboratories in Trinidad and Tobago, the ELISA should be preferred because its sensitivity and specificity were higher than the Panbio and SD Bioline kits. Besides, more samples were tested giving an effective TAT for amounts of samples completed despite a higher cost.

Key words: Dengue fever, Panbio, SD Bioline, enzyme linked immunosorbent assays (ELISA), Trinidad and Tobago.

INTRODUCTION

Dengue is a major public health problem in more than 100 tropical countries including Trinidad and Tobago (Anderson et al., 1956; Carrington et al., 2005; Simmons et al., 2012). It is the most common arthropod transmitted disease of mankind with over 2.5 billion people worldwide at risk of being infected. Early and rapid laboratory

diagnosis is essential so as to institute a timely and appropriate clinical management procedures; and avoid misdiagnosis since dengue infections can be mistaken with other febrile illnesses such as influenza, measles, leptospirosis, West Nile fever, yellow fever, Zika or even chikungunya (Gubler, 2002; Calisher, 2005; Bhatt et al., 2013; Mustafa et al., 2015). In many developing countries, viral culture and molecular testing methods are lacking or non-existent. Diagnosis of dengue viral infection is therefore dependent on the clinical acumen of the medical staff. If they get it wrong, the condition of such patient deteriorates into severe forms of dengue such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). The fatality and cost management of these complications – DHF and DSS then becomes unimaginable and unbearable (Teelucksingh et al., 1997, 1999). The need to have a rapid and accurate diagnosis of these viral infections and a test to rule out dengue cannot be a luxury. In settings where there are limited resources available, a timely and reliable test method in terms of sensitivity and cost will therefore be paramount and most welcome.

Available data suggest that dengue is endemic in several countries across all regions of Africa and more detailed epidemiological data are still required to assess the impact of dengue in Africa (Amarasinghe et al., 2011). According to most reports of dengue cases or frequency in the Caribbean countries including Trinidad and Tobago, the data are that of probable reported cases of dengue (Pal et al., 2015). Apparently, there are no confirmed cases of dengue data or information since most of these Caribbean countries may not have access to techniques or methods used to confirm dengue diagnosis due to lack of resources. Several years ago, Campbell et al. (2007) used a rapid test kit to determine the sero-prevalence of dengue from a cord blood survey. There seems to be no follow up of this survey in the country. Confirmatory diagnosis of dengue involves molecular amplification of dengue virus (DENV) RNA by reverse transcriptase polymerase chain reaction (RT-PCR), immunoassay to detect DENV non-structural protein 1 (NS1) and virus isolation (Hunsperger et al., 2009; WHO, 2009; Muller et al., 2017). Use of molecular methods is not readily available in economy strapped countries. The virus isolation in these settings is not feasible because there are no cell culture facilities. Besides, these require longer time to complete and may not be fully sensitive if compared to immunoassay or molecular methods (Chadee et al., 2005; Sahadeo et al., 2015). There is therefore the need in these countries to have an easy to use and reliable diagnostic method that is readily available, give rapid results, sensitive and specific and also cost effective. This study was carried out to simply investigate the usefulness of two rapid immuno-chromatographic tests (ICTs) for dengue virus by comparing their effectiveness, turnaround times and costs for dengue testing in a low economic setting or

country such as ours. An enzyme linked immunosorbent assay (ELISA) was used a gold standard method to compare the performances of these ICTs.

MATERIALS AND METHODS

Study design

This laboratory based cross-sectional observational study employed the use of serum samples obtained from 100 patients with suspected dengue infection. As previously reported Kallap et al. (2018), blood samples of patients who presented to health care facilities with suspected dengue infection were used for this analysis. These patients must have fever along with the following symptoms – anorexia, skin rash, aches and pains, vomiting and nausea, abdominal pains and warning signs which include positive tourniquet test, leukopenia etc. (Kallap et al., 2018). These samples were subjected to the same serological tests (for IgM, IgG and NS1 antigen). These tests were then compared to ELISA as the gold standard method for dengue detection since this is what is commonly and readily available in low economic settings such as ours.

Ethics approval and permissions

Ethics approval for this study was obtained from the University of the West Indies St. Augustine Campus and the Regional Health Authority Ethics Committees. Informed written consent was obtained from each participant, along with assent from children. Participant under the age of 18 was considered as a child for this study.

Study sample collections

Several blood samples (n=100) were collected from participants and were analyzed for performance of tests and sample analysis, but only 93 samples were used to calculate the TAT because the ELISA kits can accommodate 93 samples during a one-time set up. All the blood samples were allowed to clot at room temperature, centrifuged, separated and tested the same day for the rapid kits (Panbio and SD Bioline). The remaining samples were then stored at 2-8°C for a maximum of two days or stored frozen at -30°C for the ELISA tests that was performed in a batch at a go or one time. These tests were performed on each blood sample that was classified as acute or convalescent based on duration of patient's reported symptoms. Acute samples were from patients whose symptoms were within the last five days and convalescent were from patients whose symptoms were over five days.

Inclusion criteria

All samples used for this analysis fulfilled the criteria previously enumerated in a previous publication (Kallap et al., 2018) and above.

Laboratory analysis

Rapid immuno-chromatographic tests (ICTs)

Samples were collected in red top tubes and were tested using rapid immune-chromatographic tests (ICT) for IgM and IgG antibodies, and also for the presence of dengue non-structural

protein 1 (NS1) antigen which is consistent with acute-phase infection with dengue virus. The SD Bioline Dengue Duo Kit (Standard Diagnostics Inc., Seoul, Korea) and Panbio Dengue Duo Cassette (Panbio Diagnostics, Sinnamon Hill, Australia) were used to test the blood samples for NS1, IgM and IgG assays. These kits are meant to identify the presence of dengue specific IgM and IgG antibodies. In the SD Dengue Duo kit, IgM and IgG antibodies along with NS1 antigen are determined simultaneously using a single addition of serum. The Panbio test can only identify dengue-specific IgM and IgG antibodies. Both rapid kits were used for each sample of sera collected adhering strictly to the manufacturer's instructions and the results were recorded.

Enzyme linked immunosorbent assay (ELISA)

For this study and analysis, the ELISA was used for the detection of human serum IgM and IgG antibodies in dengue virus (DENV) infections in the diagnosis of acute dengue, the Dengue Virus IgM/IgG capture DxSelect ELISA (Focus Diagnostics, Cypress, PA, USA) was used as a reference method to the rapid ICTs in the diagnosis of dengue and the assessment of acute or convalescent sample. According to excerpt from the kit itself from the manufacturers, the sensitivity was 100 and 95%, CI was 80.5 – 100% for paired acute sera; and its specificity for sera from non-endemic normal sites was 99%. It must also be stated however that this product is not for distribution in the United States. ELISA test was also carried out for dengue diagnosis, noting index values for both IgG and IgM. All tests kits were performed according to the manufacturer's guidelines.

Turnaround time (TAT)

The TAT was calculated and taken as the time required or expended in completing each test in the laboratory once the blood sample was taken to the laboratory and analysis using the kits commenced. It did not include the time of sample collection, transportation or storage.

Cost for the tests

Cost per test method was calculated by determining the cost of consumable materials and labour. This included the cost of each individual item or kit used to perform and complete each test method. The cost of labour was calculated based on the basic monthly salary of a research assistant technical staff time and hands on duty that was approximately US$ 5.65/ h ($ 35.00/ h in Trinidad and Tobago dollars). The cost did not include the capital costs of major equipment or infrastructure.

Quality controls

Controls for both the IgM/IgG ELISA kits were provided as follows; detectable controls (human sera), non-detectable controls (human sera) and cut-off calibrators (human sera). Samples that were collected from asymptomatic and healthy individuals during the time of the study were used as controls for both rapid ICT tests. Controls were run every time test procedures were carried out.

Statistical analysis

All data entry from the study were done using the Microsoft Excel and data analysis was performed using SPSS (Statistical Package for the Social Sciences) 23.0 software. Chi-square test and Fisher's exact test were used to compare categorical variables. The Chi-square was chosen for determination of association, that is, association between a tested variable and a positive dengue result. If a relationship existed between any of the variables, the Chi-square value (p value) would reflect the strength of the association. The Fisher's exact test is used in place of the Chi-square to measure the same association for smaller sample sizes. In cases where the frequency counts are fewer than five in a two by two table, the test statistics (p) used is the Fisher's exact value. A probability value (p) of < 0.05 was considered statistically significant.

The sensitivity, specificity and predictive values were calculated as previously described in literature (Lalkhen and McCluskey, 2008).

RESULTS AND DISCUSSION

The laboratory tests for the determination of the sensitivity, specificity, cost effectiveness and rapidity of dengue infection in a resource strapped setting such as ours, all performed well in relative to each test method employed. Overall diagnostic performance of the sensitivities of the Standard Diagnostics Bioline Dengue Duo (SDB DD) rapid test for IgM and IgG were 14.3% and 38.8% ($p<0.0001$), respectively. IgM demonstrated a very high negative predictive value (NPV) of 93.1% and a very low positive predictive value (PPV) of 16.7%. The IgG showed the highest specificity of 100% with the lowest sensitivity of 13.3%. The manufacturer's values for sensitivity and specificity for IgM/IgG in SDB DD are 99.4 and 93%, respectively (Table 1).

The diagnostic sensitivities of the Panbio rapid test for IgM and IgG were 14.3 and 21.2%, ($p=0.2$), respectively. The IgG demonstrated a very high PPV of 100% and a very low NPV of 10.7%. The IgM value in this Panbio rapid test showed specificity of 91.9% with a low sensitivity of 14.3%. The manufacturer's values for sensitivity and specificity for IgM/IgG in Panbio rapid are 96.3 and 95%, respectively. Sensitivity of SDB DD test revealed that there were no significant differences in IgM or IgG detection with either acute or convalescent samples as the IgG detection in acute samples showed the highest sensitivity of 40% compared to a 38.8% in convalescent samples. Even combination of biomarkers (IgM/IgG) showed a sensitivity of 40% for acute samples and 39.5% for convalescent samples. The IgG was shown to be more sensitive in convalescent samples (47.1%) and sensitivities remained the same for combination of IgM/IgG biomarkers.

Analysis of the sensitivity of Panbio Duo Cassette test revealed that there were no significant differences in IgM or IgG detection with either acute or convalescent samples. The IgG detection in convalescent samples showed the highest sensitivity of 21% compared to 20% in acute samples. There was a very slight increase in sensitivity for IgM/IgG combination in convalescent samples (22.2%). Detection of IgG was shown to be more sensitive in convalescent samples (29.4%), and sensitivity for combination of IgM/IgG was higher (35.3%) in convalescent samples, but none of these sensitivities

Table 1. Diagnostic performance of rapid tests against reference ELISAs including test performance stratified by phase of illness.

Test parameter	SDB DD			Panbio			ELISA		
	IgM	*IgG*	*IgM/IgG*	*IgM*	*IgG*	*IgM/IgG*	*IgM*	*IgG*	*IgM/IgG*
Positives	1	33	34	1	18	19	7	85	86
Negatives	81	8	7	19	8	6	86	8	7
Sensitivity (%)	14.3	38.8	39.5	14.3	21.2	22.1	100	100	100
Specificity (%)	94.2	100	100	91.9	100	100	100	100	100
PPV (%)	16.7	100	100	12.5	100	95	100	100	100
NPV (%)	93.1	13.3	11.9	92.9	10.7	8.2	100	100	100
	Acute	**Convales**		**Acute**	**Convales**		**Acute**	**Convales**	
TP	26	8		13	6		69	17	
FN	8	26		6	13		17	69	
FP	43	9		56	11		0	0	
TN	16	50		18	63		7	7	
Sensitivity (%)	76.5	23.5		68.4	31.6		80.2	19.8	
Specificity (%)	27.1	84.7		24.3	85.1		100	100	

SDB DD, Standard Diagnostics Bioline Dengue Duo; ELISA, enzyme-linked immunosorbent assay; PPV, positive predictive value; NPV, negative predictive value; convales = convalscent; TP, true positives; FN, false negatives; FP, false positives; TN, true negatives; IgM/IgG, IgM and/or IgG antibodies present; IgM, IgM antibodies present (regardless of IgG result); IgG, IgG antibodies present (regardless of IgM result).

Table 2. Comparison of costs and turnaround times between dengue rapid diagnostic tests and dengue ELISAs.

Test kit	N	Total cost ($US)	Unit cost (US$)	TAT (Mins)	Labor cost (US$)
Panbio	93	695.64	7.48	15	1.41
SDB DD	93	829.56	8.92	15	1.41
ELISA IgM kit	93*	397.19**	4.27	200	18.83
ELISA IgG kit	93*	353.40**	3.80	180	16.95

SDB DD, Standard Diagnostics Bioline Dengue Duo; IgG, Immunoglobulin G; IgM, Immunoglobulin M; ELISA, Enzyme-linked immunosorbent assay; N, number of blood samples tested; $US, United States dollars. *There were 93 test samples for determination plus 3 controls to make up the 96 wells. But unit cost was calculated based on 93 samples tested. **Total price for the test kits were IgM ELISA $410 (for 96 test samples); IgG ELISA $365 (for 96 test samples).

were significant.

Positive samples confirmed with ELISA revealed that dengue IgM antibodies are more a marker of acute infection than they are of recent infection. Hence, more attention was paid to the optical density values that were acquired upon completion of the dengue IgG capture ELISA. According to the ELISA product guidelines, all positive samples of IgG antibodies were taken as having an OD>1, and this was noted for 92.5% of the study samples. The SDB DD showed positives with IgG OD values that were 3.01 and higher, were recorded for 35.5% of the study population as positive with the majority of positives (19.7%) having ODs between 4.01 and 5.00. In comparison, the Panbio rapid test showed positives with IgG OD values that were 4.01 and higher, recording 19.4% of the study population as positive with equal amounts (9.68%) of positives between 4.01-5.00 and 5.01 and higher.

The TAT and costs results are outlined on Table 2 and reveals that the total costs for material and labour to carry out the different detection tests were highest for IgM

ELISA test amounting to US$ 4.27 and $ 18.83 respectively per single sample. This was followed by IgG ELISA (US$ 3.80 and $ 16.95) (p<0.005). The unit material costs for each sample for Panbio and SDB DD test kits were at US$ 7.48 and $ 8.92 respectively, with the same labour cost of US$ 1.41.

The TAT results for each assay method are shown in Table 2. While the TAT for a single set up was 15 min each for the Panbio and SDB DD, appearing shorter for that of the ELISA tests that expended 200 and 180 min for ELISA IgM and IgG respectively for a one time set up of 93 samples. If the TAT is to be calculated for 93 samples, it will take more time by the Panbio or the SDB DD tests (1,395 min). Average time for ELISA IgM and IgG would be 2.08 (200/96) and 1.9 min (180/96) respectively.

A major aim of this study was to investigate the usefulness of two rapid ICTs by determining their sensitivity and specificity in making presumptive diagnosis of dengue. The analysis from this study revealed that the IgG test component of the SDB DD

rapid kit had the highest individual sensitivity of 38.8% when compared to the reference ELISA for the diagnosis of dengue. This SBD DD sensitivity is almost similar (39%) to what was reported in Jamaica (Vickers et al., 2015). The IgM sensitivities were however significantly lower in this study with reported values of 14.3% sensitivity when compared to 49.3% reported in the Jamaican study (Vickers et al, 2015). This low sensitivity in this current study may be due to the fact that the majority of samples collected in this current study were convalescent unlike that of Vickers et al. (2015) study in Jamaica that were acute samples. It should also be noted that the study in Jamaica also used ELISA as comparators like in this present study.

Parkash and Shueb (2015) listed the evaluation of several tests, both ELISAs and rapid tests and their sensitivities and specificities. The Focus Diagnostics IgM capture ELISA showed a sensitivity of 98.6% and a specificity of 79.9%. The authors mentioned however, that the major draw-backs of all IgM antibody-based assays are that they can cross-react with other flaviviruses and which may provide inaccurate results if patients had a recent infection (Parkash and Shueb, 2015). As a result, recent infection may have been confused with past infection showing the presence of IgG antibodies. Both rapid tests recorded positives with a much higher optical density (OD). This needs to be considered when choosing rapid tests for use in such hospital settings of low economy.

The studies which found lower sensitivities of NS1 antigen in samples attributed this finding to the possible presence of high IgG antibody titres (Vaughn et al., 2000). It was hypothesized that dengue viral antigens, including NS1, may form immune complexes with high levels of dengue IgG antibodies and thus become undetectable (Vickers et al., 2015). There exists however other reasons that may be responsible for the low sensitivity or absence, in this case, of NS1 antigen. No gold-standard procedure was conducted for NS1 antigen detection, and so, if any or lower amounts of NS1 was present in any one sample, it may have gone unnoticed by the rapid test. As a result of this fact, a new combination of biomarkers (IgM/IgG) was used to compare sensitivities and specificities. The Panbio Duo Cassette measured these same parameters; for both rapid kits, it was found that using the combination of dengue biomarkers increased sensitivities as SDB DD recorded a higher sensitivity of 39.5%.

In 2009, the WHO attempted to evaluate the use of commercially available anti-dengue virus immunoglobulin M tests (Hunsperger et al., 2009) in the following countries; Thailand, Cambodia, Malaysia, Vietnam, Puerto Rico, Argentina and Cuba. Sensitivities and specificities for several ELISA tests as well as the rapid tests were determined and compared, and it was noted that the sensitivities for the Panbio Duo Cassette were much higher in this evaluation; the highest being 85.6%

in Cambodia and the lowest 65.2% in Thailand. Sensitivities for the SD Bioline were lower than those of the Duo Casette (Hunsperger et al., 2009), the highest recorded was 72.9% in Cambodia and the lowest 47% in Puerto Rico is closer to the values of those recorded in this current study in Trinidad. Geographically, Puerto Rico is much closer to Trinidad and Tobago, and has similar climate and demographic patterns than those in Cambodia. During a study conducted by Pal et al. (2015), with samples from several clinics in Peru, Cambodia, Venezuela and the United States, four diagnostic tests were evaluated, including the two rapid diagnostic tests (SDB DD and Panbio) used in this study. The tests performances were evaluated using serum for all sites and stratified by days post-symptom onset.

The Panbio showed the highest sensitivity (98.5%) between 9-14 days post-symptom onset and the lowest (48.0%) 0 – 3 days post-symptom onset. The SDB DD showed the highest sensitivity (98.6%) in the same category as well as the lowest (80.2% - 0-3 days post-symptom onset), which was notably higher than the sensitivity of the Panbio (Pal et al., 2015). Also, in this instance the sensitivities for Panbio and SDB DD were higher in the convalescent phases (> 5 days) and lower during acute phases (< 5 days), though the actual values were much different, the same trends were observed. This may be accounted for by the smaller sample size in this study as opposed to the one carried out by Pal et al. (2015). With such low sensitivities, these rapid kits may not be valuable tools for presumptive diagnosis in an economy strapped setting. But if the health care providers were to make use of either one of these, a better suggestion should be the SD Bioline because of the higher recorded sensitivities. No sample was found positive for the NS1 antigen, in this study, and sensitivities of SDB DD NS1 generally trend higher in the absence of IgM/IgG (Vickers et al., 2015), maybe owing to or affected by the elevated presence of IgG antibodies in serum samples.

The Pan American Health Organization (PAHO) has issued a release of the number of reported cases of Dengue and severe dengue in the Americas by country for the year 2018. In Trinidad and Tobago, the numbers of cases of dengue were 123, zero was reported as severe dengue and there was no record of death caused by dengue (PAHO, 2018). It is of utmost importance that all probable cases not only be reported but confirmed, especially if headway is to be made on curbing infection and development/implementation of a vaccine. The cost for SDB DD rapid test, which includes an extra test strip to detect the NS1 antigen in addition to IgM and IgG, was US$ 1.14 more than the Panbio with each test method or kit taking the same time to run a complete test on one sample. According to Mitra et al. (2016), the cost of the SDB DD was much lower than that of the Panbio, the cost per test (as per manufacturer's quoted price in India) for Panbio and SDB DD were US$ 6.90 and $ 4.27,

respectively. This study appears to be the only one found to assess the price of each kit, while the difference in price may be attributed to its place of manufacture.

While it took less time to complete just one sample for the Panbio or the SDB DD tests, it was actually more cost effective to complete the tests for more samples with the ELISA kits. The difference between the time in performing 93 samples with Panbio, SDB DD and ELISA was actually very significant (200 min verses 1,395 min, p=0.001). This therefore will favour the use of the ELISA than the Panbio and SDB DD if the TAT were to be considered for all these test kits.

Conclusion

This study had some major draw backs or limitations that included comparing tests kits or methods that were generally imperfect, including the non-confirmation of dengue infection with PCR or culture methods. This study was performed with what is on the ground in so many laboratories in several developing countries. But despite this limitation in the study, the evaluation of these rapid tests revealed that the SD Bioline Dengue Duo Rapid Test (39.9%) appears more sensitive than the Panbio Duo Cassette (22.1%). While the specificities are 100% in each of the three kits, the better option for presumptive diagnosis in clinical settings could be the SDB DD, though both show very low sensitivities. But in a resource strapped setting like the laboratory for the current study, the ELISA should be preferred because its sensitivity and specificity were higher than the Panbio and SDB DD kits. Besides, more samples were tested giving an effective TAT for number of samples completed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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