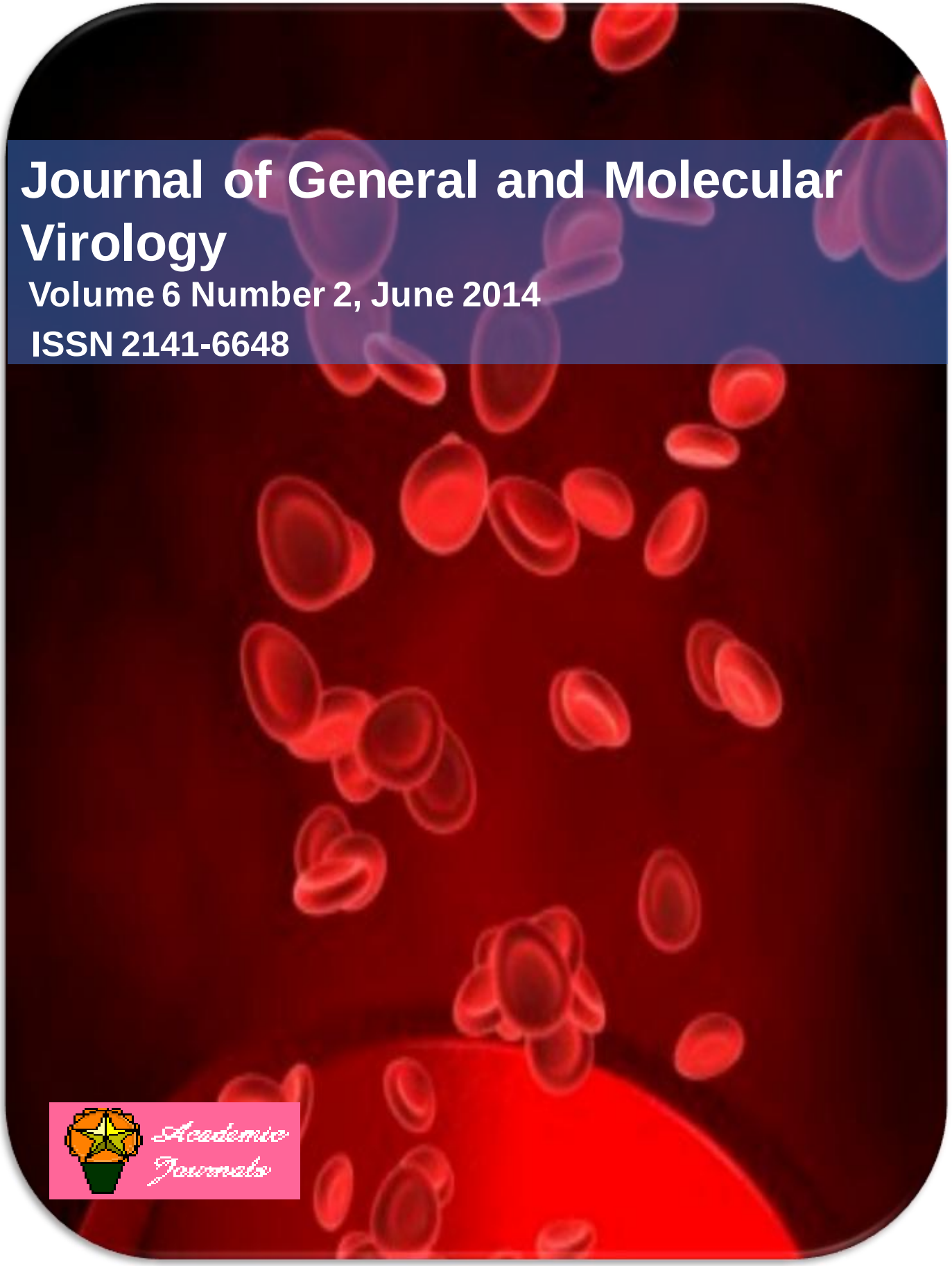

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Full Length Research Paper

Phenotypic and molecular screening of cassava (*Manihot esculentum* Crantz) genotypes for resistance to cassava mosaic disease

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Cassava mosaic disease (CMD), caused by cassava mosaic geminivirus (CMG) is the most-important disease threatening production of cassava (*Manihot esculenta*) in Ghana. The disease is best managed through host-plant resistance. The study was conducted to assess resistance of 38 cassava genotypes to CMD, determine the associated resistance gene, and to identify the strains of CMG infecting cassava in Ghana. Both morphological and molecular markers were used to screen 38 cassava accessions against CMG infection. Morphological studies revealed one genotype (Capevars) as highly resistant whilst three others (Adehye, Nkabom and KW085) were tolerant, showing mild symptoms. PCR analyses using strain specific primers, however, detected the virus in all the three tolerant genotypes, but absent in Capevars. However, the dominant CMD resistance gene, *CMD2*, was detected in both the resistant and the tolerant genotypes. Apart from Capevars, the other 37 cassava genotypes were infected by, at least, one of the four ACMV variants of ACMV1, ACMV2, ACMV-AL and ACMV3. It is, therefore, concluded that field screening for CMD resistance, should integrate phenotypic evaluation and detection of the virus.

Key words: Cassava, African cassava mosaic virus, simple sequence repeats, resistance.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), an Euphorbiaceae (Webster, 1994), is the sixth world food crop for more than 500 million people in tropical and sub-tropical Africa, Asia and Latin America (FAO, 2008). Cassava is the number one staple food crop for majority of Ghanaians, with per capita consumption of 152.9 kg/head/year (MOFA, 2011) and has played a key role in food security in Ghana. It contributes 22% of Agricultural Gross

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Abbreviations: ACMV, African cassava mosaic virus; **CMD,** cassava mosaic disease; **EACMV,** East African cassava mosaic virus; **EACMV-Ug**, East African cassava mosaic virus-Uganda variant; **PCR,** polymerase chain reaction; **SSR,** Simple sequence repeats; **CMG,** Cassava mosaic geminivirus; **WAP,** weeks after planting.

Domestic Product (AGDP) (FAO, 2014) and is also fast becoming an important crop for industries because of its high starch content. In Ghana, cassava is grown across all agro-ecological zones and ranks first in the area under cultivation (MOFA, 2011). However, the average yield of the crop in the country, which is 13.8 Mt ha^{-1}, is far below an achievable yield of 48.7 Mt ha^{-1} (MOFA, 2011). Pests and diseases are a major contributing factor to the low yield of the crop (Akinlosotu, 1985; Thresh et al., 1994). Major pests of cassava include the cassava mealybug (*Phenacoccus manihoti*), green spider mite (*Mononychellus tanajoa*) (Akinlosotu, 1985) and whitefly (*Bemisia tabaci*) (Perrings, 2001).

Cassava mosaic disease (CMD), caused by cassava mosaic geminiviruses of the family *Geminiviridae* and genus *Begomovirus* (Fauquet and Stanley, 2003; Fauquet et al., 2005), is the most important factor limiting cassava yields in many parts of Africa (Fauquet and Fargette, 1990; Legg and Fauquet, 2004). CMD is responsible for an estimated loss of yield of over 1.5 billion US dollars a year (Thresh et al., 1994). It is undoubtedly the most important constraint to the production of cassava in Ghana (Lamptey et al., 1998). The characteristic severe distortion and stunting of leaf and entire plant associated with the disease, especially on local genotypes, indicates how serious yields could be affected (Lamptey et al., 2000). ACMV has been reported to cause 80% yield loss in susceptible cultivars in Ghana (Moses et al., 2007). Losses due to ACMV disease reported elsewhere range from 20 to 95% (Fargette et al., 1988; Hahn et al., 1989; Terry and Hahn, 1990; Otim-Nape et al., 1994; Braima et al., 2000).

The mosaic virus spread is highly linked with its whitefly (*Bemisia tabaci*) vector (Fargette et al., 1985). The virus can also be transmitted from infected planting materials. Plants grown from infected cuttings are much more seriously affected than those infested later by the whitefly vector (*Bemisia tabaci*) and plants infected at a late stage of crop growth are almost unaffected (Thresh et al., 1994).

Nine distinct cassava mosaic viruses have been characterized worldwide from CMD-affected cassava plants and seven of them are from sub-Saharan Africa (Fauquet and Stanley 2003; Alabi et al., 2011). These viruses are African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), East African cassava mosaic Cameroon virus (EACMCV) (Fondong et al., 2000), East African cassava mosaic Kenya virus (EACMKV) (Bull et al., 2006), East African cassava mosaic Malawi virus (EACMMV) (Zhou, et al., 1998), East African cassava mosaic Zanzibar virus (EACMZV) (Maruthi et al., 2004) and South African cassava mosaic (SACMV) (Berrie et al., 1998). Two other viruses, Indian cassava mosaic virus (ICMV) (Matthew and Muniyappa, 1992; Saunders et al., 2002) and Sri Lankan cassava mosaic virus (SLCMV) (Saunders et al., 2002), were reported from the Indian sub-continent.

Cassava mosaic geminivirus (CMG) strains reported so far in Ghana are ACMV (Clerk, 1974; Lamptey et al., 1998) and EACMV (Offei et al., 1999). ACMD was first observed near Accra in 1926 (Doku, 1966) and its spread was more significant in the coastal areas of the country around 1930 (Leather, 1959; Clerk, 1974). At present, ACMD is widespread and found in all the agro-ecological zones in Ghana (Lamptey et al., 1998). The EACMV was first reported in Ghana in 1999 (Offei et al., 1999). The emergence of EACMV, which has its origin from East Africa but has been documented in Central and West Africa (Fondong et al., 1998; Offei et al., 1999; Ogbe et al., 1999), raises a lot of concern to cassava growers in the sub-region including Ghana.

Effective management of the CMD-pandemic in Ghana is quite important in order to improve yields. The most effective means of controlling CMD is by the deployment of resistant varieties (Thresh et al., 1997). CMD-resistant cassava had been developed through integration of resistance traits from *Manihot glaziovii* by interspecific hybridization (Nicholas, 1947), which has become the major source dominating CMD resistance in Africa (Fargette et al., 1996). Two CMD resistance genes *CMD1* (recessive gene) and *CMD2* (major dominant gene) have so far been placed on the map and important molecular markers associated with the *CMD2* gene have been identified (Fregene et al., 2001; Akano et al., 2002). Through cassava breeding programmes, these markers are very useful and hold great promise in fast-tracking the identification of CMD-resistant germplasms (Bi et al., 2010). Knowledge of genetic diversity or an understanding of which viral strain, and strain combinations and how they are distributed, is important to such breeding programmes for resistance.

This work was, therefore, aimed at assessing the genetic diversity of ACMV currently infecting cassava in Ghana, identifying resistant cassava cultivars and determining the presence of the *CMD2* resistance gene using its associated simple sequence repeats (SSR) markers.

MATERIALS AND METHODS

Collection of cassava planting materials

Thirty-eight (38) distinct cassava genotypes were used for the study. Thirty (30) of them were obtained from the Plant Genetic Resources Research Institute (PGRRI), Bunso, Ghana and the remaining eight from the University of Cape Coast (U.C.C.) Teaching and Research Farm, Cape Coast, Ghana. Three of the materials (Capevars, Adehye, and Nkabom) have been released as cultivars for farmers.

Field experiment

Experimental site and field layout

The 38 cassava genotypes were evaluated in 2007/2008 and 2008/2009 growing seasons, on the Teaching and Research Farm,

Table 1. Disease rating and the corresponding symptom expression for cassava mosaic disease (CMD).

Rating	Symptom
1	No symptoms observed
2	Mild chlorotic pattern on entire leaflets or mild distortion at base of leaflets appearing green and healthy
3	Strong mosaic pattern on entire leaf, and narrowing cum distortion of lower one-thirds of leaflets
4	Severe mosaic distortion of two-thirds of leaflets and general reduction of leaf size
5	Severe mosaic distortion of four-fifths or more of leaflets, twisted and misshapen leaves.

U.C.C., Ghana. The location (5.1000° N, 1.2500° W) is a coastal savanna zone with a ferric luvisol soil type and is a high pressure (highly endemic) site for CMD. The soil has been described by Asamoa (1973) as Atabadze, equivalent to Ultisol in the United States Department of Agriculture, (USDA) classification. Cape Coast has a typical climate of the coastal savannah lowland characterized by an annual rainfall range of 800 to 1000 mm and mean monthly temperature of about 26.5°C.

A 380 m^2 land (38 × 10 m) was ploughed, harrowed and divided into 10-m rows with 1.0 m between rows in the 2007 and 2008 major planting seasons. A total of 38 cassava genotypes were planted in single rows in completely randomised plots. Ten 20 cm-long cuttings (bearing three to four nodes) were planted per genotype, in single rows at a spacing of 1 m within rows and 1 m between rows.

Cultural practices

The ploughed and harrowed field was lined and pegged before planting. The experiment was set out under rain-fed conditions and weeding was done manually using a hoe or cutlass when necessary.

Morphological screening of the cassava genotypes for CMD resistance

The 38 cassava genotypes were evaluated at 6, 12, 20 and 48 weeks after planting (WAP) in both 2007/8 and 2008/9 growing seasons to ascertain the resistance status of each genotype to CMD. Each plant was examined for symptom severity of the whole plant. Plants were assigned disease severity scores based on the standard 1-5 disease rating (Hahn, 1980; IITA, 1990; Ariyo et al., 2005), where 1 represents no disease symptom and 5 being the presence of the most severe symptoms, including severe chlorosis, leaf distortion and plant stunting (Table 1).

Five plants for each genotype were scored and the mean ordinal score determined. Plants with a mean CMD severity score of “1” were then classified as highly resistant (HR), those with a score of “2” were moderately resistant (MR), those with a score of “3” were classified as susceptible (S) and those with scores of “4” and “5” were classified as highly susceptible (HS), according to Lokko et al. (2005)

Determination of population of whitefly

Since whiteflies are the vectors of CMD, their population on cassava plants were determined in order to assess their relationship with the severity of the CMD disease infection. Direct counts of adult whiteflies on the crop were made as previously described (Hill, 1968; Fargette et al., 1985; Abdullahi et al., 2003). Whitefly counting was usually done between 0600 and 0800 h when the environment was cooler and whiteflies were relatively immobile compared to later in the day as reported by Fauquet et al. (1987). Adult whitefly populations on the five topmost fully expanded leaves of the selected cassava cultivars were counted according to Otim-Nape et al. (2005) and Ariyo et al. (2005).

Whitefly count was often carried out on the five topmost fully expanded leaves. The counts were done one month after planting and were repeated at three and six months after planting. Five plants were randomly selected for each cassava genotype. On each plant, leaves were carefully turned over and the number of adult whiteflies on the abaxial leaf surfaces were counted and recorded. The mean number of whiteflies per 5 top leaves was then determined.

Screening for CMD resistance using molecular markers

Collection of cassava leaf samples

Young leaves from the 38 cassava genotypes were collected from both CMD-infected plants (symptomatic) and uninfected (non-symptomatic) plants at the experimental site.

DNA extraction and purification

Genomic DNA was extracted from the fresh samples, according to the method described by Dellaporta et al. (1983) with slight modifications. The leaf tissues were lysed using a lysis buffer, followed by extraction of DNA from the leaf tissues and DNA precipitation. DNA pellets from precipitation were washed with 700 µl of 80% ethanol, air-dried on tissue paper at room temperature (25-30°C) re-dissolved in 100 µl of 1x TE buffer and stored at -20°C until required.

PCR amplification

The ACMV strains or variants causing the mosaic symptoms in the 38 accessions were detected using the PCR method described by Zhou et al. (1997). The DNA samples of the cassava genotypes were tested for presence or absence of CMG using primers that could detect the four variants of ACMV (ACMV1, ACMV2, ACMV-AL and AVMV3). Four pairs of primer sequences designed by Zhou et al. (1997) were used (Table 2). The PCR reactions were conducted using Applied Biosystems® 2720 Thermal Cycler in 96-well plates (Life Technologies, New York, USA). The reaction mixture composed of 10 µl, which consists of AccuPower® PCR Premix (BIONEER Inc., Alameda, USA), genomic DNA, sterile distilled water (SDW) and primers. The PCR mixture contained 9 µl of PCR premix and primers and genomic DNA (10 ng μl^{-1}). The PCR programme consisted of an initial denaturation for 4 min at 94°C and then 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s depending on the annealing temperature of the primer, and

Table 2. Primers for PCR amplification and strain differentiation of cassava mosaic virus diseases.

Virus strain	Name of primer	Primer sequence (5' - 3')	Reference
ACMV1	ACMV-F1	TTC AGT TAT CAG GGC TCG TAA (F)	Zhou et al. (1997)
	ACMV-R1	GAG TG AAG TTG ACT CAT GA (R)	Zhou et al. (1997)
ACMV2	ACMV-F2	GTG AGA AAG ACA TTC TTG GC (F)	Zhou et al. (1997)
	ACMV-R2	CCT GCA ATT ATA TAG TGG CC (R)	Zhou et al. (1997)
ACMV-AL	ACMV-AL1/F	GCG GAA TCC CTA ACA TAA TC (F)	Zhou et al. (1997)
	ACMV-ARO/R	GCT CGT ATG TAT CCT CTA AGG CCT (R)	Zhou et al. (1997)
ACMV3	ACMV-1	GCTC AAC TGG AGA CAC ACT TG (F)	Zhou et al. (1997)
	ACMV-2	CCT GCA ACA TAC TTA CGC TT (R)	Zhou et al. (1997)

extension at 72°C for 1 min and final extension of 5 min at 72°C. The PCR products were separated by electrophoresis in a 1% agarose gel at 100 V for 1.5 h. The gel was stained with ethidium bromide and viewed under UV light.

Detection of *CMD2* resistance gene in ACMD-resistant cassava genotypes

Plant DNA samples that did not show presence of any of the strains of cassava mosaic virus following PCR amplification with strain specific primers were further amplified with specific SSR markers (SSRY28, NS158, NS169 and RME1) associated with the *CMD2* gene, the dominant gene, which confers resistance to ACMD. PCR amplification and gel electrophoresis were carried out as described earlier.

Data analysis

Scatter plots showing the relationship between mean whitefly population and mean CMD severity scores during 2007 and 2008 crop seasons were drawn using MICROSOFT EXCEL (Microsoft Corporation, USA). The corresponding correlation coefficients were also determined using GenStat statistical software version 12 (Payne et al., 2009).

The relationships among cassava accessions, with respect to their susceptibility to the four ACMV strains were determined based on band patterns produced in the gel. Bands of alleles were scored as 1 for presence of virus or infection, and 0 as absence of alleles, denoting no infection or healthy, for various primers-cassava accessions combinations. The band scores were then used to calculate genetic distances (Nei, 1983) between pairs of cassava accessions. Then, using the unweighted pair-group mean average (UPGMA) cluster method of Nei's genetic distance (Sneath and Sokal, 1973), a dendrogram of genetic similarity was constructed using the Power Marker software version 3.5 (Liu and Muse, 2005).

RESULTS

Cassava mosaic disease (CMD) severity

The mean CMD severity scores recorded for the cassava genotypes planted during 2007 and 2008 growing seasons showed a varying and an interesting pattern (Table 3). At 6 weeks after planting (WAP) in 2007 the mean score for all the cassava genotypes on the field was 2.8, with a range score of 1-5.

With this range of scores, five accessions had a score of 1, 12 had a score of 2, 14 had a score of 3, nine were scored 4 while three accessions registered the highest score of 5. Thus, DMA 002, ADW 004 and OFF 029, which had the highest score of 5, were the most susceptible to ACMV infection at 6 WAP.

AT 12 WAP, four genotypes had a score of 1, twelve a score of 2, sixteen a score of 3, nine a score of 4 and three had a score of 5. The mean severity score was 2.9 for 2007. In 2008 the severity scores at 12 WAP were 1, 2, 3, 4 and 5 for four, nine, seven, twenty and four accessions, respectively, with a mean score of 3.3. This indicates that the severity of infection of the cassava genotypes by the ACMV was higher in 2008 than in 2007. This indicates that the cassava genotypes were more susceptible to the ACMV infection in 2008 than in 2007.

At 20 WAP in 2007, the mean score was 2.6 and that of 2008 was 3.4 with severity scores for both years ranging between 1 and 5. At 48 WAP, which was the harvest time, ACMD severity score was recorded to assess the degree of recovery from the disease among the accessions. The mean scores reduced to 1.7 and 1.9 for 2007 and 2008, respectively.

However, in both years, 23 had severity score of 1, 12 were scored 2, five had a score of 3 while three of them had a score of 4. None of the accessions was scored the most severity score of 5.

The overall mean CMD severity responses recorded for all the 38 cassava accessions at different sampling dates and time revealed varying levels of resistance or susceptibility (Figure 1). The accessions were thus grouped into the five disease severity classes. Three genotypes were classified as highly resistant (HR) with a mean score of 1, nine as resistant (R) with a mean score of 2, 12 as susceptible (S) with a mean score of 3 and 14 as highly susceptible (HS) with mean scores of 4 and 5.

Whitefly population

At six weeks after planting (WAP), the overall mean adult whitefly population was 9.7 whiteflies plant^{-1}, with a range of 1.8 to 28.4 whiteflies plant^{-1} in 2007 (Table 4). More

Table 3. Severity of cassava mosaic disease (CMD) infections on 38 cassava accessions during 2007 and 2008 cropping seasons.

Cassava accession	2007				2008			
	WAP				WAP			
	6	12	20	48	6	12	20	48
OFF 146	3.7	4.2	3.1	2.1	5.0	4.2	3.7	3.1
AFS 136	3.0	2.7	2.2	1.9	4.7	3.7	3.1	1.4
ADW 063	4.0	3.1	3.2	1.2	5.0	4.4	2.8	1.2
DMA 002	4.7	5.0	4.0	1.0	5.0	4.1	4.1	1.3
AFS 001	4.0	3.8	2.7	2.8	4.2	4.0	5.0	4.3
AFS 027	3.1	4.1	3.0	2.2	3.1	4.4	4.3	1.0
OFF 058	4.3	2.7	2.8	2.1	4.0	3.2	4.1	3.1
DMA 066	3.1	4.1	3.1	1.3	4.1	3.0	3.3	1.0
ADW 004	4.6	5.0	4.1	4.0	5.0	4.1	4.4	1.2
AFS 131	4.4	4.3	3.2	3.2	5.0	4.0	3.6	1.1
KW 148	2.1	3.1	2.0	1.0	3.8	3.2	2.8	1.2
KW 181	3.3	3.3	3.0	2.1	4.7	4.8	4.2	1.4
ADW 051	3.1	3.1	2.1	1.0	3.3	2.4	3.1	2.4
KW 001	1.5	2.8	1.8	1.0	4.0	2.1	3.3	1.1
KW 085	1.0	1.0	1.0	1.0	1.0	1.0	2.2	1.0
OFF 029	4.6	4.8	3.5	1.8	5.0	5.0	5.0	3.2
ADW 053	2.9	3.1	1.6	1.0	3.1	2.3	4.3	1.3
OFF 086	3.1	2.6	3.0	2.0	4.7	3.4	3.1	2.2
OFF 145	2.2	3.3	4.0	3.7	4.0	4.0	5.0	4.1
KW 161	3.1	2.4	3.1	1.0	4.2	3.1	4.1	2.0
OFF 025	1.8	3.9	4.3	2.0	5.0	4.3	4.8	3.2
OFF 023	2.8	2.6	2.0	2.0	3.1	4.0	1.7	1.3
OFF 063	1.0	1.7	2.1	1.0	2.3	2.0	1.8	1.2
AFS 048	2.1	2.1	1.7	1.0	2.1	2.3	2.1	2.3
KW 070	3.8	3.0	5.0	1.0	4.3	4.6	4.8	1.0
AFS 041	2.0	2.1	2.0	2.7	1.5	2.4	2.2	2.1
OFF 093	3.0	3.0	2.8	1.0	3.2	4.3	4.2	1.0
OFF 019	2.3	2.0	2.1	1.0	3.3	2.8	2.4	2.0
AFS 126	4.1	3.7	3.9	1.0	5.0	4.1	5.0	4.4
NKABOM[a]	2.4	2.3	1.5	1.1	1.9	1.9	2.0	2.0
OFF 136	2.1	3.0	2.1	1.7	2.2	2.1	2.1	2.0
UCC 517	2.7	2.1	2.0	1.8	3.4	4.1	3.2	2.0
UCC506	2.2	2.0	1.3	1.1	1.6	3.2	4.1	1.3
B. BOTAN[a]	1.0	1.0	2.0	1.0	1.6	3.5	2.6	2.4
CAPEVARS[a]	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0
ADEHYE	1.0	1.0	1.2	1.0	1.0	1.0	1.0	1.0
UCC 470	2.0	2.2	2.0	1.2	2.2	1.4	2.0	2.0
UCC 153	1.8	2.0	3.0	1.3	3.0	2.2	2.3	2.0
Mean	2.8	2.9	2.6	1.6	3.4	3.2	3.3	1.9
Range	1 - 5	1 - 5	1 - 5	1- 4	1 - 5	1 - 5	1 - 5	1 - 4
%CV	39.3	35.9	38.5	52.9	37.1	33.3	35.3	52.6

WAP = Weeks after planting.

than 50% of the cassava accessions had values below the overall mean value for 2007. However, in 2008 at 6 WAP, the overall mean was 93.2 whiteflies plant^{-1} with a range of 25.4 to 209.9. The mean in 2008 was almost 10 times higher than that for 2007. Capevars had the highest mean number of whiteflies plant^{-1}, being 28.4 and 209.9

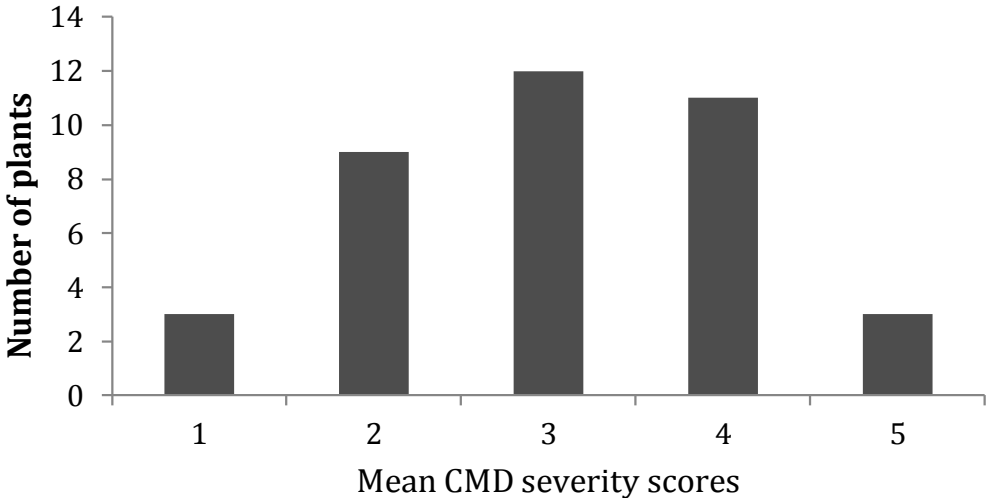

Figure 1. Distribution of 38 cassava accessions in CMD severity classes of 1 to 5. A score of 1 denotes no symptom while 5 indicates a display of severe mosaic symptoms, based on the mean CMD severity responses.

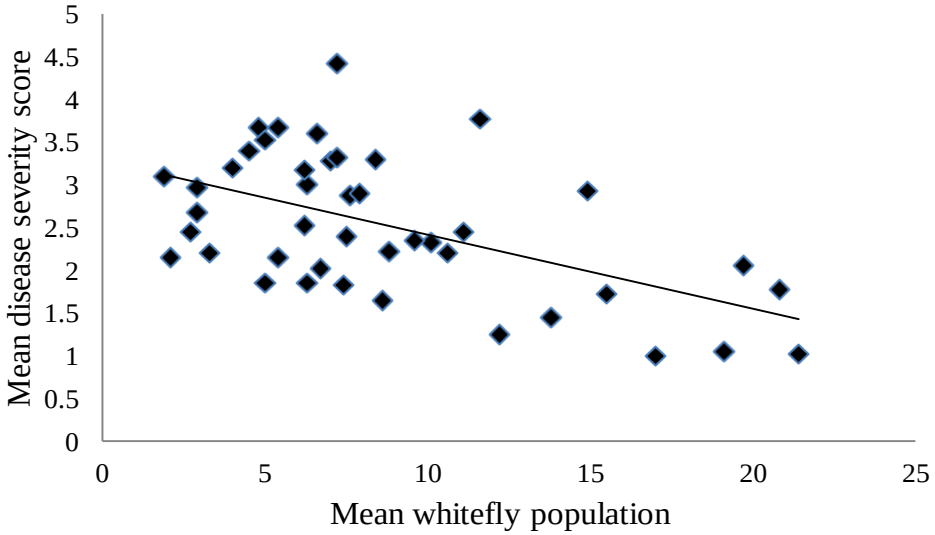

Figure 2. Relationship between mean whitefly population and mean score of cassava mosaic disease (CMD) during 2007 crop season (r = -0.543; $P < 0.05$).

for 2007 and 2008, respectively (Table 4). The lowest count was recorded on OFF 086 with a mean value of 1.8 and 25.4 for 2007 and 2008, respectively.

The whitefly population for most accessions reduced at 8WAP for both years. The mean values were 8.7 for 2007 and 33.9 for 2008. Adehye (24.4) and AFS 001 (52.7) had the highest mean counts for 2007 and 2008, respectively.

The whitefly population reduced further for most of the genotypes at 10 WAP. The mean counts ranged from 1.1 to 18.6 and 5.5 to 35.3 for 2007 and 2008, respectively (Table 4). The most infested genotypes were KW 148 for 2007 and AFS 001 for 2008. Overall, AFS 027 was the least infested by whiteflies and Capevars was the most infested in 2007. However, in 2008, genotype AFS136 was the least infested and Capevars cultivar was again the most infested. The infestation in 2008 also was clearly higher than in 2007.

Relationships between whitefly population and disease severity score

Interestingly, in both 2007 and 2008 crop seasons (Figures 2 and 3), the mean whitefly populations

Table 4. Mean number of adult whiteflies on 38 genotypes of cassava during 2007 and 2008 crop seasons.

Cassava accession	2007				2008			
	WAP				WAP			
	6	8	10	Mean	6	8	10	Mean
OFF 146	7.6	9.0	4.5	7.0	53.9	31.4	26.1	37.1
AFS 136	18.0	11.4	3.8	11.1	28.0	29.0	5.6	20.8
ADW 063	12.6	5.6	4.7	7.6	55.0	25.1	15.8	32.0
DMA 002	3.0	5.6	5.7	4.8	43.0	46.1	12.5	33.9
AFS 001	10.6	7.6	3.4	7.2	31.4	52.7	35.3	39.8
AFS 027	2.2	2.4	1.1	1.9	49.6	33.0	9.3	30.6
OFF 058	3.4	3.0	2.4	2.9	56.6	23.2	15.4	31.7
DMA 066	11.6	8.4	3.8	7.9	79.9	46.9	27.0	51.2
ADW 004	8.8	8.0	4.8	7.2	49.6	31.9	27.5	36.3
AFS 131	16.2	13.3	5.2	11.6	39.2	27.4	11.8	26.1
KW 148	25.2	15.3	18.6	19.7	98.7	38.5	9.7	48.9
KW 181	26.4	10.8	7.5	14.9	51.7	42.2	25.8	39.9
ADW 051	13.6	8.2	8.6	10.1	70.3	33.6	13.6	39.2
KW 001	28.4	17.2	16.9	20.8	90.3	44.5	25.7	53.5
KW 085	5.2	6.0	4.9	5.4	90.0	28.4	8.6	42.3
OFF 029	6.2	5.6	4.5	5.4	97.6	33.3	11.8	47.6
ADW 053	20.4	16.6	14.0	17.0	71.7	42.9	12.3	42.3
OFF 086	1.8	3.6	3.4	2.9	25.4	30.0	27.2	27.5
OFF 145	10.0	8.8	6.3	8.4	81.8	25.4	11.7	39.6
KW 161	6.5	8.6	7.5	7.5	106.8	31.7	24.4	54.3
OFF 025	5.8	8.2	4.9	6.3	47.0	27.1	12.3	28.8
OFF 023	10.3	10.8	7.8	9.6	127.5	26.5	12.4	55.4
OFF 063	14.0	13.4	14.0	13.8	89.7	45.5	5.5	46.9
AFS 048	14.2	16.6	15.6	15.5	163.8	35.9	15.0	71.6
KW 070	3.4	4.6	3.9	4.0	55.6	21.3	15.0	30.6
AFS 041	11.2	7.6	12.9	10.6	162.8	33.5	10.0	68.8
OFF 093	2.6	2.2	3.3	2.7	134.9	31.6	24.1	63.5
OFF 019	6.6	7.6	4.7	6.3	143.7	34.3	10.1	62.7
AFS 126	4.6	8.6	5.5	6.2	105.6	41.5	14.6	53.9
NKABOM[a]	8.0	8.8	9.6	8.8	165.7	32.6	11.9	70.1
OFF 136	5.8	8.2	8.2	7.4	175.9	32.7	12.1	73.5
UCC 517	3.0	1.0	2.2	2.1	128.2	35.1	9.1	57.5
UCC506	10.2	6.0	9.7	8.6	161.5	22.4	14.7	66.2
B. BOTAN[a]	11.6	16.2	8.9	12.2	162.5	26.5	17.0	68.6
CAPEVARS[a]	16.8	24.4	16.1	19.1	140.3	38.4	19.6	66.1
ADEHYE	26.6	21.6	16.0	21.4	209.9	33.9	26.7	90.2
UCC 470	7.4	1.4	6.2	5.0	93.6	43.9	27.0	54.8
UCC 153	2.2	7.0	10.8	6.7	97.9	30.1	26.2	51.4
Mean	9.7	8.7	7.6	8.7	93.2	33.9	16.4	47.8
Range	1.8 -28.4	1.0 - 24.4	1.1 - 18.6	1.9 - 21.4	25.4-209.9	21.3- 52.7	5.5 - 35.3	20.8-90.2
% CV	75.3	60.9	59.2	59.8	49.5	33.9	44.5	32.4

[a] Released varieties; WAP=weeks after planting.

significantly (P <0.05) negatively correlated with mean CMD severity scores. That is, on the average, higher populations of whitefly were found on the resistant cultivars than on the susceptible cultivars.

Detection by PCR of 4 variants of ACMV

All four ACMV-specific primer pairs (associated with the four variants of ACMV), produced allelic bands in the

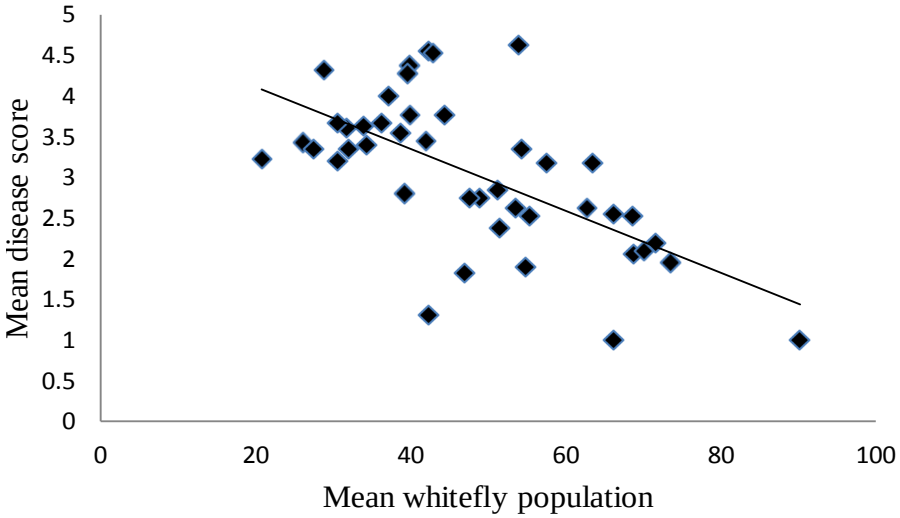

Figure 3. Relationship between mean whitefly population and mean score of cassava mosaic disease (CMD) during 2008 crop season. (r = -0.634; *P* < 0.05).

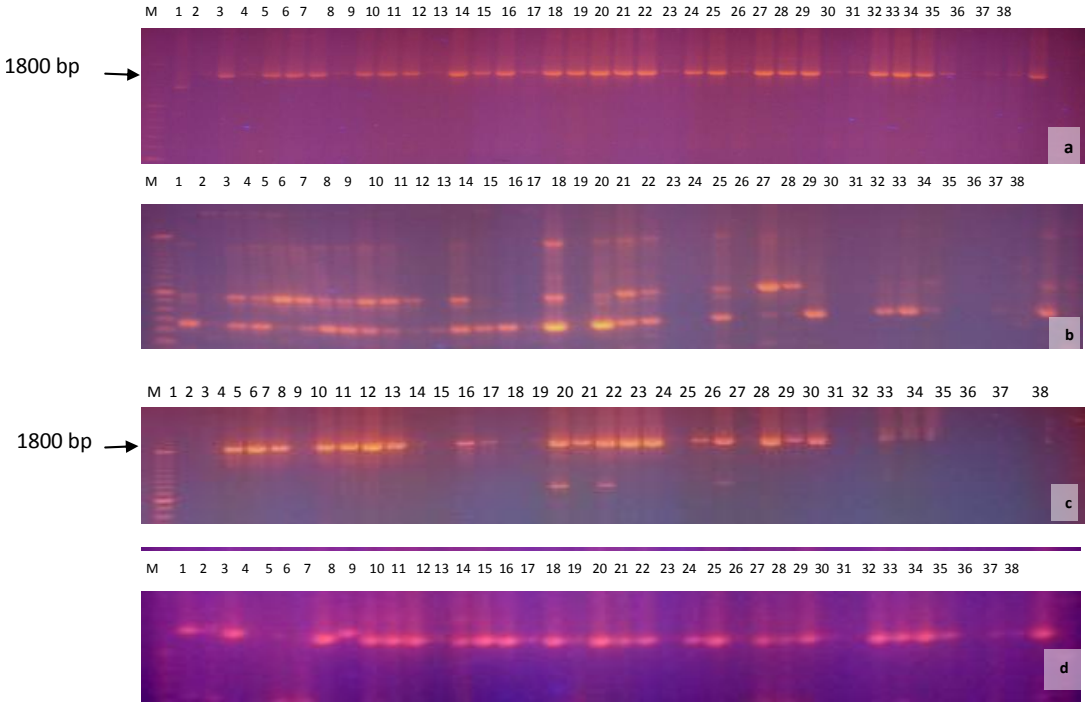

Figure 4. PCR amplification products for ACMV-specific primers: ACMV-F1/ACMV-R1 (a), ACMV-F2/ACMV-R2 (b), ACMV-AL1/F/ACMV-ARO/R(c) and ACMV-1/ACMV-2 (d) - resolved by PAGE and stained with ethidium bromide. M = 1kb+ ladder; 1-38 represent the various cassava accessions. Arrow indicates specific band for ACMV resistance.

accessions. The ACMV-specific primer pair that was most efficient in detecting the virus was ACMVF1/ACMV-R1, which detected the virus in 34 (89.5%) out of the 38 cassava accessions, whilst the primers ACMV-1/ ACMV-2, ACMV-F2/ACMV-R2, and ACMV-AL1/F/ACMV-ARO/R detected the virus in 26(68.4%), 24(63.2%) and 22(57.9%) accessions, respectively (Figure 4). With the exception of genotype Capevars, all the samples were

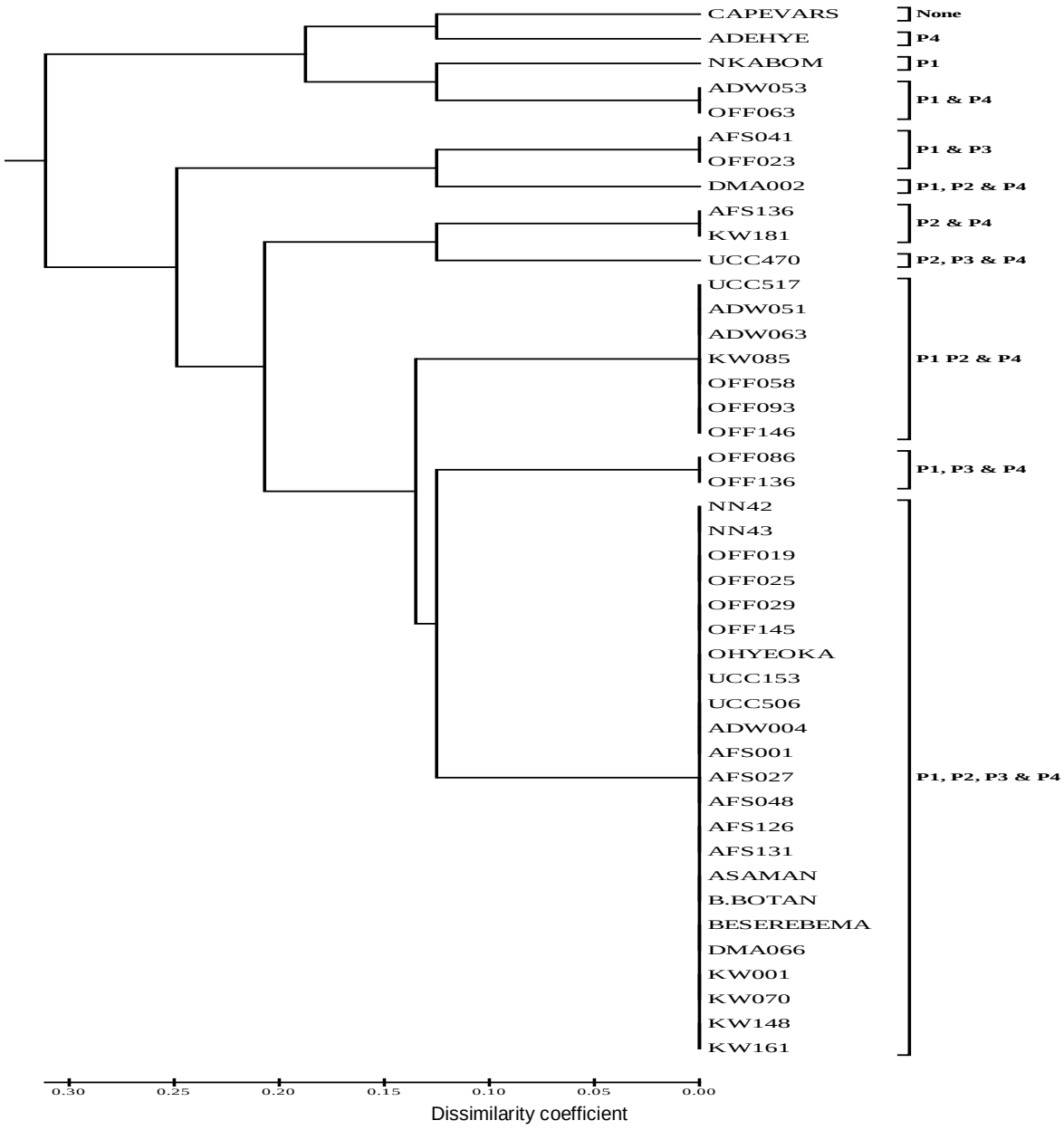

Figure 5. Genetic differences among the 38 cassava accessions based on PCR products of four ACMV primer pairs using the unweighted pair group method with arithmetic averages. P1, P2, P3 and P4 represent ACMV variants ACMV1, ACMV2, ACMV-AL, and AMCV3, respectively.

infected with one or more of the ACMV strains. The cassava genotypes were infected with two or more of the ACMV variants, with the exception of Adehye and Nkabom, which were infected with only one ACMV variant (ACMV1 and ACMV3, respectively).

The cassava genotypes were clustered into 11 groups at a similarity coefficient of 0.13 based on the PCR amplification products, indicating that the cassava genotypes were genetically diverse (Figure 5). The cluster size ranged from 1 to 23 cassava accessions. Cluster 11 had the highest number of accessions (Figure 5).

Detection of *CMD2* resistance gene

From the results obtained from PCR reactions with ACMV-specific primers and field screening for CMD resistance, four genotypes were selected for further screening with markers associated with the *CMD2* gene that confers resistance to CMD to ascertain their source of resistance. All the four accessions selected had bands of alleles of all the four markers associated with the *CMD2* gene (Figure 6). However, the bands present were more intense in two markers (NS169 and RME1), which

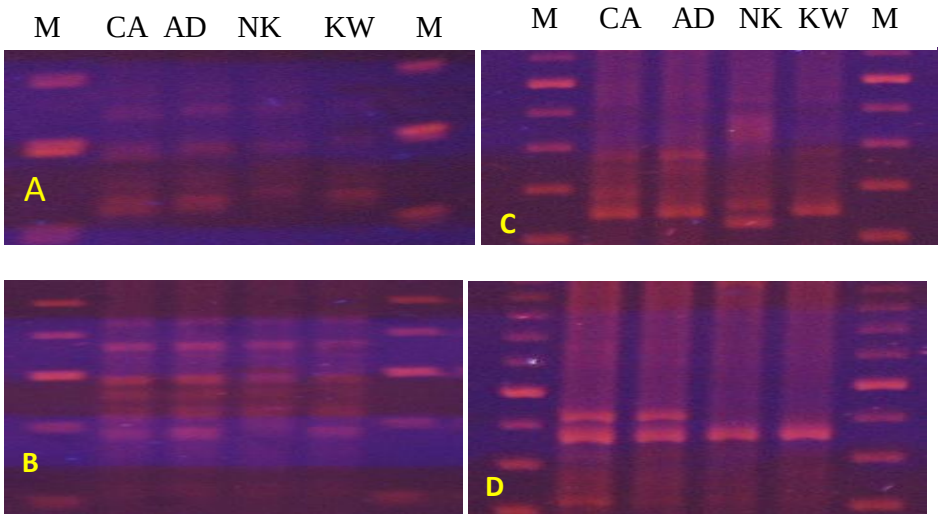

Figure 6. PCR amplification products of four markers associated with *CMD2* resistance gene (SSRY28 (A), NS158 (B), NS169 (C) and RME1 (D) resolved by PAGE stained with ethidium bromide among 4 cassava accessions - Capevars (CA), Adehye (AD), Nkabom (NK) and KW085 (KW). M is the standard marker.

are closer to the gene than the SSRY28 and NS158 markers, indicating that they were more efficient in detecting the *CMD2* gene than the latter two.

DISCUSSION

Morphological screening of the 38 cassava genotypes for CMD resistance based on the 1-5 disease rating (IITA, 1990; Ariyo et al., 2005) and classification according to Lokko et al. (2005) revealed one highly resistant genotype (Capevars) and three moderately resistant genotypes (Adehye, Nkabom and KW 085) (Table 3). However, the subsequent resistance screening using PCR with CMG strain-specific primers showed that only one genotype, Capevars, was resistant whilst the others were infected with ACMV (Figures 4 and 5). This suggests that the three genotypes (Adehye, Nkabom and KW 085) are tolerant to ACMV infection whereas Capevars was a resistant genotype. Thus, field selection of resistance should be complemented with virus detection methods such as PCR. The reason could be that the field resistance, as shown by lack of symptoms, is not necessarily an indication of resistance to virus infection as has been reported by Ogbe (2001). Therefore, the mean symptom severity scores calculated for breeding lines has a limitation, in that, the virus incidence and symptom severity are not clearly distinguished; and symptomless plants plants could be CMD-free 'escapes', or they could be extremely tolerant (Thresh and Cooter, 2005). Moreover, a low average score for a progeny or selection could mean that a few plants are infected and show severe symptoms, or that many succumb but are only slightly affected.

The ACMV-specific primer ACMVF1/ACMV-R1 was more efficient in detecting the virus in the cassava genotypes, since it detected the virus in more samples than the primers ACMV-1/ ACMV-2, ACMV-F2/ACMV-R2, and ACMV-AL1/F/ACMV-ARO/R. Whilst primer ACMVF1/ACMV-R1 detected the virus in 34 (89.5%) out of the 38 cassava accessions, the primers ACMV-1/ACMV-2, ACMV-F2/ACMV-R2, and ACMV-AL1/F/ACMV-ARO/R detected the virus in 26 (68.4%), 24 (63.2%) and 22 (57.9%) accessions respectively. In screening F_1 progeny of cassava against CMD infection, Lokko et al. (2005) also observed that the ACMV primer ACMV-F1/ACMV-R1 detected the virus in more samples than the primer ACMV-AL F/ACMV-AROR. This suggests that the ACMV1 strain detected by the primer ACMVF1/ACMV-R1 as reported by Zhou et al. (1997) is the most dominant virus among the ACMV variants detected in the study.

The detection of the resistance gene (*CMD2*) using linked SSR markers, in the four field-resistant cassava genotypes (Capevar, Adehye, KW058 and Nkabom) suggests that the *CMD2* gene is, at least, partly responsible for both CMD resistance and field tolerance. In this case Capevars can be said to be a highly resistant genotype, whereas Adehye, KW058 and Nkabom, which showed mild field symptoms are tolerant genotypes. The dominant nature of *CMD2* and its effectiveness against a wide spectrum of viral strains makes its deployment very appealing in protecting cassava against the actual or potential ravages of CMD in Africa (Boateng, 2010). Knowledge of the markers associated with this resistance gene will also facilitate the use of marker- assisted selection in a cassava breeding programmes for the development of resistant lines. It was observed in this study,

that markers RMEI and NS158 were more reliable for the detection of the CMD2 resistance gene than markers SSRY28 and NS158, as the former gave more intense bands in the gel than the latter two.

Capevars, the CMD-resistant cassava cultivar has since been released (Tetteh et al., 2005). Currently, the Government of Ghana, through the Ministry of Food and Agriculture, is multiplying the Capevars cultivar to be distributed to farmers, especially, those from the Western Region (J.P. Tetteh, pers. comm.).

The highest mean severity score for 2007 was recorded at 12 WAP. This finding agrees with Leuschner (1978) and Ogbe et al. (1996) that high incidence of CMD is achieved at 12 WAP. However, in 2008 the highest mean severity was recorded at 6 WAP. It might be due to the fact that the cuttings used were obtained from the previous crop, and these might have been already infected. This confirms the reports of Fargette et al. (1988) that plants are generally more susceptible to secondary infection.

Most (35 out of 38) of the cassava genotypes showed mixed infection with the four different ACMV variants, and this can have serious consequences for the management of CMD. It has been reported that mixed infections provide the precondition for recombination, which may contribute to the appearance of more severe viral strains (Ribeiro *et al.*, 2003). Zhou et al. (1997) has shown that EACMV-Ug, associated with the severe cassava mosaic disease in Uganda, has arisen by interspecific recombination of EACMV and ACMV**.** Mixed genotypes infections have been reported in many host-pathogen interactions (Read and Taylor, 2001; Hodgson et al., 2004; Schurch and Roy, 2004).

The whitefly, *Bemisia tabaci*, is one of the most important insect pests in world agriculture, because of its direct feeding, contamination from honeydew, and ability to transmit plant viruses (Perrings, 2001). Additional evidence of differences in whitefly infestation among a range of cassava accessions at different locations in Ghana were also found in the present study. The adult whitefly population was high at six WAP in both years. A higher number of whiteflies were found on resistant genotypes in this study, which agrees with Otim Nape et al. (2005), who recorded higher populations of *B. tabaci* on the cassava mosaic disease-resistant genotypes than in susceptible ones. Similar observations have been made by Legg et al. (2003), and are attributed to the whitefly preference for the resistant varieties of cassava. The leaves of resistant plants were broader and softer than the susceptible ones, whose leaves were mis-shapen, highly reduced and showed severe mosaic symptoms. According to Sserubombwe et al. (2001), Omongo (2003) and Ariyo et al. (2005), such leaves are usually avoided by the whitefly and this might account for the whitefly preference for the resistant plants in this study. Otim-Nape et al. (1994) has also reported the lack of any significant correlation between whitefly numbers and mosaic severity when they studied the effects of African cassava mosaic geminivirus on the main cassava varieties grown in three districts of western Uganda. On the contrary, we observed a significant negative correlation between the whitefly population and the CMD severity scores. This further supports the findings earlier made by Sserubombwe et al. (2001), Omongo (2003) and Ariyo et al. (2005).

Conclusion

Out of 38 cassava genotypes screened against CMG infection, three tolerant cassava genotypes (Adehye, KW058 and Nkabom) and a highly resistant genotype, (Capevars) were identified. Apart from Capevars, between 1 and 4 variants of ACMV (ACMV1, ACMV2, ACMV-AL, and ACMV3) were detected in the cassava genotypes including the tolerant ones. This suggests that field selection of resistance should be complemented with virus detection methods such as PCR test. Most (35 out of 38) of the cassava genotypes showed mixed infections with two or more ACMV variants, which could have serious consequences for the management of the CMD in Ghana. A higher number of whiteflies were found on resistant genotypes than the susceptible genotypes in this study, which confirms that the presence of whiteflies per se may not be an indication of possible infection with the ACMV.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Molecular characterization of influenza A (H7N9) virus from the first imported H7N9 infection case in Malaysia

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H7N9 is an avian strain of the species Influenza virus that circulates among avian populations. Occasionally, some variants of this strain were known to infect humans. On March 30, 2013, a novel avian influenza A H7N9 virus that infects human beings was identified in China. In February 2014, the first case of H7N9 infection outside China was reported in Malaysia involving a Chinese tourist. This study was aimed to characterize the first case of H7N9 in Malaysia by means of molecular identification, sequencing of hemagglutinin (HA) and neuraminidase (NA) genes, and phylogenetic analysis. The patient was confirmed positive for H7N9 virus by real-time RT-PCR (rRT-PCR). Subsequently, the samples were sequenced and mutation analysis identified R65K, E122K, L186I and N285D mutations in *HA* gene and M26I, R78K and V345I mutations in *NA* gene. We reported the emergence of a new mutation L186I, not found in the current database of any H7N9 sequences. Mutations associated with drug resistance were not found in this patient. Phylogenetic analysis revealed that the *HA* gene is closely related to the group of strains from Guangzhou, whereas *NA* gene is closely related to the group of strains from Guangdong. The present study provides crucial information on the first case of H7N9 outside China and the diversity of this strain from other reported H7N9 strains by molecular analysis.

Key words: H7N9, avian strain, China, molecular analysis, influenza virus.

INTRODUCTION

In March 2013, cases of novel Influenza A (H7N9) were first identified in China involving three urban residents of Shanghai and Anhui (Shuihua et al., 2013). The novel avian-origin reassortant influenza A (H7N9) virus was identified in patients who were hospitalized due to severe lower respiratory tract disease of unknown cause (Gao et al., 2013). As at 3 March 2014, the fatality rate was 379 cases (WHO, 2013). Most of these infections are

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believed to have occurred due to exposure to infected poultry or contaminated environments. To date, no evidence of sustained person-to-person transmission of H7N9 has been reported (WPRO, 2014). However, there were several family-clustered cases with unsustained person-to-person transmission of H7N9 reported. As of 1st December 2013, four family-clusters had been identified in three areas in China (Li et al., 2014).

On February 12, 2014, Malaysia confirmed and reported the first case of influenza A (H7N9) outside China (Centre for Disease Control, 2014). The import case involved a 67-year-old female Chinese tourist, who had travelled from Guangdong, China, to Kuala Lumpur on February 4, then to Sandakan, Sabah the next day before going to Kota Kinabalu, Sabah on February 6. The patient was previously treated by a general practitioner (GP) for symptoms of fever, cough, fatigue and joint pain in China on January 30, 2014, four days before travelling to Malaysia. On February 5, she sought treatment at a GP in Sandakan for similar complaints and was given symptomatic treatment. On February 7, as her condition worsened, she was brought to a district hospital in Sabah and upon family request; she was referred to a private hospital in Sabah and admitted to intensive care unit (ICU). On February 9, the first specimen was tested for suspected avian influenza A (H7N9) and on February 11, our team at Institute for Medical Research (IMR) in Kuala Lumpur tested her second specimens to be positive for H7N9 avian influenza virus nucleic acid. Currently, patient has recovered and was discharged from the hospital. In this study, we report the identification of the first case of influenza A (H7N9) Malaysia, which is also the first case outside China and molecular characterization of the virus by direct sequencing of the hemagglutinin (*HA*) and neuraminidase (*NA*) genes.

MATERIALS AND METHODS

Clinical specimen

Two types of clinical specimens from the patient which include throat swab (TS) and tracheal aspirate (Tasp) were obtained from the private hospital, Kota Kinabalu, Sabah where the patient was admitted to ICU. The first batch of specimens consisting of 2TS (TS1 and TS2) were received at ambient temperature on February 9, 2014. The second batch consisting of 2 Tasp and 1 TS (Tasp1, Tasp2 and TS3) received on February 11, was sent in ice.

Isolation of viral nucleic acid

Viral RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. The isolation procedure was based on spin-column method. A final elution volume of 50 µL containing viral RNA from each specimen was used as template in the one step Real-time RT- PCR amplification.

Real-time reverse transcriptase PCR (rRT-PCR)

The clinical specimens received on February 9, were tested for flu A, Flu B, H1, H3, H7 CNIC, N9 CNIC, pdmA and pdmH1 by real-time RT-PCR assay using sets of specific primers and probes obtained from Centre of Disease Control (CDC), Atlanta and Chinese National Influenza Center (CNIC). Clinical specimens received on February 11 were tested together with the previous specimens using Flu A, H7 CDC, H9 CDC, H7 CNIC and H9 CNIC primers and probes. Positive controls, extraction controls and reagent controls were included in each run. All amplification reactions were performed using the SuperScript III one-step RT-PCR kit (Invitrogen, USA) in a 96-well real-time PCR thermal cycler (Bio Rad, USA). The assay was undertaken at 50°C for 30 min, 95°C for 2 min and 45 cycles of 95°C for 15 s and 55°C for 30 s. Reaction setup was composed of 12.5 µL of 2x RT-PCR Mix, 0.5 µL of each primers (40 µM), 0.5 µL of respective probes (20 µM), 0.5 µL of RT enzyme, 5.5 µL of sterile distilled water and 5 µL of extracted RNA.

cDNA synthesis

The isolated RNA of Influenza A (H7N9) virus was subjected to cDNA synthesis using Super Script III First Strand Synthesis kit (Invitrogen, USA) according to manufacturer's instruction. An influenza specific universal reverse transcriptase oligonucleotide, uni12 (5'AGC AAA AGC AGG 3') was used in this assay (Hoffmann et al., 2001). The cDNA was then used as template in the amplification of HA and *NA* gene by conventional PCR.

Conventional PCR amplification of HA and NA

Amplification of the HA and NA using the WHO Collaborating Centre (WHOCC), Melbourne primers was performed in a thermal cycler (BioRad, USA) with the following condition: 95°C for 2 min; 40 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, and a final heating at 72°C for 10 min. Annealing temperature for amplification of NA was optimized to 60°C. Assay reactions were carried out in a final volume of 25 µL containing 5 µL of 1x Buffer (Promega, USA), 4 µL of $MgCl_2$ (Promega, USA), 0.5 µL dNTP (Promega, USA), 1.0 µL of each primers (10 µM), 0.5 µL of Taq Polymerase (Promega, USA), 8 µL of distilled water and 5 µL of cDNA. Amplification of the HA and NA using the primer sequences obtained from CDC was performed with the following condition: 94°C for 3 min; 35 cycles at 94°C for 20 s, 60°C for 30 s and 72°C for 30 s, and a final heating at 72°C for 1 min. Assay reaction was composed of 12.5 µL of MiFi Mix (Bioline, UK), 1.2 µL of each primers (10 µM), 1.0 µL of $MgCl_2$ (Promega, USA), 6.1 µL of distilled water and 3 µL of cDNA.

Agarose gel electrophoresis

The 25 µL of each amplified PCR products were analyzed using 2.0% agarose gel (Promega, USA) pre-stained with Red Safe dye (Intron Biotech, Korea). Gel electrophoresis was performed in 1x TBE buffer at 90 V for 40 min and visualized under UV illumination. The expected amplicons were extracted from the agarose gel by Gel Extraction Kit (Qiagen, USA) according to manufacturer's instruction. Final elution contained 15 µL of purified PCR amplicons from which 2 µL was reanalyzed on 2% agarose gel to confirm that the purification step was performed precisely.

Sequencing

Prior to sequencing, the purified amplicons were subjected to cycle sequencing under the following condition: 40 cycles of 96°C for 2 min, 50°C for 5 s and 60°C for 4 min. The assay setup composed a Final volume of 10 µL consisting of 2 µL of Big Dye Terminator

(Applied Biosystem, USA), 2 µL of Buffer (Applied Biosystem, USA), 1 µL of either sense or antisense primers (4 µM), 4 µL of purified PCR amplicon and 1 µL of distilled water. The PCR amplicons which were amplified by WHOCC, Melbourne primers were cycle sequenced using universal primers (M13F: 5'TGTAAAACGACGGCCAGT3' and M13R: 5'CAGGAAACAGCTATGACC3'), whereas the amplicons which were amplified by CDC primers were cycle sequenced using the same primers in the PCR amplification step. All reactions were purified by dye-ex purification kit (Qiagen, USA) according to the manufacturer's instruction after which was concentrated by vacuum spin. A 10 uL of HIDI formamide (Applied Biosystem, USA) was added to each concentrated reaction tubes. Subsequently, these reaction mix were transferred to a 96 well plate, sealed and denatured at 95°C for 2 min and finally subjected to sequencing in genetic analyzer ABI 3730 (Applied Biosystem, USA).

Data analysis

All sequencing raw data were first processed and analyzed by Cromas Lite 2.1.1 software. Sense and antisense sequences were then aligned to produce full length of HA and NA gene sequences using CLUSTAL Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The reference sequences used in the alignment were influenza A virus (A/Guangdong/1/2013(H7N9)) segment 4 hemagglutinin (HA) gene (GenBank Accession number: KF662943.1) and influenza A virus (A/Guangdong/1/2013(H7N9)) segment 6 neuraminidase (*NA*) gene (GenBank Accession number: KF662949.1). A BLAST search was performed for the aligned sequences in NCBI database to indicate the closest match. The assembled sequences were also analyzed in FluSurver database (http://flusurver.bii.a-star.edu.sg/) for the presence of mutation.

Phylogenetic tree

Phylogenetic trees were constructed using neighbor joining method (bootstrap replication 1000x) to display the relationship and genetic variation of the *HA* and *NA* genes among the various influenza A (H7N9) isolates available in GenBank database. This was performed using MEGA 6.06 software.

RESULTS

Real-time RT-PCR data

The amplification is regarded positive when CT value is ≤38. The first batch of patient specimens (TS 1 and TS 2) tested on February 9 were positive for flu A and H7 but was negative for N9. Thus, a second batch of specimens was requested from the clinician considering the possibility for degradation of specimens due to broken cold-chain during transportation of the first batch.

The second batch of specimens which was tested on February 11 clearly indicated a positive result for H7N9 with a strong CT value for tracheal aspirate (Tasp). The CT values and the amplification curves of the second test are shown in Figure 1. In all run, positive controls were successfully amplified and negative controls showed no amplification.

HA and *NA* genes amplified by conventional PCR

Amplification of *HA* gene using WHOCC Melbourne primers were successful for both HA1 (~860 bp) and HA2 (~890 bp) segments using cDNA synthesized from Tasp 2. The CDC primers failed to amplify the HA region. The NA1 segment (~800 bp) was successfully amplified by WHOCC Melbourne primers using cDNA of Tasp2 but failed to amplify the NA2 segment. Therefore, CDC primers were used as an alternative targeting four segments and all yielded the expected amplicons: NA1 (~290 bp), NA2 (~550 bp), NA3 (~520 bp) and NA4 (~270 bp).

Sequencing data

Sequencing and alignment of the HA and NA genes produced a length of 1664 and 1321 bp respectively. The sequences were deposited in GISAID (Accession numbers: EPI 509111 for influenza A virus (A/Malaysia/228/2014(H7N9)) segment 6 neuraminidase (*NA*) gene and EPI 509205 for influenza A virus (A/Malaysia/228/2014(H7N9)) segment 4 hemagglutinin (*HA*) gene. The BLAST search of these sequences revealed the closest match with influenza A virus (A/environment/Guangzhou/1/2014(H7N9)) segment 4 *HA* gene for HA and Influenza A virus (A/Guangdong/05/2013(H7N9)) segment 6 *NA* gene for NA.

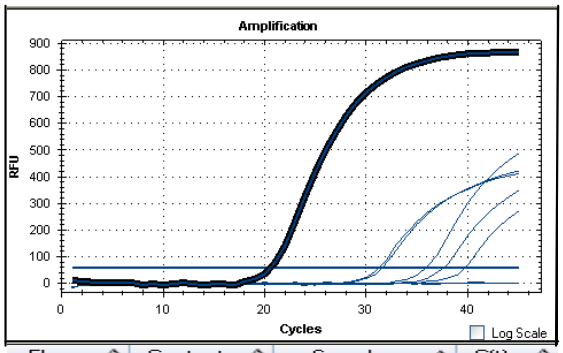
Phylogenetic analysis

The phylogram as shown in Figure 2 clustered the Influenza A Virus (A/Malaysia/228/2014(H7N9)) segment 4 hemagglutinin (*HA*) gene into the group of Guangzhou strains whereas the Influenza A Virus (A/Malaysia/228/2014(H7N9)) segment 6 neuraminidase (NA) gene was clustered into the group of Guangdong strains (Figure 3). In both phylogram, these strains were observed to be highly divergent from the A/Shanghai/1/2013 which is the isolate from the first case of H7N9 in China during the 2013 outbreak.

Mutation analysis

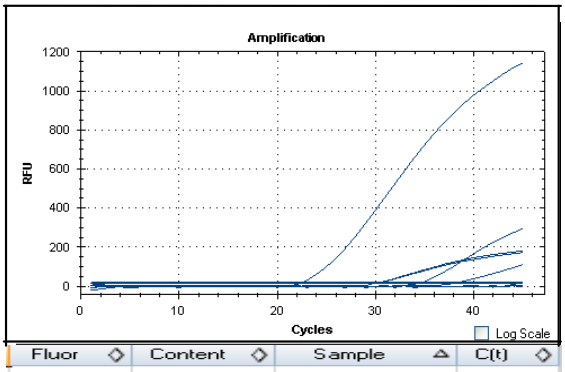
The FluSurver computed all mutations detected in our sequences. Results displayed include details such as position, involvement of mutation, frequency of appearance and previous literature reviews of the particular mutation. It was found that the A/Malaysia/228/2014(H7N9) segment 4 *HA* gene contained mutations R65K, E122K, L186I and N285D whereas A/Malaysia/228/2014(H7N9) segment 6 neuraminidase (*NA*) gene contained mutations M26I, R78K and V345I. The HA mutations found in this study were mostly involved in viral oligomerization and NA

Amplification of FLU A



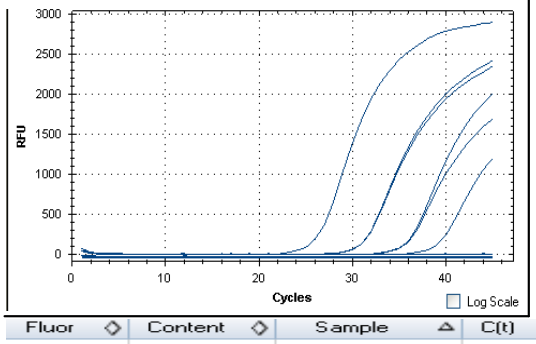
Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	20.18
FAM	Unkn	RM039 Swab 1	39.30
FAM	Unkn	RM039 Swab 2	37.05
FAM	Unkn	RM039 Swab 3	35.40
FAM	Unkn	RM039 T.Asp 1	30.80
FAM	Unkn	RM039 T.Asp 2	31.20

Amplification of H7 with CDC



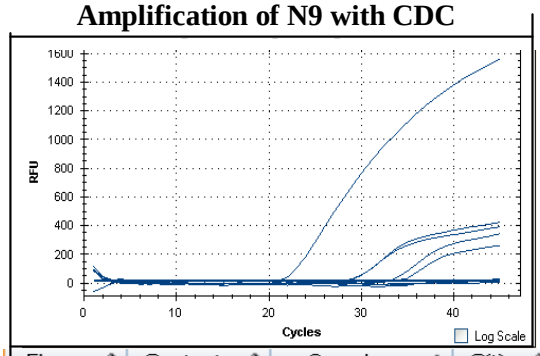
Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	23.43
FAM	Unkn	RM039 Swab 1	N/A
FAM	Unkn	RM039 Swab 2	35.83
FAM	Unkn	RM039 Swab 3	40.53
FAM	Unkn	RM039 T.Asp 1	32.40
FAM	Unkn	RM039 T.Asp 2	32.31

Amplification of H7 with CNIC oligonucleotides



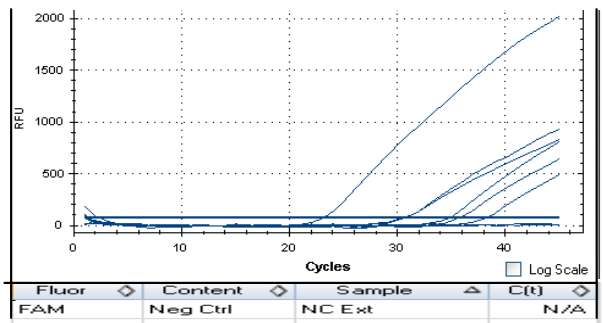
Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	25.76
FAM	Unkn	RM039 Swab 1	39.43
FAM	Unkn	RM039 Swab 2	36.07
FAM	Unkn	RM039 Swab 3	36.00
FAM	Unkn	RM039 T.Asp 1	31.21
FAM	Unkn	RM039 T.Asp 2	31.24

Amplification of N9 with CDC



Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	22.52
FAM	Unkn	RM039 Swab 1	N/A
FAM	Unkn	RM039 Swab 2	35.94
FAM	Unkn	RM039 Swab 3	34.75
FAM	Unkn	RM039 T.Asp 1	30.46
FAM	Unkn	RM039 T.Asp 2	30.45

Amplification of N9 with CNIC



Fluor	Content	Sample	C(t)
FAM	Neg Ctrl	NC Ext	N/A
FAM	Neg Ctrl	NC Rgt	N/A
FAM	Pos Ctrl	PC	23.58
FAM	Unkn	RM039 Swab 1	38.87
FAM	Unkn	RM039 Swab 2	36.34
FAM	Unkn	RM039 Swab 3	35.34
FAM	Unkn	RM039 T.Asp 1	31.43
FAM	Unkn	RM039 T.Asp 2	31.34

Figure 1. Real-time RT-PCR results for amplification of H7N9 virus (all amplification were performed in a single run).

Figure 2. Phylogram showing the divergence of influenza A virus (A/Malaysia/228/2014(H7N9)) segment 4 hemagglutinin (HA) gene from other strain.

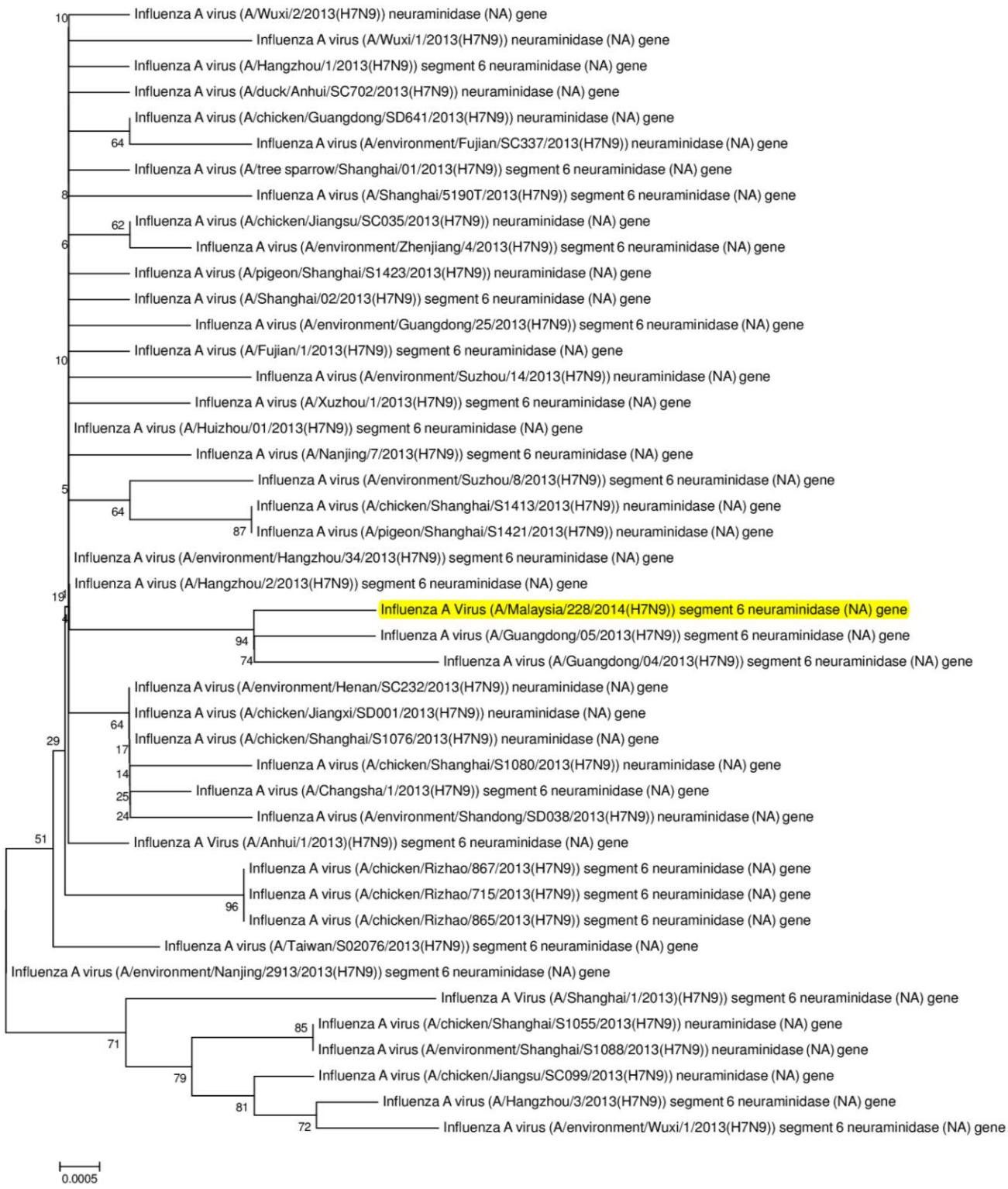


Figure 3. Phylogram showing the divergence of influenza A virus (A/Malaysia/228/2014(H7N9)) segment 6 neuraminidase (NA) gene from other strain.

mutations were mainly involved in small ligand binding. None of the neuraminidase inhibitor resistant mutations were found in these strains. The details of the mutations are summarized in Table 1.

Table 1. Mutations found in this study.

HA mutation	Frequency of mutation found globally	Involvement of mutation
E122K	Frequency of 1.12% of all samples with HA sequence. Occurred one time in one country in the strainA/Chicken/Jiangxi/SD001/2013(H7N9).	Involved in viral oligomerization interfaces and in a T- cell epitope presented by MHC molecules
L186I	New mutation not found in any of the H7N9 sequences in current GenBank database.	Unknown
N285D	Frequency of 2.25% of all samples with HA sequence. Occurred two times in one country in strains A/Huizhou/01/2013(H7N9) and A/Guangdong/1/2013/(H7N9).	Involved in viral oligomerization interfaces, binding small ligands and antibody recognition sites
R65K	Frequency of 17.20% of all samples with HA sequence. Occurred 16 times in one country. The first strain with this mutation was A/chicken/Zhiejiang/DTID-ZJUO1/2013/(H7N9) collected in April 2013 and the most recently occurred in strain A/Guangzhou/2/2014/(H7N9) collected in Jan 2014.	Involved in viral oligomerization interfaces and binding small ligands
NA mutations	**Frequency of mutation found globally**	**Significance of mutation**
M26I	Frequency of 95.40% of all samples with NA sequence. Occurred 83 times in three countries. The first strain with this mutation was A/Changsa/1/2013/(H7N9) collected in March 2013 and recent occurrence in A/Guangdong/05/2013/(H7N9).	Involved in binding small ligand
R78K	Frequency of 3.45% of all samples with NA sequence. Occurred three times in one country. First occurrence is in the strain A/Guangdong/02/2013/ (H7N9) and most recent presence was in A/Guangdong/05/2013/(H7N9).	Unknown
V345I	Frequency of 3.45% of all samples with NA sequence. Occurred three times in one country. First occurrence is in the strain A/chicken/ Shanghai/S1055/2013/(H7N9) and most recently found in A/chicken/Shanghai/S1053/2013/(H7N9).	Involved in small ligand binding

Information on the distribution of the mutations and the involvement in biological process were extracted from FluSurver database (http://flusurver.bii.a-star.edu.sg).

DISCUSSION

In this study, we reported an imported case of Influenza A(H7N9) in Malaysia. Molecular characterization of the H7N9 virus extracted from clinical specimen of the patient were carried out by real-time RT-PCR detection, amplification by conventional PCR, sequencing of the HA and NA genes and phylogenetic analysis. The real-time RT-PCR data showed a substantially strong CT value for tracheal aspirate specimens (Tasp1 and Tasp2) as compared to throat swabs (TS1, TS2 and TS3). This indicated that tracheal aspirate has higher viral RNA yield, a reason why it is generally regarded as the specimen of choice for detection of lower respiratory infection (Drosten et al., 2013). Lower respiratory tract specimens such as tracheal aspirate can produce high viral load because influenza virus shedding is no longer in the upper respiratory tract as the duration of infectiousness prolongs. Therefore, a negative viral yield on upper respiratory tract specimens does not necessarily conclude absence of the virus. To increase the likelihood of detecting the virus, multiple samples from multiple sites should be collected over the course of the illness. Moreover, it is noted that the first batch of specimens was not received in an optimum condition, whereby the cold-chain was not maintained. This could have triggered the false negative result for N9 during the first real-time RT-PCR amplification. The requested second batch of samples was properly shipped and real-time RT-PCR clearly indicated a positive result for H7N9.

Due to unavailability of culture isolate in our study, amplification of the *HA* and *NA* genes by means of conventional PCR from direct specimen was laborious and time consuming as it required optimization from many aspects. The primers used in this step showed variability in amplifying the H7N9 virus from the clinical specimens. For instance, the WHOCC Melbourne primers for NA amplification could not amplify the N2 segment whereas the CDC primers failed to amplify the HA segments. This could be mainly due to some variation that had occurred in the new strain that had prevented the primer to bind to the sequences. It has been reported that the novel H7N9 strains could be mutating up to eight times faster than an average flu virus

(Cheepsattayakorn and Cheepsattayakorn, 2013). Apart from that, some of the primers have been validated by amplification with culture but have not been tested with clinical specimens. Therefore, the efficiency in amplifying viral genetic material from clinical specimen is questionable.

The BLAST search and phylogenetic analysis suggested that the *NA* and *HA* genes of the new strain of A/Malaysia/228/2014/(H7N9) clustered to the Guangdong and Guangzhou group of strains respectively. This finding is consistent with the fact that the patient originated from the Guangdong province and Guangzhou being the capital of this province. Initially, the *HA* gene sequence of A/Malaysia/228/2014 were found to have clustered into the Guangdong group of isolates, however, with the recent addition of the Guangzhou strains to the GenBank database, a reconstructed phylogram showed that it was more closely related to the Guangzhou strains.

The mutation analysis in the HA and NA sequences of A/Malaysia/228/2014/(H7N9) did not discover drug resistant associated mutations. The patient was initially given oseltamivir treatment, and recently switched to zanamivir, gradually recovering and reported to be in stable condition. This unlikely have produced drug resistant mutations within a short period of time. The R292K (R294K in N9 numbering) mutation is one of the most commonly identified mutations among seasonal H3N2 isolates with dramatically reduced sensitivity to oseltamivir, intermediate resistance to peramivir, and slightly reduced sensitivity to zanamivir (Gubareva, 2004). This mutation was also discovered in A/Shanghai/1/2013/(H7N9), the first case of H7N9 infection in China. However, surveillance study suggested that the emergence of NA mutations conferring resistance to NA inhibitors has reportedly been low, with the exception of the naturally emergent H274Y NA mutation in H1N1 seasonal influenza (Whitley et al., 2013).

Some other mutations were found in the HA and NA sequences of the A/Malaysia/228/2014 as shown in Table 1. All mutations except one have been discovered in other strains of H7N9 at least once. Among the HA mutations discovered in this strain, the R65K was found to have occurred more commonly in other strains of H7N9 reported thus far, whereas M261 had higher prevalence of occurrence in *NA* gene. The significance and function of these reported mutations were not well understood, however most are thought to be involved in viral oligomerization and ligand binding. Previous study had demonstrated that evolutionary variation involved in an oligomerization interface of the influenza A virus neuraminidase were essential for viral survival (Mok et al., 2013). Involvement in ligand binding mechanism is crucial for the virus to substantially interact with host receptor, sialic acid (Taylor and von Itzstein, 1994).

The novel mutation found in the *HA* gene of our new strain was L186I, a substitution of leucine to isoleucine (CTA→ATA). The occurrence of this mutation globally was not documented, however, our alignment results with other influenza strains, revealed that L186I has previously occurred in Influenza A virus (A/chicken/Wenzhou/323/2013(H7N7)) segment 4 *HA* gene and Influenza A virus (A/chicken/Wenzhou/299/2013(H7N7)) segment 4 *HA* gene. This may suggest that the H7N9 and H7N7 Wenzhou viruses have similar, but independent evolutionary origins. Surveillance study showed that the hemagglutinin genes from these two lineages originated from H7 viruses that have been introduced to and established among the domestic ducks in China since 2010 (Lam et al., 2013).

In conclusion, the present study provides crucial information on the first case of H7N9 outside China and the diversity of this strain from other reported H7N9 strains by molecular analysis.

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Conflict of Interest(s)

Authors have no financial interests related to the material in the manuscript.

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