

Report

First Report of Cowpea Polero Virus 1 (CPPV1) Infecting Cowpea in Kenya

Orakha Odhiambo Patrick^{1, *}, Hassan Were², Millicent Ndonga¹, Mukoye Benard¹

¹Department of Biological Sciences, Masinde Muliro University of Science and Technology (MMUST), Kakamega, Kenya

²Department of Agriculture and Land Use Management, Masinde Muliro University of Science and Technology (MMUST), Kakamega, Kenya

Email address:

orakhapk@gmail.com (O. O. Patrick), hwere@mmust.ac.ke (H. Were), mndonga@mmust.ac.ke (M. Ndonga),

btemukoye@gmail.com (M. Benard)

*Corresponding author

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Abstract: Cowpea is an important legume crop for nutritional security, livestock feed and source of income in Kenya. Cowpea yield in Kenya is very low and declining further due to high incidences of pests and diseases among other factors. Like other pathogens, viruses have continued to cause yield losses of up to 100% depending on the prevailing epidemiological factors. This study had sought to determine the distribution, prevalence and genetic diversity of Cowpea Mild Mottle Virus (CPMMV) in western Kenya. Two surveys were conducted in the main legume growing areas of western Kenya. A total of 139 legume leafy samples were collected from 5 Counties; Bungoma, Nandi, Vihiga, Kakamega and Busia in June and October 2016. Collected samples were analyzed by Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) for detection CPMMV. ELISA was not carried out to test for CPPV1. Total RNA was extracted from the symptomatic leaf samples using RNeasy Mini Kit (Qiagen) according to the manufacturers' protocol. Next Generation Sequencing (NGS) protocol proceeded. Assembled contigs were then subjected to a BLASTn search against the Genbank database. Phylogenetic analyses and comparisons were performed using MEGA X. Symptoms observed on legumes in the fields were mosaic, severe yellowing, chlorotic blotches and downward leaf curling. Some of the groundnut, cowpea and common bean samples collected in June and October tested CPMMV positive by DAS-ELISA. Legume samples of soybean, lablab, green grams, bambara nuts and legume weed collected tested negative for CPMMV. Results from Next Generation Sequencing technique revealed the presence of Cowpea Polero Virus 1 (CPPV1), the first full-length CPPV1 report and sequence to be reported in Eastern Africa. Phylogenetic analysis of VCP1 Kenyan isolate sequence revealed 98.27% similarity with that of Burkina faso isolate BE 167 (KY364846.1) (Palanga et al., 2017) and they clustered together. Kenyan isolate VCP1 and the Burkina faso BE 167 isolate are closely related. It is not clear if the spread of CPPV1 is through international trade from either country. Revelation of CPPV1 show that there could be more viruses affecting legume crops in the region whose identities are still unknown. There is need to conduct more diagnosis using NGS technique to detect more unknown but occurring viruses in the region. CPPV1 has been found infecting cowpea in Burkina faso, to the best of our knowledge this is the first report of CPPV1 in Kenya.

Keywords: Cowpea Mild Mottle Virus (CPMMV), Cowpea Polero Virus 1 (CPPV1), Next Generation Sequencing

1. Introduction

Cowpea is among the most important legume food crop also used as livestock feed and source of income for both producers and consumers [1, 2]. The level at which cowpea leaf is

consumed in western Kenya has heightened to un-ignorable magnitude. Virtually, all western Kenya rural households consume cowpea leaves for vegetables and plant cowpea during various seasons of the year, alternating drought tolerant to heavy rain tolerant varieties. However, its production is

very low compared to other cowpea producing countries due to pests and diseases basically fungal, bacterial and viral as well as poor agronomic practices. Under normal circumstances, cowpea yield in Kenya is supposed to be higher than it actually is as demonstrated by the comparison between Kenya and her neighbors as well as the rest of the world. Yield of cowpea in USA is 2.1 tons/ha per year compared to Kenya's 0.5 tons/ha annually [2, 3] Cowpea grain yield has been effectively evaluated [3] as opposed to cowpea leaf yields in Kenya. Cowpea grows well in warm weather and is tolerant to drought. It is adapted to grow in drier parts of the tropics, areas where performance of other legumes consumed as food performs decimally [1, 4].

Cowpea Polero Virus 1 is classified under the family *Luteoviridae*, genus *Polerovirus*. Other genera of the family include; *Enamovirus* and *Luteovirus* [5]. Members of *Luteoviridae* family infect both monocotyledonous and dicotyledonous crops around the world [6]. They are made up of positive sense single-stranded RNA genomes encapsidated in icosahedral particles and encode 5 to 7 ORFs [7]. The three genera differ in genome organization; members of genus *Enamovirus* lack an ORF4, members of *Luteovirus* lack an ORF0 while members of *Polerovirus* encode both ORF0 and ORF4 [7]. Members of genus *Enamovirus* lack non-AUG- initiated gene (ORF3a) which is found in both *Polerovirus* and *Luteovirus* [7].

2. Materials and Methods

2.1. Survey

Two surveys of CPMMD were conducted during long rains in June and short rains in October 2016. Counties of Western Kenya including; Busia, covering agro ecological zones LM1, LM2 and LM3, Bungoma covering LM2 and LM3, Kakamega covering LM4, Vihiga covering UM1 and Nandi covering LH1 and UM1 were surveyed.

Fields representing sampling units in the five counties (Bungoma, Nandi, Vihiga, Kakamega and Busia) were identified randomly along motorable roads where legume plants were growing. Sampling of legume farms was done by stopping at initially predetermined intervals of three to five kilometers along passable roads. Both leafy symptomatic samples with virus-like symptoms and asymptomatic samples from common bean, cowpea, ground nuts, soybeans, green grams, bambara nuts and some leguminous weeds were collected and used for serological and molecular analysis. At least 1-3 plants per field (depending on the size of the field) were sampled along field diagonals. Survey process was conducted by walking through fields in which legumes were growing and inspecting legume plants visually for presence Cowpea mild mottle disease (CPMMD) related symptoms. Quadrat of dimensions 1.5m by 1.5m was used in the determination of disease incidence and severity along the diagonals by line transects approach at quadrat interval spacing of two meters. Disease incidence and severity was recorded by use of a disease score sheet in each farm.

Symptoms observed on legumes in the fields were leaf mosaic, overall leaf yellowing, inter-veinal yellowing, chlorotic blotches, stunted growth and downward leaf curling. Collected samples were wrapped in paper towels then placed in a cool box and finally transferred to a fridge at Masinde Muliro University of Science and Technology Micro biology laboratory at 4°C until use. Data on disease incidence in legumes was determined using the Nono-Womdim *approach* [8], where the proportion of diseased plants in an area is expressed as a percentage.

$$\text{Disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Disease incidence was scored using a rating scale as developed by Nono Womdim [8] where: Low incidence; 1-20%, Moderate incidence; 21-49% and High incidence; 50-100%.

Disease symptom severity was scored on a scale of 0-3 where: 0 = No disease symptoms on plants, 1 = Mild foliar disease symptoms, 2 = Moderate foliar disease symptoms, 3 = Severe distortion, malformation of leaves or stem and stunting. Field target viral symptoms included; leaf mosaic, overall leaf yellowing, inter-veinal yellowing, chlorotic blotches, stunted growth and downward leaf curling.

2.2. Double Antibody Sandwich ELISA (DAS-ELISA)

ELISA test was not conducted for detection of CPPV1 because the CPPV1 antisera were not available to facilitate the process and the virus had not been targeted. The initial target CPMMV DAS-ELISA was conducted following the DSMZ manufacturers' protocol. Microtiter plates were coated with CPMMV 1Gg diluted 1:1000 (v/v) in coating buffer and incubated for 2h at 37°C. Sample extracts were added and incubated overnight at 4°C. Extracts from healthy plants and of plants infected with CPMMV were used as negative and positive controls respectively. 1Gg- alkaline phosphatase conjugate, diluted 1:1000 (v/v) in conjugate buffer, was added and incubated for 2h at 37°C. Assessment of results was done by visual observation.

2.3. Extraction of RNA, cDNA Library Preparation and Sequencing

All Prep RNA Mini Kit (Qiagen RNeasy) was used in the extraction of total RNA from semi purified virions according to the manufacturers' recommendation. Quantification of RNA was done using Nano-drop and 1.5% agarose gel electrophoresis.

Double stranded cDNA synthesis was done using the total RNA extracted using the superscript II (Thermo Fisher Scientific, Waltham, USA) kit. Column-purification of the cDNA was done with the DNA Clean & concentrator TM-5 – DNA kit (Zymo Research, Irvine, USA) followed by quantification with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The transposon-based chemistry library preparation kit (Nextera XT, Illumina) was used to process samples following manufacturer's instructions. Agilent 2100

Bioanalyzer (Agilent Technologies, Santa Clara, USA) was used for assessment of fragment sizes and structure of the DNA libraries. Sequencing of the indexed denatured DNA libraries (200-bp paired-end sequencing) was done on the Illumina MiSeq platform (Illumina).

FastQC (Version 0.11.5) was used to check reads quality; reads were then trimmed to remove poor quality sequences. The trimmed reads [9] were used for de novo assembly and contigs aligned to the viral genomes database (<ftp://ftp.ncbi.nih.gov/genomes/Viruses/all.fna.tar.gz/>, downloaded on January 2018) using CLC Genomics workbench 10.1.2. The resultant assembled contigs were subjected to BLASTn search against the GenBank database. Complete and partial CPPV1 sequences used for comparison and Phylogenetic analyses were retrieved from GenBank. (<http://www.ncbi.nlm.nih.gov/>). MEGA X [10] and DnaSP v. 5 [11] programs were used in the phylogenetic analyses and comparisons to infer relatedness of the revealed virus to the rest in the Genbank.

3. Results

3.1. Symptoms Observed in the Fields

Different crops exhibited varied virus-like symptoms on the field. Some of the symptoms observed in the field included; mosaics, downward leaf curling, chlorosis and severe leaf yellowing (Figures 1A and B). Severe yellowing symptom was very prominent and it could be identified from a distance in the field. Cowpea plants infected had their whole leaves colored severely yellow including the young developing leaves (Figure 1A). The symptom existed in the field in patches showing “stand alone symptoms”. It was also noted that most of the cowpea plants that exhibited the “severe yellowing symptom” did not have other common virus-like symptoms such as down-ward leaf curling, leaf deformation, stunting among others, even though the leaf area of the severe yellowed plants were observed to be reduced a little.

3.2. DAS-ELISA

Serological test (DAS-ELISA) was not carried out to test for CPPV1 since it was not the initial target of study virus. The CPMMV DAS-ELISA test carried out on the many samples collected in the field tested positive in most samples including the VCP1 sample whose photo is shown in (Figure 1A).

3.3. Next Generation Sequencing

Results from Next Generation sequencing (NGS) technique revealed a complete genome sequence for Cowpea Polero Virus 1 (CPPV1) with 5319 nucleotides from (VCP1) isolate collected from Vihiga County, at N-00.12725, E-034.78904 and Elevation 1638m above sea-level. The VCP1 isolate had tested positive for CPMMV by DAS-ELISA. Revelation of CPPV1 complete sequence by NGS technique was contrary to the expected CPMMV sequence.

3.4. Phylogenetic Analysis

Phylogenetic analysis of sequences using Maximum likelihood method with bootstrap values 1000 yielded a consensus tree (Figure 3). Contrary to the expectation, no CPMMV sequence was found in the sample when NGS process took effect



A



B

Figure 1. (A) Severe yellowing in cowpea and (B) downward leaf curling in common beans.

The two samples above (A & B) tested positive for CPMMV by DAS-ELISA, they were among the samples selected for further analysis by NGS, and both samples had been expected to yield CPMMV complete genomes. The severe yellow symptom is a characteristic that identifies with Poleroviruses [12, 13] (Figure 1A) Identified in the field as VCP1 with the severe yellow symptom yielded a complete Polero virus genome sequence.

Sequencing and Sequence analysis

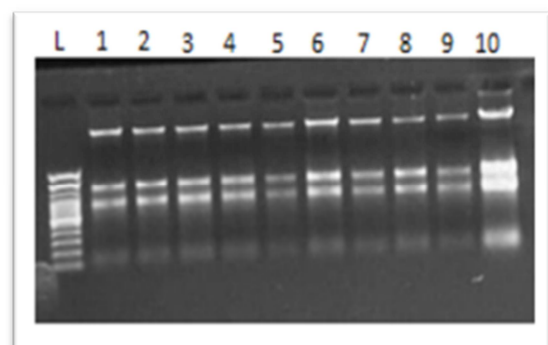


Figure 2. Gel electrophoresis (1.5% TAE) of total RNA extracted from 10 cowpea samples L - 100bp ladder, lanes 1-10 extracted samples.

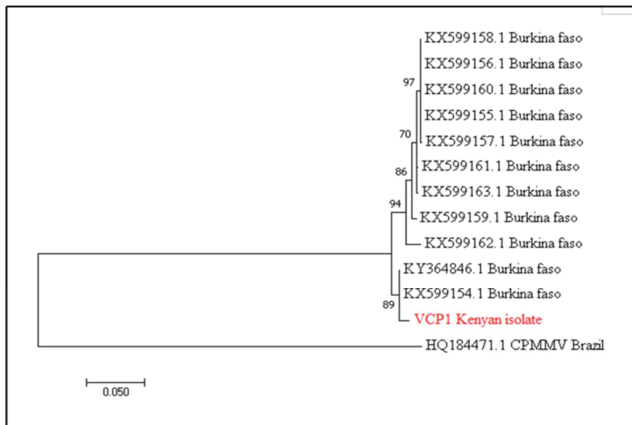


Figure 3. Phylogenetic tree depicting the relationship between VCP1 and established members of the family Luteoviridae based on full length CPPV1 genome sequence.

Analysis of cowpea Polero virus 1 sequence isolated from the Kenyan isolate (VCP1) revealed that it was 98.27% identical to the Burkina faso isolate BE 167 (KY364846.1) [17] despite their different geographical origin and isolation hosts. Consensus tree also displayed close clustering of the isolate VCP1 with the Burkina faso isolate BE 167 (KY364846.1) as shown in (Figure 3) above.

The above phylogenetic analysis show that VCP1 isolate from Kenya clusters together with isolate BE167 from Burkina faso (KY364846.1.).

4. Discussion

The main symptoms observed in the field including; severe leaf yellowing, Chlorotic spots, early stage deformation of plant leaves, downward leaf curling, chlorotic blotches and leaf mosaic [14], were symptoms consistent with the main CPMMD symptoms documented elsewhere [15, 16]. The key symptom associated with presence of CPPV1 is severe yellowing, [12, 13] the symptom which has been linked to CPPV1 by various studies carried out on the virus. The “stand-alone severe yellow symptoms” associated with CPPV1 quite discourages consumption of cowpea since cowpea in the region is consumed as leaf vegetable. When the leaf color changes to yellow, get deformed, reduce in shape and size, cowpea yield automatically reduces by virtue of the part of the crop consumed in the region. Results showing “stand alone severe yellowing symptom” without showing other virus like symptoms could mean that CPPV1 does not cause other complex morphological symptoms on the cowpea crop. This was observed in the field with reference to (Figure 1A) showing whole cowpea leaves with their conventional shape well maintained yet severely yellowed. Biological characterization of CPPV1 could not be carried out on various test crops since the virus cannot be mechanically transmitted.

It is not clear how CPPV1 spread to or from either country (Kenya and Burkina faso) but it may have been as a result of international trade of seeds infected with the Cowpea Polero Virus. Results from this complete genome analysis implies that the viral isolate VCP1 belong to the CPPV1 species and is

one of the causal agent of severe yellowing symptom in cowpea. To the best of our knowledge, this is the first report of CPPV1 infecting cowpea in Kenya.

5. Conclusion

The severe yellowing symptom “standing alone” without any other common virus-like symptoms, yet the same sample yielding complete CPPV1 complete sequence could be an indication that CPPV1 is not responsible for the many known conventional plant viral symptoms. Un-targeted detection and diagnosis of CPPV1 may also imply that there exists many more legume plant infecting viruses whose occurrence in the region has not been noticed.

This report forms basis for more molecular approach studies on CPPV1 in the region.

It is therefore recommended that diagnostic studies using NGS be carried out in the region to help identify more viruses affecting legumes so as to initiate control measures.

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