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Full Length Article

Rapid Identification and Detection of *Xanthomonas phaseoli* pv. *manihotis*, Causing Bacterial Blight Disease in Cassava by Real-Time PCR using LNA Probe

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Abstract

Effective detection and early monitoring of *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*) causing Cassava Bacterial Blight (CBB) disease using rapid DNA-based method is paramount in promoting curative management of the disease. The traditional methods of identification such as pathogen culturing-based upon morphological, biochemical and physiological approaches, traditional PCR involving post-PCR reaction processing gel electrophoresis are more often labour intensive, time-consuming and require exceptional classical taxonomical dexterity. Owing to these limitations of the traditional detection methods, we developed primer and Locked Nucleic Acid probe (LNA probe), and with the aid of Real-time PCR, *Xpm* was reliably detected from the extracted DNA and directly from the infected cassava plant tissues without DNA extraction within a relatively shorter time (20–25 min). The designed probe has a fluorescent amide (FAM) as reporter dye and carboxytetramethylrodamine (TAMRA) as repressive dye. The selectivity of the developed probe and primer sets were also tested against different *Xanthomonas* species, and other plant pathogenic bacteria from different genera as well as host cassava genomic DNA. In the end, only *Xpm* was sensitively and selectively detected by the developed probe, confirming reliable detection and identification of *Xpm* as the true causal agent of CBB of the infected cassava samples taken from various agro-ecological zones of Ghana. This is the first study, which uses LNA probe, which is mostly used for human pathogens in the identification of cassava plant pathogen.

Keywords: Cassava Bacterial Blight; Detection; Real-time PCR; LNA probe; *Xanthomonas phaseoli* pv. *manihotis*

Introduction

Cassava is a major staple food in Ghana and the crop plays a key role in import substitution (IITA 1997). Cassava bacterial blight disease (CBB) is caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) (Vauterin *et al*. 1995) and now reassessed taxonomically and named *X. phaseoli* pv. *manihotis* (*Xpm*) (Constantin *et al*. 2016). Accurate and reliable detection of the bacterium is a vital tool for disease management (Acero *et al*. 2011; Garrido *et al*. 2012).

In recent times, CBB was found to be distributed and established in the various ecological zones of Ghana, Nigeria, Cameroon and Republic of Benin with varied degrees of incidence and severity (Wydra and Msikita 1998). *Xpm* is considered as one of the most important agents limiting cassava yield in Ghana. Infected cassava plants show symptoms such as angular leaf spots (water-soaked), wilting, blighting, defoliation, production of exudates on leaves, petioles or stems, vascular necrosis and dieback of the stem (Lozano 1986). Cassava tuber yield loss above 50 to 75% or complete yield loss in severe situations as a result of this pathogen has been documented (Wydra and Rudolph 1999; Wydra 2002; Zinsou *et al*. 2004).

Reliable and efficient identification of this pathogen requires a strong molecular diagnostic technique and among such methods is Real-time PCR technique. Although the traditional PCR is also a strong tool for pathogen diagnosis, it has not been used extensively in the quantification of plant pathogens because it is inaccurate and labour intensive (Schena *et al*. 2013).

The Real-time PCR also called quantitative PCR (qPCR) is a laboratory method and an advanced form of the traditional PCR used to amplify and at the same time quantify the gene of interest in a stretch of DNA. Here, the reaction is being monitored by a detector in real-time *via* the discharge of fluorescence as it progresses to finish (Heid *et al*. 1996). The qPCR serves as a fundamental diagnostic tool and gold standard for early pathogen detection in plant

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tissues and environmental samples. The Real-time PCR unlike the traditional PCR can amplify short DNA fragments (70–100 bp), which can increase the sensitivity and efficiency of the detection (Garrido *et al.* 2009).

The Real-time PCR has several other advantages as compared to the traditional PCR and it is gaining widespread acceptance in the diagnostic and molecular arena because it allows the amplified products to be monitored in the real-time during the exponential phase of the reaction leading to accurate determination of the starting material. It is a versatile method for typing between closely related organisms, because of its higher throughput in the quantification of gene of interest in biological and environmental samples (Cooke *et al*. 2007; Schena *et al*. 2013). It also removes the post-reaction processing steps such as gel casting and electrophoresis, exposure to harmful ethidium bromide chemical and UV-radiation, which are carcinogenic in nature. Additionally, it reduces the level of contamination in the samples (Paplomatas 2006; Capote *et al*. 2012).

Generally, there are two common Real-time PCR chemistry for the detection of the amplified PCR products and these can be clustered into one: Amplicon sequence non-specific fluorescent dyes (*e.g*., SYBR Green), which binds to any doubled-stranded DNA (dsDNA) in the reaction and two: Sequence-specific method, which is based on the fluorescent labelled oligonucleotides such as Molecular Beacons or Scorpions and 5´nuclease commonly known as TaqMan DNA probes (the first fluorescent signal probe to be designed for real-time PCR), that permits the detection of the target gene when the probe hybridised with its complementary sequence (McCartney *et al.* 2003; Schena *et al*. 2004; Capote *et al.* 2012).

In the Amplicon sequence non-specific methods, the dye emit fluorescent light during the reaction when it binds to the dsDNA. The most commonly used fluorescence dye is SYBR Green I, which has high compatibility with dsDNA. Other related intercalating dyes, which include ethidium bromide has also been successfully utilized (Capote *et al.* 2012). The major problem with the amplicon sequence non-specific method is that, the dyes are not selective and therefore bind to any dsDNA in the reaction, leading to the development of non-specific amplification products as well as the formation of primer dimmers. Meanwhile, in the Amplicon sequence specific method, the reaction involves the use of fluorescent probes labelled with a donor fluorophore and an acceptor quencher dye. The probes are pieces of synthetic DNA molecules complementary to the target gene in a stretch of DNA, which are labelled with a fluorescent dye. The most commonly used or types of probes are TaqMan, Molecular beacons or Scorpion (Schena *et al*. 2004; Capote *et al*. 2012).

In most cases, the labelled probes and primers are designed based on either nucleic acid or some of their synthetic derivatives such as Locked Nucleic Acid (LNA) (Costa *et al*. 2004) and Peptide Nucleic Acids (PNA) (Egholm *et al*. 1992). The LNA probes are modified nucleotides and also forms methylene bridges after binding to the sequence of interest within a stretch of a DNA (Braasch and Corey 2001). There are two kinds of dye labels, which include: fluorophores with inherent vigorous fluorescence, *e.g.*, fluorescein and products derived from Rhodamine, which structurally are designed to come into contact with the quencher particle (Sjöback *et al*. 1995). The second one has a fluorophores, which change their fluorescence semblance when binds to the nucleic acids.

During the PCR reaction, the probe intercalated to the target gene is cleaved (TaqMan) by the enzyme, leading to physical dissociation of the reporter from the quencher dye, which increases the fluorescence (Schaad *et al*. 2003; Capote *et al*. 2012). The fluorophore is quenched by FRET in TaqMan, but in Scorpion and Molecular beacons-PCR, due to the closeness between fluorophore and quencher, the fluorescence quenching is proximal (Schena *et al*. 2004). Normally the TaqMan probes can be specified with various reporter dyes (TAMRA, TET, FAM, VIC, Texas Red, JOE, Cy5, ROX, HEX, *etc*.) that can allow multitudinal detection of more than one distinct pathogen simultaneously in the same reaction with high accuracy (Aroca *et al*. 2008; Bilodeau *et al*. 2009).

A step from being a substitute to many well-known laboratory methods, Real- time PCR has some exceptional features or chemistry, which makes it a method of choice in different fields of study as compared to several other laboratory techniques currently available. Up to now, the sensitivity, selectivity and the reliability of Real-time PCR in the pathogen detection has never been questioned or in doubt, making pathogen detection much simpler and faster with several benefits for agriculture, especially in Africa where pathogen identification and detection are mostly based on traditional methods. This study was conducted to design specific primer and probe (LNA probe) for rapid, reliable, sensitive and selective detection of *Xpm* in the CBB infected cassava samples collected from various agro-ecological zones of Ghana. To the best of our knowledge based on the available data searched, this is the first study on *Xpm*, using designed VNTR probe and primer for specific detection and identification of this pathogen in cassava samples.

Materials and Methods

Bacteria isolation from cassava samples

The bacteria were isolated from the blighted cassava leaves and other tissues collected from different agro zones in Ghana (Table 1). The method of isolation was as described by Bradbury (1970). The acronyms provided in Table 1 under the heading bacterial isolates are explained as follows: As = Ashanti Region, BA= Brong-Ahafo Region, E = Eastern Region, GAR = Great Accra Region and VR = Volta Regions of Ghana where the samples were collected for bacterial isolation.

Table 1: The bacterial isolates used for the study

Host plant	Bacteria isolates	Location/Region
Cassava	As 2/1	Ashanti Region
Cassava	As 3/1	Ashanti Region
Cassava	As 4/3	Ashanti Region
Cassava	As 5/2	Ashanti Region
Cassava	As 6/1	Ashanti Region
Cassava	As 7/2	Ashanti Region
Cassava	As 8/1	Ashanti Region
Cassava	As 10/1	Ashanti Region
Cassava	As 11/1	Ashanti Region
Cassava	As 12/1	Ashanti Region
Cassava	As 13/1	Ashanti Region
Cassava	As 14/1	Ashanti Region
Cassava	As 15/1	Ashanti Region
Cassava	As 16/1	Ashanti Region
Cassava	As 17/2	Ashanti Region
Cassava	BA 14	Brong Ahafo Region
Cassava	BA16	Brong Ahafo Region
Cassava	BA 29	Brong Ahafo Region
Cassava	BA 30	Brong Ahafo Region
Cassava	E15	Eastern Region
Cassava	E17	Eastern Region
Cassava	E19	Eastern Region
Cassava	E 23	Eastern Region
Cassava	ER26	Eastern Region
Cassava	GAR 13	G. Accra Region
Cassava	GAR 24	G. Accra Region
Cassava	VR8	Volta Region
Cassava	VR13	Volta Region
Cassava	VR 14	Volta Region
Cassava	VR 31	Volta Region
Cassava	VR 33	Volta Region
Cassava	NCPPB 2965	Brazil

Bacterial culturing and molecular identification of isolates

All the bacterial isolates were recovered and grown on the Cefazolin Trehalose Agar (CTA) media for 48 h at 27°C. The bacterial suspension used for the PCR was prepared by mixing freshly grown bacterial colonies with sterile distilled water, vortex, and 2 μL was used for each of the Real-time PCR run.

The Real-time PCR reaction was carried out using Cepheid Smart Cycler II, in a final volume of 27.6 μL, that contained 3.0 μL 10x *Taq* buffer with KCl, 7.2 μL of 2.5 m*M* $MgCl_2$, 4.8 μL of 100 m*M* dNTPs, 1.2 μL of each primer, 0.36 μL of 5 U/μL *Taq* DNA polymerase-recombinant, 0.3 μL LNA probe (HBXamL/HBXamR), 9.54 μL of sterile distilled water and 2.0 μL of *Xpm* suspension as DNA template. The temperature profile of the PCR included, an initial denaturation for 2 min at 94°C followed by 35 cycles of 10 sec at 94°C, 10 sec at 61°C, 10 sec at 72°C and a final extension 2 min at 72°C. A number of non-template controls (NTC, *i.e.*, water) were used in each PCR run.

Designing of probe and primer

The primer and probe were developed using the variable number tandem repeat (VNTR) sequence region of *X. phaseoli* pv. *manihotis*. In designing the primer and the probe, the pathogen was first of all identified with the classical PCR, using *Xpm* VNTR specific primer-XaG1_67F/XaG1_67R (Arrieta-Ortiz *et al.* 2013) to amplify the desired region. The amplified region was then sequenced and was used to search for similar *Xpm* sequences in the NCBI GenBank. After showing high homology with those sequences in the GenBank, we then proceeded to design the primer and probe. The designed probe has fluorescent amide (FAM) as a reporter dye, and carboxytetramethylrod amine (TAMRA) as the repressive dye. To perform PCR, the lyophilized primer was diluted with molecular biology grade sterile distilled water based on the manufacture's specifications to obtain a final concentration of 50 mol/μL. The primer and probe were designed using Roche Universal ProbeLibrary System Assay Design found at (https://lifescience.roche.com).

Primer and probe specificity assay

In order to determine the specificity of the designed primer and probe sets for the detection of *Xpm*, eleven (11) other plant pathogenic bacterial strains namely: *X. axonopodis* pv. *malvacearum*, *X. axonopodis* pv. *vitians*, *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *vesicatoria*, *Xylella fastidiosa*, *Erwinia amylovora*, *Pseudomonas tomato* pv. *tomato*, *Rhizobium vitis*, *Dickeya chrysanthemi*, *Acidovorax citrulli*, *Clavibacter michiganensis subsp. michiganensis* were taken from our bacterial stock collection stored at (-80^{o}C), cultured on the nutrient agar (NA) and incubated at 27^{o}C for 24 h. The bacterial suspension was then prepared from the developed pure colonies using steriled distilled water. The spectrophotometric determination of the suspension concentration was checked using spectrophotometer Eppendorf Biophotometer 6131 (Eppendorf AG, Hamburg, Germany). The optical density of the suspension was adjusted to OD600 = 0.1 corresponding to 1×10^8 cfu/mL and 2 μL of each of the strain suspension used for the PCR anlysis.

Sensitivity of primer and probe from pure DNA

The sensitivity of the designed primer and probe was tested in this study. To study the lowest amount of DNA to be detected by Real-time PCR using the designed probe and primer, pure DNA isolated from the *X. phaseoli* pv. *manihotis* was used. The DNA was extracted by using Thermo FastPrep FP120A-230 multi-tube Homogenizer (Thermo Scientific, MA, USA) and Qbiogene/BIO101 Fast DNA molecular isolation kit (MP Biomedicals, California, USA). The purity and the concentration of the isolated DNA from *X. phaseoli* pv. *manihotis* were determined at nanogram (ng) and picogram (pg) levels, using Thermo NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA) and Invitrogen Qubit fluorometer (Invitrogen, CA, USA), respectively. The determination of the sensitivity of the Real-time PCR to detect *Xpm* was carried

out at 51 pg, 40 pg, 34 pg, 20 pg and 13 pg DNA levels. Deionized sterile distilled water was used for dilutions of the DNA from nanogram to the picogram level and 2 μL of the DNA of each level used for the reaction.

Determination of sensitivity of primer and probe from bacterial cell

To determine the lowest number of *X. phaseoli* pv. *manihotis* cell that can be detected by Real-time PCR, the isolates were sub-cultured on fresh CTA media and stock solution made from freshly grown pure colonies. From the stock solution, 10 serial dilutions (10^{-1}–10^{-10}) were prepared. All the solutions were kept in separate tubes and replicated three times. For each of the solutions, 2 μL was used for the Real-time PCR reaction.

Since it was aimed at determining the lowest number of bacterial cells to be detected by Real-time PCR at this stage, 2 μL of the serially diluted suspensions was simultaneously taken, mixed with 48 μL of sterile deionized water and spread on the surface of the CTA media in the Petri dishes using a flamed glass spreader. The Petri dishes were then incubated at 27^{o}C for 4 days to determine the number of bacterial cells grown in the medium that can be detected (*i.e.*, detection limit). For reliability of getting accurate bacteria count, the counting was done three times and the mean standard values determined.

Determination of selectivity of primer and probe

To determine the selectivity of the developed primer and probe for *X. phaseoli* pv. *manihotis*, the primer-probe set was first tested against other *Xanthomonas* pathogens and then different other plant pathogenic bacterial species. The different bacterial strains tested in this study beside *X. phaseoli* pv. *manihotis* were: *X. axonopodis* pv. *malvacearum*, *X. axonopodis* pv. *vesicatoria*, *X. axonopodis* pv. *vitians*, *X. axonopodis* pv. *phaseoli*, *Acidovorax avenae* subsp. *citrulli*, *C. michiganensis* subp. *michiganensis*, *Dickeya chrysanthemi*, *Erwinia amylovora*, *Pseudomonas tomato* pv. *tomato*, *Rhizobium vitis* and *Xylella fastidiosa* (taken from our storage stock at -80^{o}C). All these bacteria were cultured on the nutrient agar in the Petri dishes and bacterial suspension solutions prepared using sterilized distilled water from each of them after colony growth at 27^{o}C for 72 h. The concentration of the bacterial suspension was determined by using spectrophotometric Eppendorf Biophotometer 6131. The optical density of the suspension was adjusted to OD600 = 0.1 corresponding to 1×10^{8} cfu/mL. The Real-time PCR was performed by taking 2 μL of the suspension from each bacterial suspension.

Detection of *X. phaseoli* pv. *manihotis* directly from diseased plant tissues

In order to detect *X. phaseoli* pv. *manihotis* directly from infected cassava plant tissues with Real-time PCR, one-year-old Esam cassava seedlings were inoculated with the pathogen and replicated three times. The pathogen used for the inoculation was freshly grown on the CTA media and incubated at 27°C for 72 h. The bacterial colonies developed in this medium were suspended with sterile deionized water for the preparation of bacterial suspension. The optical density of the suspension was adjusted to OD600 = 0.1 corresponding to $1x10^{8}$ cfu/mL using spectrophotometric Eppendorf Biophotometer 6131.

The prepared suspension was then used to inoculate healthy, susceptible Esam cassava plant parts. For the development of symptoms to be observed, the inoculated plants were transferred to the controlled-room in the Department of Plant Protection - Akdeniz University at 30^{o}C and 50–70% relative humidity. CBB symptoms were observed two weeks after inoculation. The diseased symptomatic leaves and stems were collected from the inoculated plants and subjected to surface sterilization with 70% ethanol. The surface sterilized blight tissues were grinded in a sterile medium phosphate buffer (NaCl 7.2 g, Na_2HPO_4 1.48 g, K_2PO4 0.43 g, ddH_2O 1000 mL) for the preparation of the stock solutions to be used in the direct detection of the pathogen from the diseased plant tissues. Due to high concentration of the phenolic and other inhibitory substances in the crashed plant tissues, which could hamper the success of the PCR reaction, the prepared stock solution was further diluted in three-fold and 2 μL from each of the solutions used to perform the Real-time PCR.

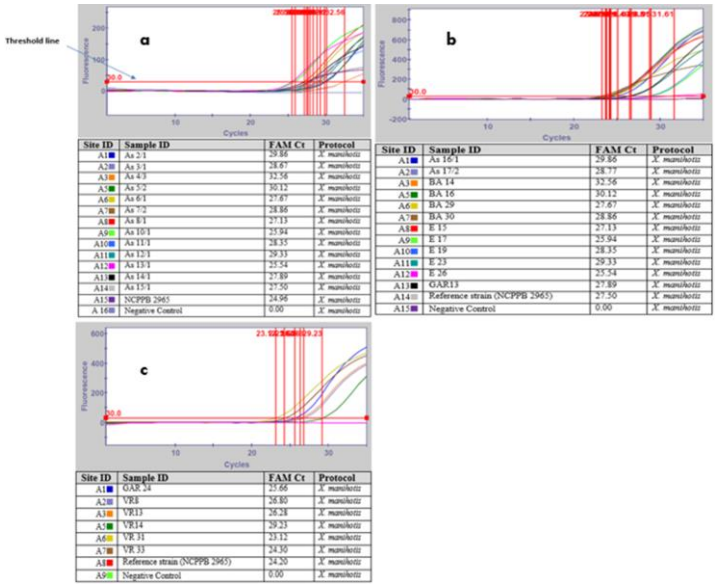
In order to check whether or not the designed primer and probe can amplify or hybridize with cassava DNA, plant total genomic DNA was extracted from healthy cassava plants and used as a check for the designed primer and probe. The total DNA was extracted using Thermo FastPrep FP120A-230 multi-tube Homogenizer and Qbiogene/ BIO101 Fast DNA molecular isolation kit. The purity of DNA isolated from cassava plants and concentration at the nanogram level was determined by using Thermo NanoDrop ND-1000 spectrophotometer, and the DNA concentration diluted to approximately 100–150 ng/μL.

Results

Primer and probe design and detection of isolates by real-time PCR

The isolated putative pathogen isolated from blighted cassava tissues across the agro-zones of Ghana and cultured on the CTA medium was subjected to Real-time PCR analysis. The detection of the pathogen was done by using amplicon sequence specific LNA probe developed in this study, based on the variable number tandem repeats (VNTRs) sequence region of *X. phaseoli* pv. *manihotis*. The probe was designed to anneal to the sequence of interest (VNTRs) between the traditional left (L) and right (R) primer. The designed primer and probe were coded with acronyms HBXamL/ HBXamR (Fig. 1). The HBXamL/

Fig. 1: The binding sites of the HXAML and HBXAMR primers and the probe (5' 3') on the VNTRs gene (Green color: L and R primers, Red color: probe) used for the identification of *Xpm*



Site ID	Sample ID	FAM Ct	Protocol
A1	As 2/1	29.86	*X. manihotis*
A2	As 3/1	28.67	*X. manihotis*
A3	As 4/3	32.56	*X. manihotis*
A5	As 5/2	30.12	*X. manihotis*
A6	As 6/1	27.67	*X. manihotis*
A7	As 7/2	28.86	*X. manihotis*
A8	As 8/1	27.13	*X. manihotis*
A9	As 10/1	25.94	*X. manihotis*
A10	As 11/1	28.35	*X. manihotis*
A11	As 12/1	29.33	*X. manihotis*
A12	As 13/1	25.54	*X. manihotis*
A13	As 14/1	27.89	*X. manihotis*
A14	As 15/1	27.50	*X. manihotis*
A15	NCPPB 2965	24.96	*X. manihotis*
A 16	Negative Control	0.00	*X. manihotis*

Site ID	Sample ID	FAM Ct	Protocol
A1	As 16/1	29.86	*X. manihotis*
A2	As 17/2	28.77	*X. manihotis*
A3	BA 14	32.56	*X. manihotis*
A5	BA 16	30.12	*X. manihotis*
A6	BA 29	27.67	*X. manihotis*
A7	BA 30	28.86	*X. manihotis*
A8	E 15	27.13	*X. manihotis*
A9	E 17	25.94	*X. manihotis*
A10	E 19	28.35	*X. manihotis*
A11	E 23	29.33	*X. manihotis*
A12	E 26	25.54	*X. manihotis*
A13	GAR13	27.89	*X. manihotis*
A14	Reference strain (NCPPB 2965)	27.50	*X. manihotis*
A15	Negative Control	0.00	*X. manihotis*

Site ID	Sample ID	FAM Ct	Protocol
A1	GAR 24	25.66	*X. manihotis*
A2	VR8	26.80	*X. manihotis*
A3	VR13	26.28	*X. manihotis*
A5	VR14	29.23	*X. manihotis*
A6	VR 31	23.12	*X. manihotis*
A7	VR 33	24.30	*X. manihotis*
A8	Reference strain (NCPPB 2965)	24.20	*X. manihotis*
A9	Negative Control	0.00	*X. manihotis*

Fig. 2A-C**:** Specificity of primer and probe in the detection of *X. phaseoli* pv. *manihotis* isolates by Real-time PCR with associate FAM Ct values

HBXamR probe and primer were developed to operate at high annealing temperature of 61°C after optimization in order to prevent the co-amplification of non-specific DNA or gene of interest. The probe and the primer designed in this study, relaiblly, specifically, selectively and sensitively detected *Xpm* as well as the reference strain (NCPPB 2965, Fera-UK) from purely extracted DNA, bacteria cell without DNA isolation and directly from the diseased cassava plant tissues as shown below.

Primer and probe specificity assay

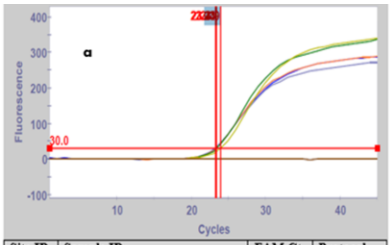
The specificity of the probe and primer tested on the *Xpm* isolates, including the reference strain (NCPPB 2965, Fera-UK) and other plant pathogenic bacteria showed that the designed primer and probe were specific to only *X. phaseoli* pv. *manihotis*. The assays with the developed probe and primer produced positive results (Ct values > 0) only from suspected CBB infected samples, but negative results (Ct values = 0) for all the other strains, which failed to cross the baseline (Fig. 2 A–C). The starter DNA in a given sample of a reaction is related to the number of PCR cycles that is required for the fluorescence signal to get to a specific threshold cycle called Ct value, which is defined as the number of cycles needed for a signal to be detected at threshold level.

Sensitivity of primer and probe from pure DNA

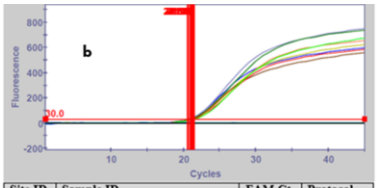
In order to determine the sensitivity of the developed primer and probe by Real-time PCR, the genomic DNA isolated from *X. phaseoli* pv. *manihotis* was first measured and tested at the nanogram level and then reduced to picogram level. The dilutions of the DNA were made at approximately 51 pg, 40 pg, 34 pg, 20 pg and 13 pg level measurements. The detection limit of the primer and probe set was 13 pg. There was no difference in the FAM Ct values. The negative control as expected, yielded zero Ct value (Fig. 3A).

Sensitivity of primer and probe from bacterial cell

The results of the sensitivity of the primer and probe from direct bacteria cell grown on the CTA in the Petri dish and incubated at 27°C showed the detection limit of *X. phaseoli* pv. *manihotis* from the counted bacterial colonies to be one cell (Plate. 1) corresponding to 10^{-7}. After the bacteria made from 10-fold serially diluted (10^{-1}–10^{-10}) suspensions have grown, they were counted and the mean standard deviation computed from the three replicates (Table 2). Serial dilutions from 10^{-1}–10^{-7} produced positive results (Ct > 0), whereas dilutions from (10^{-8}–10^{-10}) yielded negative results (Ct = 0) and therefore could not produce any signal or cross the threshold line (Fig. 3B).



Site ID	Sample ID	FAM Ct	Protocol
A1	51 pg	23.23	*X. manihotis*
A2	40 pg	23.24	*X. manihotis*
A3	34 pg	23.30	*X. manihotis*
A5	20 pg	23.41	*X. manihotis*
A6	13 pg	23.90	*X. manihotis*
A7	Negative Control	00.00	*X. manihotis*



Site ID	Sample ID	FAM Ct	Protocol
A1	Stock Solution	20.62	*X. manihotis*
A2	10^{-1}	20.66	*X. manihotis*
A3	10^{-2}	21.08	*X. manihotis*
A5	10^{-3}	20.91	*X. manihotis*
A6	10^{-4}	21.12	*X. manihotis*
A7	10^{-5}	21.14	*X. manihotis*
A8	10^{-6}	21.43	*X. manihotis*
A9	10^{-7}	21.54	*X. manihotis*
A10	10^{-8}	00.00	*X. manihotis*
A11	10^{-9}	00.00	*X. manihotis*
A12	10^{-10}	00.00	*X. manihotis*
A13	Negative Control	00.00	*X. manihotis*

Fig. 3: (**A**) Sensitivity of the primer and probe set used in the detection of *X. phaseoli* pv. *manihotis* from pure DNA based on picogram level (**B**) Detection of serially diluted bacterial cell in the sensitivity assay by Real-time PCR

Primer and probe selectivity

The selectivity of the probe and primer designed in this study was tested on other plant pathogenic bacteria in addition to *X. phaseoli* pv. *manihotis*, which causes CBB in cassava plants. The results indicated that the designed probe and primer were selective only to *X. phaseoli* pv. *manihotis* isolates, but not the other tested plant pathogenic bacteria used in this study. The *X. phaseoli* pv. *manihotis* selectively yielded positive result (Ct > 0) with FAM Ct value of 23.17, whereas all the other plant pathogens produced negative results (Ct = 0) (Fig. 4A).

Detection of *X. phaseoli* pv. *manihotis* from diseased plant tissues

The symptoms of CBB disease caused by *X. phaseoli* pv. *manihotis* appeared two weeks after artificial inoculation. The bacteria were detected directly from the serially diluted aliquot of grinded diseased cassava plant tissues, including the reference strain, which produced signal curves that crossed the threshold line (*i.e.*, Ct > 0). The negative control with sterilized distilled water and the cassava genomic DNA could however not be hybridized with the probe and primer, thereby recording zero (*i.e.*, Ct = 0) (Fig. 4B).

Discussion

Cassava tuber yield loss due to *X. phaseoli* pv. *manihotis*, which causes cassava bacteria blight disease is a persistent threat to cassava production for centuries in many parts of the world and Ghana is not an exception. In order to minimize this threat during growth, harvest and maximize yield productivity and ensure crop biosecurity and prevent them from bioterrorism, an advanced pathogen detection method which is robust, accurate, rapid, sensitive and selective is imperative. Detection and identification of *Xpm* based on physiological and morphological examinations through culturing on the media are time consuming, labor intensive and require in-depth taxonomic knowledge in the identification of the pathogen (Lo´Pez *et al.* 2003; Michailides *et al.* 2005; Naqvi *et al.* 2015). The study, therefore illustrate the importance of designing a specific probe and primer for rapid and specific detection of putative *Xpm* isolates collected from various regions of Ghana.

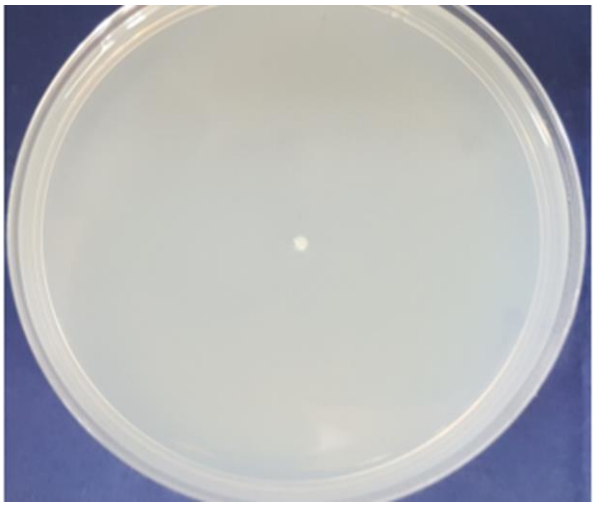


Plate 1: Sensitivity assay of *X. phaseoli* pv. *manihotis* grown on CTA with detection limit of 1 bacterium cell

The Real-time PCR can detect pathogen within relatively shorter time (15–20 min) as compared to the conventional method, which can take about three to four hours to give a result. The Real-time PCR has all-round empirical application in the laboratory diagnostics of plant pathogens, not only it can be used to detect and identify the absence or presence of the pathogen of interest, but also carry out a quantitative assessment of the target pathogen in a given sample. This will help the farmer employ the appropriate preventive or curative measures to stop further spread of the disease. Verdier *et al.* (2001) reported different methods that include nested-PCR assay, dot-blot hybridization, and ELISA tests for detection of *Xpm* in cassava tissues. In southeastern Nigeria, a method like

Table 2: Sensitivity limits and direct bacterial cell (*Xpm*) detection by Real -time PCR

Concentration	RT-PCR Results FAM (Ct)	Bacterial count (cfu/ml)					
	X. phaseoli pv. *manihotis*	*X. phaseoli* pv. *manihotis* 3 replications			$\bar{x}$	std	P/N
Stock	15.47	*	*	*	-	-	+
10^{-1}	17.71	*	*	*	-	-	+
10^{-2}	20.25	*	*	*	-	-	+
10^{-3}	19.93	*	*	*	-	-	+
10^{-4}	20.36	600	314	668	527,3	2.35	+
10^{-5}	19.80	66	82	76	71,3	2.13	+
10^{-6}	20.16	11	11	12	11,3	2.10	+
10^{-7}	20.09	1	3	1	1.6	0.43	+
10^{-8}	00.00	0	0	0	0	0	-
10^{-9}	00.00	0	0	0	0	0	-
10^{-10}	00.00	0	0	0	0	0	-
Negative control	00.00	0	0	0	0	0	-

*Not counted due to intense bacterial growth
std: Standard deviation
P/N: Positive/Negative
$\bar{x}$: Arithmetic mean

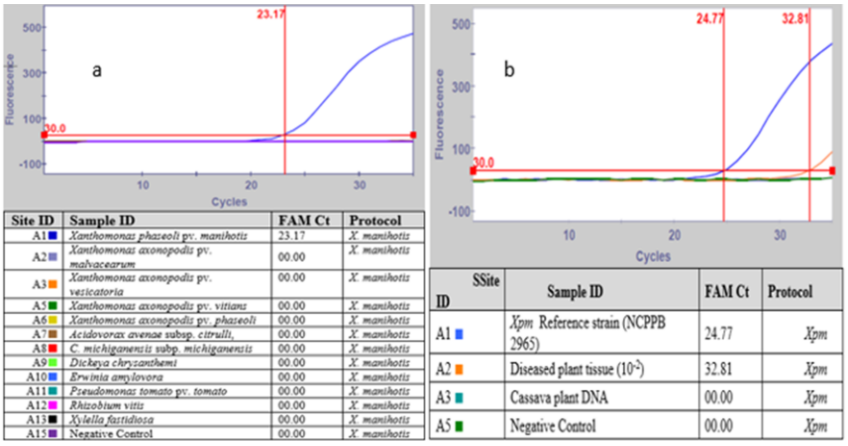


Fig. 4: (**A**) Selectivity of primer and probe sets for the detection of *Xpm* isolates against other plant pathogenic bacterial species (**B**) Direct detection of *Xpm* from diseased plant tissue without bacterial isolation

Random amplified polymorphic DNA (RAPD) have been applied by Ogunjobi *et al*. (2001) to assess the genetic variability in the population structure of *Xpm* collected from different zones of the country. Also in Columbia, geographical differentiation of *Xpm* population using restriction fragment length polymorphism was conducted by Restrepo and Verdier (1997).

The Real-time PCR chemistries can be categorized into amplicon sequence non-specific and sequence specific methods. In this study, we used sequence specific methods for detection and identification of *Xpm* causing CBB disease in Ghana. The sequence specific methods include: TaqMan probe (Holland *et al*. 1991; Livak *et al*. 1995), Scorpion PCR (Whitcombe *et al*. 1999), Molecular beacons (Tyagi and Kramer 1996), LNA probes (Tomlinson *et al*., 2007) *etc*. The LNA probes have been used for specific and multiplex detection of other group of pathogens such as *P. ramorum* and *Phytophthora* (Tomlinson *et al*. 2007; Bilodeau *et al*. 2009).

The probe (HBXamL/HBXamR) designed in this study was labelled with a fluorophore donor and an acceptor dye (quencher), which binds to the *Xpm* VNTR sequence and is cleaved by the 5'–3' Taq DNA polymerase during extension activity from an upstream primer into probe region. This causes fluorophore to separate from the acceptor and thus allowed the fluorescence signal to be emitted by the reporter dye as seen in the reaction from the tested *Xpm* isolates. The fluorescence signal detected in the reaction by the Real-time PCR is directly proportional to the quantity of template DNA in the reaction and the fluorophore emitted. These fluorogenic probes have an advantage over nonspecific DNA binding dyes such as SYBR Green I, in that, there was specific hybridization between probe and a target DNA sequence of *Xpm* needed to emit a fluorescent signal.

The primer and the probe were developed to enhance the specificity of the PCR reaction for *Xpm*, as reliable detection of this pathogen needs high complementarity of the probe with the target sequence, which in this case is VNTRs. The designed probe and primer specifically, sensitively and selectively detected *Xpm* isolates, including the reference strain. The probe was found to be specific in

the detection of *X. phaseoli* pv. *manihotis* in the specificity assay test, because all the tested isolates produced numerous Ct values, which crossed the threshold line after the designed probe binded to the VNTRs sequence of *Xpm.* Diagnostic specificity is defined as the capacity of a given method to detect pathogen of interest in the absence of false negative and positive results (Lo´Pez *et al*. 2003). Usually, amplicon specific sequence methods warrants greater specificity levels that are of particular significance when used for detection and quantification of organism in a given sample (Schena *et al*. 2004). The negative control using non-template DNA could not hybridize with the designed probe and therefore couldn't produce any fluorescent signal in the specificity test, thereby recording Ct values of zero. The zero Ct values meant that, the probe and primer could not bind with the non-template DNA and hence failed to produce fluorescence signal or better still, it could be that, it produced weak signal, which could not cross the threshold line. The Ct values generated depend on the starting materials of the target DNA used and is defined as the number of cycles required for amplified signal to reach threshold level and first be detected. From the results, it could be seen that, some of the samples yielded early Ct values whereas others late. The late Ct values may likely be due to poor amplification efficiency, which in most situations comes from technical biases internal to PCR (Mehle *et al*. 2014; McMullen and Petter 2014).

Based on the specificity results, Ashanti region, which represented Semi-deciduous forest of Ghana, recorded the highest prevalence in percentage of CBB of the infected cassava samples analysed, with Greater Accra region (Coastal savannah) being the least. The samples that did not yield any fluorescent signals were considered non-*Xpm* pathogen. The high prevalence of CBB in the Semi-deciduous forest of Ghana could be attributed to the favourable bimodal rainfall pattern of this zone that favours the pathogen growth and establishment, this is consistent with the views of (Fanou *et al*. 2001), who claimed that favourable climatic conditions favours better survival and establishment of the pathogen.

In a related survey, CBB was reported in different agro-ecological zones of four West Africa countries with varied degree of severity across the zones, where higher CBB incidence was found to occur in the savannah than in the transitional forest zones (Wydra and Msikita 1998; Wydra and Verdier 2002). In a survey conducted by Wydra and Verdier (2002), CBB was not observed in the Sudan savannah (upper regions) - zones of Ghana, this assertion was promoted by this study during our survey in the various agro-ecological zones of Ghana. The CBB incidence was also not seen in the Guinea savannah (Northern region area) and Rain forest - Moist and Wet evergreen zones (Western region area) of Ghana during our survey. Low CBB (10%) incidences were reported in the few deforested areas of the Rainforest zones of southern Nigeria (Wydra and Msikita 1998). In the Central Africa and Congo Republic, higher CBB severity was observed in the savannah area than found in Rainforest and Forest transition zones (Daniel *et al*. 1981).

The developed primer and probe were found to be sensitive when the extracted diluted genomic DNA was tested at the picogram level. The assay was of high sensitivity, which enabled detection of low levels of heavily diluted extracts DNA in the master mix. Diagnostic sensitivity is defined as the measure of degree of detection or the lowest or minimum number of organisms reliably detected per sample (Lo´Pez *et al*. 2003). The DNA concentration at the nanogram level all things being equal, was perceived high, and so any good or bad primer or probe can sensitively detect it at this level, because of that, we further diluted the original concentrations of the extracted DNA from nanogram level to picogram level to see whether our designed primer and probe could detect the presence of this bacterium in the reaction. In the end, we found that, the designed primer and probe were successful in detection of this pathogen at a very low concentration level of 13pg (detection limit). By diluting the DNA further below 13pg, our designed primer and probe could not detect the pathogen, meaning the detection limit for these primer and probe using extracted purified DNA was 13pg.

The designed probe was very sensitive and could also detect as low as one bacterial cell (cfu/mL) in the reaction. The PCR was able to detect serially diluted bacterial suspension within the ranges of 10^{-1}–$10^{-}7$, but failed to do so with an increasing serial dilution (10^{-8}–10^{-10}). Verdier *et al*. (2001) detected *Xpm* in the stem and leaf lesion extracts with the lowest level of cells detected ranging from 3×10^{2} to 10^{4} cfu per mL. They also reported highest detection quantity of 1–2 viable cells per run and total dot-blot method sensitivity of 10^{3} cfu per reaction.

To determine how selective our designed LNA probe was, we subjected all the *Xpm* isolates together with non-*Xpm* pathogens to PCR analyses. Of all the 11 other plant pathogenic bacteria tested together with *Xpm*, the probe was found to be selective to only *Xpm* in the selectivity assay test, resulting in the reliable and successful identification of this pathogen. The *Xpm* DNA hybridized with the designed probe, producing numerous fluorescence signals that crossed the threshold line. All the other pathogens failed to yield any positive fluorescent signal curves.

To also find out whether we could detect *Xpm* directly from the inoculated cassava tissues without laborious culturing of the isolated pathogen on the media and DNA extraction, we artificially inoculated the cassava plants and after the symptoms have developed, infected tissues were taken, crushed and diluted crude extract from the tissues used for the PCR reaction. The crude extracts were serially diluted so as to do away with the phenolic compounds which could hinder or inhibit the amplification process. Sometimes designed probe can also hybridized with the content of the host plant from which the pathogen is isolated and for that reason, we decided to include extracts cassava

DNA during the PCR reaction. From the results, it was seen that, only the positive control, which constituted the reference strain and the direct crude extract from *Xpm* inoculated tissues produced fluorescent signal curves proportionally during the amplification of the reaction products. The cassava DNA and the negative control as expected could not yield any fluorescent signals. What this tells us is that, the *Xpm* can be detected directly from crude extract of the cassava without DNA extraction or pathogen culturing on media. This finding is promoted by Verdier *et al*. (2001) who detected this pathogen in the crude extracts of the stem and leaf lesions of the infected cassava plant.

Conclusion

The designed probe and primer in this study detected all the isolates of *X. phaseoli* pv. *manihotis* in the cassava tissues. The designed probe was found to be accurate, sensitive and selective. The developed probe also enabled the Real-time PCR to be specific and efficient. The probe was able to detect this vascular pathogen from extracted pure DNA, direct cultured bacteria colony grown on NA and CTA media and as well as from direct cassava crude extract without DNA extraction.

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