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Development of a lateral flow device for in-field detection and evaluation of PCR based diagnostic methods for *Xanthomonas campestris* pathovar *musacearum*, the causal agent of banana Xanthomonas wilt.

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Abstract

Xanthomonas campestris pathovar *musacearum* (*Xcm*) is the causal agent of banana Xanthomonas wilt, a major threat to banana production in eastern and central Africa. The pathogen is present in very high levels within infected plants and can be transmitted by a broad range of mechanisms; therefore early specific detection is vital for effective disease management. In this study we have developed a polyclonal antibody (pAb) and deployed this in a lateral flow device (LFD) format to allow rapid in-field detection of *Xcm*. We also independently assessed published *Xcm* PCR assays: only two assays gave specific amplification of *Xcm*, whilst others cross-reacted with non-target *Xanthomonas* species. Pure cultures of *Xcm* were used to immunise a rabbit, the IgG antibodies purified from the serum and the resulting polyclonal antibodies tested using ELISA and LFD. Testing against a wide range of bacterial species showed the pAb detected all strains of *Xcm*, representing isolates from seven countries and the known genetic diversity of *Xcm*. The pAb also detected the closely related *Xanthomonas axonopodis* pathovar *vasculorum* (*Xav*), primarily a sugarcane pathogen. Detection was successful in both naturally and experimentally infected banana plants, and the LFD limit of detection was 10^5 cells/ml. Whilst the pAb is not fully specific for *Xcm*, *Xav* has never been found in banana. Therefore the LFD can be used as a first line screening tool to detect *Xcm* in the field. Testing by LFD requires no equipment, can be performed by non-scientists and is cost-effective. Therefore this LFD provides a vital tool to aid in the management and control of *Xcm*.

Introduction

Xanthomonas campestris pv. *musacearum* (*Xcm*) is a Gram negative member of the gamma-Proteobacteria that is the causal agent of Banana Xanthomonas Wilt (BXW), considered to be one of the greatest threats to banana productivity in the great lakes region of Eastern Africa

(Tripathi *et al.* 2009). It was first found in Ethiopia around 40 years ago on enset (Yirgou and Bradbury, 1978) and is now wide spread in east and central Africa. Since the early 2000's the disease has been reported in Uganda, the Democratic Republic of the Congo, Rwanda, Tanzania, Kenya and Burundi (Adikini *et al.* 2011).

Xcm causes symptoms of foliar wilting and yellowing, leading to uneven fruit ripening and death of the plant. Symptom development is rapid, typically less than one month after inoculation, and leads to high levels of the pathogen within the plant typified by pockets of bacterial ooze when the plants are cut (Tripathi *et al.* 2009; Adikini *et al.* 2011). The economic impact of *Xcm* is high due to total loss of yield and rapid death of the mother plant, preventing the creation of more plants by division of suckers, a primary mode of cultivation (Tripathi *et al.* 2007). The disease affects almost all banana cultivars with only a small number able to avoid insect borne infection due to the differing morphological structure of the male buds (Adikini *et al.* 2013). Therefore recent approaches to control have included genetic engineering to create transgenic bananas with resistance to *Xcm* (Tripathi *et al.*2010; Namukwaya *et al.* 2012) and the development of *in vitro* screening methods for rapid determination of cultivar resistance/susceptibility to *Xcm* infection (Tripathi *et al.* 2008).

Xcm can be transmitted in numerous ways including insect vectors, the use of contaminated farm equipment, transmission within the mat to suckers, soil borne infection and planting of infected asymptomatic plantlets (Tripahti *et al.* 2008; Adikini *et al.* 2013). A range of management strategies have been developed to limit *Xcm* spread. These have been demonstrated to be highly effective when employed properly, causing reduction of disease incidence to less than 10% per year in regions of Uganda. However the approaches, including restricting movement, tool sterilisation, removal of male buds and burying infected plants are

often poorly taken up by farmers due the high financial and labour costs (Tushemereirwe *et al.* 2006).

The *Xanthomonas* genus is large, composed of many species and pathovars which cause disease in several hundred plant species. The genus is composed of two groups, with *Xcm* sitting within Group 2 along with many other important plant pathogens (Rodriguez-R *et al.* 2012). The taxonomy of species and pathovars is very complex and still evolving, meaning confusion can exist over the identity of isolates within culture collections and that species identification can be problematic for diagnostic laboratories (Parkinson *et al.* 2009). *Xcm* was initially described as *Xanthomonas musacearum* and was re-classified *Xcm* in 1978 (Young *et al.* 1978). It has recently been proposed that *Xcm* should be re-classified as *X. vasicola* pv *musacearum* (*Xvm*) due to its close relatedness to strains of *X. vasicola*. Indeed, partial *gyrB* sequences have shown 100% nucleotide sequence identity between *Xcm* and some *X. vasicola* isolates, and FAME and rep-PCR methods group *Xcm* with *X. vasicola* (Aritua *et al.* 2008; Tripathi *et al.* 2009).

Recent whole-genome comparisons of *Xcm* and *Xvv* are also corroborative, finding significantly greater nucleotide identity with each other than with any other sequenced xanthomonad. Importantly, all *Xcm* isolates form a clade closely related to but distinct from *Xvv* (Studholme *et al.* 2010; Wasukira *et al.* 2012). Studied *Xcm* isolates have demonstrated very little genetic diversity with comparisons of whole genomes showing at least 99.99% nucleotide identity (Wasukira *et al.* 2012). Despite this, genome-wide analyses looking for single nucleotide polymorphism (SNP) variation have proposed two major sub-lineages within *Xcm*, each corresponding to a distinct geographic region. Whilst this is based on only 86 SNPs and a relatively small number of isolates, it splits sub-lineage I containing isolates

from Ethiopia, the Democratic Republic of the Congo and Rwanda from sub-lineage II comprising isolates from Uganda, Tanzania, Burundi and Kenya (Wasukira *et al.* 2012). Numerous published data sets support the reclassification of *Xcm* as *Xvm*; however, further pathogenicity testing is required before this can be formally recognised as a pathovar designation (Aritua *et al.* 2008).

A primary driver for *Xcm* specific detection tools is to allow discrimination from numerous other diseases of banana which can cause similar wilt type symptoms, including Moko, bugtok and blood disease caused by bacteria and the fungal disease Fusarium wilt (Adikini *et al.* 2011). Typically *Xcm* detection is based upon visual symptom expression and culture based morphological assessment. Recently, semi-selective media for *Xcm* have been developed (Mwangi *et al.* 2007; Tripathi *et al.* 2007) but this approach is found to be lacking as it is time consuming, requires confirmatory testing and often misses early stages of infection (Adikini *et al.* 2011). Biochemical tests can be used in some cases to resolve species and pathovars; however approaches such as metaboloic profiling (e.g. Biolog) are time consuming, whilst fatty acid methyl ester (FAME) analysis is costly (Aritua *et al.* 2008; Adikini *et al.* 2011). As an alternative, molecular approaches have been investigated to allow the specific detection of *Xcm* including conventional PCR (Lewis Ivey *et al.* 2010; Adikini *et al.* 2011; Adriko *et al.* 2012) or rep-PCR (Versalovic *et al.* 1994). Since the recent discovery of sub-lineages within *Xcm*, Wasukira *et al.* (2012) developed a PCR-RFLP assay to allow discrimination of the lineages. Whilst these approaches can provide specific detection they require well equipped laboratories, highly trained staff and are not readily amenable to field applications.

Lateral flow devices (LFDs) are an established technology for the rapid detection of plant pathogens, and are one of the primary techniques used for 'point-of-care' or 'in-field' pathogen detection (Boonham *et al.* 2008). LFDs provide an identification in minutes, have no technological requirements and are simple to use so they are an important identification tool despite recent molecular detection advances (De Boer and López, 2012; Lane *et al.* 2007). The requirement of such diagnostic approaches for *Xcm* is essential to facilitate elimination of residual sources of infection and improve disease management (Ocimati *et al.* 2013). This is critical for diseases such as BXW where by the time symptoms are visible the pathogen has spread systemically and therefore poses a high risk of further transmission (Adikini *et al.* 2013) and where latent infection can occur with long incubation periods (Ocimati *et al.* 2013). Therefore in this study we set out to develop an antibody based diagnostic kit for *Xcm* that could be deployed in a low-resource setting, providing a cost effective diagnosis which can be performed by un-trained personnel. A further advantage of this approach for diseases of this kind is that testing can occur simultaneously with control. Divorcing sampling from testing by sending samples to the lab can potentially lead to delays and loss of urgency on the part of growers. To enable this, polyclonal antibodies were generated to *Xcm* and formatted into a lateral flow device for in-field detection.

Materials and methods

Bacterial cultures used and their maintenance

A range of *Xcm* isolates, closely related *Xanthomonas* species including *Xvv*, *Xav* and *Xanthomonas vasicola* pv *holicola*, more distantly related *Xanthomonas* species and other bacterial species known to cause disease on banana were selected from the National Collection of Plant Pathogenic Bacteria (NCPPB), held at Fera, York, UK (see Table 1). This included 32 isolates representing eight pathovars of *Xanthomonas*, and *Enterobacter* sp.,

Serratia marcescens, *Pseudomonas marginalis* pv. *marginalis* and *Ralstonia solanacearum* (all associated with diseases of banana or known endophytes of banana). *Melissococcus plutonius* was sourced from The National Bee Unit (Fera, York, UK).

All *Xanthomonas* strains were grown on yeast dextrose chalk (YDC) agar (10 g L^{-1} yeast extract, 20 g L^{-1} dextrose, 20 g L^{-1} $CaCO_3$, 12 g L^{-1} agar technical no. 3, pH 7.2) incubated at 28°C for 48 hours. *Ralsonia solanacearum* was grown on SMSA agar (6.28g L^{-1} glycerol, 10g L^{-1} peptone, 1g L^{-1} casimino acid, 18g L^{-1} bacteriological agar, supplemented immediately prior to use with 0.1 g L^{-1} polymixin B sulphate, 0.1 g L^{-1} bacitracin, 0.05 g L^{-1} 2,3,5-triphenyltetrazolium chloride, 0.005 g L^{-1} crystal violet, 0.005 g L^{-1} chloramphenicol and 0.0005 g L^{-1} penicillin G) and incubated at 25°C for 48 hours. *Melisococcus plutonius* was grown anaerobically on M110 media (2.5 g L^{-1} peptone, 10 g L^{-1} glucose, 2 g L^{-1} soluble starch, 2.5 g L^{-1} yeast extract, 5 g L^{-1} neopeptone, 2 g L^{-1} trypicase, 4.33 g L^{-1} K_2HPO_4 and 3.42 g L^{-1} KH_2PO_4, 15 g L^{-1} agar technical no. 1, pH 7.2, supplemented immediately prior to use with 1 g L^{-1} cysteine HCl) incubated at 37°C for approximately 2 weeks. *Pseudomonas marginalis* pv. *marginalis*, *Enterobacter* sp and *Serratia marcescens* were grown on Kings B media (20 g L^{-1} proteose peptone, 1.5 g L^{-1} K_2HPO_4, 1.5 g L^{-1} $MgSO_4$ $7H_2O$, 10 g L^{-1} glycerol, and 12 g L^{-1} agar technical no.3) incubated respectively at 25°C for 48 hours, 37°C for 24 hours and 37°C for 48 hours. ACDP (Advisory Committee on Dangerous Pathogens) hazard group 2 species (*Enterobacter* sp. and *Serratia marcescens*) were formalin fixed prior to use by the addition of 2.5 µl mL^{-1} 37% formalin.

Inoculation of banana plants and field infected samples

Young (8-10 inches (20-25 cm) in height) *Musa* var. Tropicana and var. Cavendish Dwarf plants were artificially inoculated with *Xcm*. *Xcm* was grown on YDC agar as above, colonies

re-suspended in sterile water and the concentration adjusted to 1 x 10^9 cells ml^{-1} by spectrophotometry. Inoculation was performed by injection of 200 µl inoculum into the pseudostem of the plant approximately 1 inch above soil level. Control plants were either un-inoculated or inoculated with sterile water. Post inoculation the plants were grown in a glasshouse maintained at 28°C day, 20°C night with 16 hours supplemented lighting. After inoculation 100µl of the inoculum was spread onto the YDC agar and incubated as above to confirm viability. Symptom development was monitored and leaf material harvested and stored at -20^0C for later use. Sections of pseudostem and leaves from 16 field grown *Musa* plants showing typical symptoms of *Xcm* infection were collected in Uganda and shipped to the UK prior to storage at 4°C.

Production of polyclonal antibody and LFD development

An eight month old female New Zealand cross rabbit was used for the production of polyclonal antibodies, and prior to immunisation a blood sample was collected. The immunogen was composed of a mix of six *Xcm* strains all collected from *Musa* sp (NCPPB4378 from Uganda, NCPPB4387 from the Democratic Republic of the Congo, NCPPB4389 from Rwanda, NCPPB4392 from Tanzania, NCPPB4433 from Burundi and NCPPB4434 from Kenya) encompassing the geographic variability of *Xcm*. To prepare the immunogen each isolate was first grown on YDC agar before being transferred to nutrient broth (Oxoid) and grown at 28°C at 200 rpm for 24 hours. Cells were pelleted by centrifugation, re-suspended in 1 x phosphate buffer saline, concentrations adjusted by spectrophotometry and equally pooled to form the immunogen. The immunogen was mixed equally with Freund's complete adjuvant to give a final concentration of 1.25 x 10^8 cells ml^{-1} and the rabbit immunised subcutaneously with 1 ml. Three further immunisations of the immunogen mixed equally with incomplete Freund's adjuvant to a final concentration of 1.25

x 10^8 cells ml^{-1} were administered subcutaneously at four week intervals. Four weeks after the final immunisation a blood sample was taken and the serum analysed for immune response by ELISA (see below). Further blood samples were taken at four week intervals, the serum separated from whole blood by centrifugation and stored at -30°C prior to use. The IgG antibodies were purified from post immunisation serum by HiTrap Protein G column (GE Healthcare) following the manufacturer's protocol. The protein concentration of the purified IgG antibody was determined by extinction coefficient calculation at 280nm. Purified IgG antibodies were provided to Forsite Diagnostics Ltd (York, UK) for the production of a lateral flow device (Danks & Barker, 2000).

ELISA testing

PTA-ELISA was performed as follows; 96-well ELISA plates were coated with 100µl of bacteria (see Table 1) at a concentration of 10^7 cells ml^{-1} in coating buffer (0.015M anhydrous sodium carbonate, 0.046M sodium hydrogen carbonate, pH 9.6), or 100µl of plant material extract (2 g plant material in 20ml coating buffer, ground using a hand homogeniser (BIOREBA)) and incubated overnight at 2 - 8°C. Plates were washed three times with phosphate buffered saline + Tween-20 (PBS-T) (1.4M sodium chloride, 0.015M potassium dihydrogen orthophosphate, 0.08M di-sodium orthophosphate dihydrate, 0.03M potassium chloride, pH 7.4, 0.05% Tween-20) and blocked with 200µl PBS-T containing 1% bovine serum albumin (BSA) for 1 hour at 33°C. Plates were washed as above and 100µl of polyclonal antibody applied, either pre-immunisation serum, post immunisation serum or purified antibody or serum from a rabbit immunised with *Cucumber vein yellowing virus* (as a negative control) at various dilutions in PBS-T containing 0.2% BSA and incubated for 1 hour at 33°C. Plates were washed as above and 100µl goat anti-rabbit alkaline phosphatase was applied, diluted 1:4000 in PBS-T containing 0.2% BSA, and incubated for 1 hour at

33°C. Plates were washed as above and 100µl of 1 mg ml^{-1} p-nitrophenyl phosphate in substrate buffer (10% diethanolamine, 0.002M magnesium chloride, pH9.8) was added. The plates were kept in the dark at room temperature for 30 minutes and the absorbance measured at 405 nm on a plate-reader (Thermomax Microplate reader) using SOFT Max Pro software.

To determine the sensitivity of the ELISA, bacterial cells were serially diluted from 10^8 cells ml^{-1} through eight log dilutions and 100µl applied per well. Wells of *Melissococcus plutonius* bacterial culture and *M. plutonius* monoclonal antibody were included in each experiment as a procedural positive control. Healthy *Musa* var. Tropicana and var. Cavendish Dwarf, *Musa* var. Tropicana experimentally inoculated with *Xcm* NCPPB4434 and 16 symptomatic (presumed *Xcm* infected) field plants were tested by ELISA, with sections of either leaf or psuedostem prepared as described above.

LFD testing

When testing LFDs, samples were prepared in buffer A provided by Forsite Diagnostics Ltd and in all cases 100µl was applied to each LFD. Each LFD was allowed to run for ten minutes before assessing the results. A positive result was indicated by both a test (T) line and control (C) line, a negative result but successfully run LFD was indicated by a C line alone, and a failed LFD run showed by no lines. Controls of buffer A and healthy *Musa* were included in all experiments.

Bacterial cultures of target and non-target species (see Table 1) were diluted to 10^8 cells ml^{-1} in buffer A. To test plant material 5 cm sections of leaf or pseudostem were cut into approximately 1 cm sections and placed in an extraction bottle (Forsite Diagnostics Ltd). This was either allowed to stand for 5 minutes to allow the sap/bacteria to ooze into the buffer, or

homogenised by shaking manually for 1 minute. To determine the sensitivity of the LFDs, *Xcm* NCPPB4378 was serially diluted from 10^8 cells ml^{-1} through six log dilutions in buffer A. To test sensitivity of the LFD in a plant exudate background cultured bacteria were diluted in healthy *Musa* leaf sap exuded into buffer A. Healthy *Musa* var. Tropicana and var. Cavendish Dwarf, healthy sugarcane, *Musa* var. Tropicana experimentally inoculated with *Xcm* NCPPB4434 and the 16 symptomatic (presumed *Xcm* infected) field plants were tested by LFD, with sections of either leaf or pseudostem prepared as described above.

Evaluation of PCR assays

PCR was performed using published protocols designed for the detection of *Xcm* (see Table 2). In all cases 2x ReddyMix PCR Master Mix with 1.5 mM $MgCl_2$ (Thermo Scientific) was used with the cycling conditions as originally described. PCR was performed in a GeneAmp® 9700 thermocycler using 1µl DNA as template (concentration as extracted). Assays used were; the BXW assay of Lewis Ivey *et al.* 2010 using 500 nM each of primers BXW-1 and BXW-3; the GspD assay of Adriko *et al.* 2012 using 400 nM each of primers Gsp DM-F2 and Gsp DM-R3; assays Xcm12, Xcm35, Xcm36, Xcm38, Xcm44, Xcm47 and Xcm48 of Adikini *et al.* 2011 using 400 nM of each forward and reverse primer. All primers were synthesised by Eurofins-MWG-Operon. PCR products (10µl) were separated by gel electrophoresis in 1.5% agarose gel in 1x Tris-borate-EDTA buffer, stained with ethidium bromide and visualised using a UV transilluminator.

Results

Specificity of the ELISA and LFD

To assess the specificity of the pAb, ELISA was conducted testing *Xcm*, a wide range of Xanthomonas species, and other bacterial species known to cause disease in banana. All

seventeen *Xcm* strains, representing isolates from seven countries (Burundi, the Democratic Republic of the Congo, Ethiopia, Kenya, Rwanda, Tanzania, and Uganda) encompassing both sub-lineages I and II, therefore representing the known genetic diversity of *Xcm*, could be readily detected. However the pAb was also found to detect all strains of *Xav* tested. All of the other *Xanthomonas* species and pathovars tested were negative, as were other bacterial species (Table 1). Control samples of sap, leaf and pseudostem from healthy *Musa* var. Tropicana and var. Cavendish Dwarf were tested by ELISA and found to give negative results in all cases.

The specificity of the LFD was found to be identical to that of the ELISA, detecting all *Xcm* strains tested along with *Xav* strains but not any other species tested (Table 1). Healthy *Musa* var. Tropicana and var. Cavendish Dwarf sap, leaf and pseudostem and healthy sugarcane leaf were tested by LFD and gave negative results in all cases. The LFD gave unambiguous easy to interpret results with clear differentiation between positive and negative samples, in both pure culture and infected plant material (Figure 1).

Sensitivity of the ELISA and LFD

The sensitivity of the ELISA with the purified pAb was determined using eight *Xcm* isolates. When testing bacteria from pure culture the limit of detection ranged from 10^6 to 10^2 cells ml^{-1}. However when testing in a sap exudate background, to mimic actual samples, the limit of detection was reduced to 10^8 cells ml^{-1} for seven of the isolates. One isolate (NCPPB4434), which was only detectable at 10^6 cells ml^{-1} in pure culture, could not be detected in sap exudate.

The sensitivity of the LFD was first determined using pure culture of *Xcm* NCPPB4378 in buffer where the limit of detection was 10^5 cells ml^{-1}. To determine the effect upon the limit of detection in a plant background *Xcm* NCPPB4433 was serially diluted in leaf exudate and whole-leaf extract where the limit of detection was decreased to 10^6 cells ml^{-1}. Six further *Xcm* isolates were tested in a sap exudate background and the limit of detection ranged from 10^6 to 10^3 cells ml^{-1}, most typically being 10^5 cells ml^{-1}. Lower dilutions yield fainter but still clearly visible test lines (Figure 2) which became easier to view upon drying of the membrane. To determine if a hook effect (Amarasiri Fernando & Wilson, 1992) may have a deleterious effect on detection by the LFD at high bacterial titres, *Xcm* NCPPB4433 was tested at 10^{10} and 10^9 cells ml^{-1} in buffer A and buffer A with sap exudate, all of which could be detected.

Testing of naturally and experimentally infected plant samples by ELISA and LFD

Leaf samples showing moderate wilt symptoms from *Musa* experimentally infected with *Xcm* NCPPB4434 could be detected by both ELISA and the LFD. Sixteen of the symptomatic *Musa* samples from Uganda, suspected of *Xcm* infection, were tested. Despite the fact that these samples were noticeably degraded to the extent that they were starting to rot following shipping to the UK, when tested by ELISA, nine samples tested positive and two samples tested negative. Five of the samples gave positive results in ELISA control wells lacking primary antibody. We speculate this may be due to the degraded nature of the samples with a component of the sample causing non-specific binding of the secondary antibody. However, when tested by LFD all 16 samples were positive.

PCR evaluation

All previously published PCR assays were tested against a wide range of bacteria, to independently evaluate their performance characteristics with regard to specificity. Our evaluation included the *Xcm* specific assays of Adikini *et al.* 2011 and Adriko *et al.* 2012 and the *Xcm*, *Xav* and *Xvh* assay of Lewis Ivey *et al.* 2010.

All primer sets were able to detect all strains of *Xcm* tested, representing isolates from seven different countries (Burundi, the Democratic Republic of the Congo, Ethiopia, Kenya, Rwanda, Tanzania, and Uganda) and both sub-lineages I and II of *Xcm* (Table 1). The assays could also detect successfully either pure bacterial culture or naturally infected plant material.

The GspD assay of Adriko *et al.* 2012 was found to provide specific amplification of only *Xcm* and no other species. In our study, the assay Xcm35 of Adikini *et al*, 2011 was found to be specific and only detected *Xcm*. However the remaining six assays cross-reacted with other species, with all detecting a minimum of *Xvv* and *Xvh*. The least specific assay, Xcm38, gave positive results with all *Xanthomonas* species tested as well as *Ralstonia solanacearum* and *Enterobacter* sp.

The Lewis Ivey *et al.* (2010) assay detected all strains of *Xcm* and *Xvh*; however, it did not amplify any strains of *Xav* but did amplify all strains of *Xvv* tested. A possibility for this anomaly is the complexity arising from the challenges with *Xanthomonas* species and pathovar taxonomy, and the proposed but not formally accepted re-naming of *Xav* as *Xvv*. In this study the species names are as listed in NCPPB, however some isolates were also studied by Lewis Ivey *et al.* (2010) and Adriko *et al.* (2012) and have contrasting species designations. For example NCPPB702 is described as *Xvv* in this study but *Xav* by Lewis

Ivey *et al.* (2010). Interestingly Lewis Ivey found that only some *Xav* strains were PCR positive, for example finding *Xav* NCPPB702 to be PCR positive and *Xav* NCPPB796 to be PCR negative. These two isolates were included in our study, where NCPPB702 were classified as *Xvv* and was PCR positive and NCPPB796 was classified as *Xav* and was PCR negative. Therefore it seems highly likely this apparent difference in PCR assay specificity is due to the species names assigned to *Xav* and *Xvv* isolates. Using the NCPPB species names the assay specificity was shown to be specific for all *Xvv* isolates (PCR positive) and that all *Xav* isolates were PCR negative and not detected.

Unclassified *Xanthomonas* isolates

The unidentified isolates NCPPB1131 and NCPPB1132 both *Xanthomonas* species isolated from Eastern and Western Samoa on *Musa* species were negative with all methods, with the exception of the very non-specific PCR assay Xcm38. Interestingly, based upon whole genome sequencing Studholme *et al.* (2011) concluded that NCPPB4393 was in fact not an *Xcm* isolate as initially described but was *X. sacchari*. However in this study NCPPB4393 has tested positive with all of the *Xcm* specific detection methods (ELISA, LFD and PCR assays) indicating that it is indeed an *Xcm* isolate.

Discussion

BXW is a devastating disease; however, it has been demonstrated that effective management can cause substantial decreases in disease prevalence. Crucial to a successful management strategy is the ability to specifically detect the pathogen and with sufficient sensitivity to identify latent infections. The antibody developed in this study has been formatted for use in both ELISA and LFD, therefore providing an alternate laboratory test if used in ELISA, and allowing in-field testing with the LFD. The substantial reduction of the ELISA limit of

detection when testing in a plant background compared to pure bacterial culture is likely due to the much larger number of proteins competing for binding sites and therefore reducing the sensitivity, an effect considerably reduced when testing by LFD. Interestingly five samples were found to fail under ELISA testing due to degradation of samples during shipping and two samples were negative, presumably due to levels of infection below the limit of detection, whereas all of these were positive when tested by LFD. This is particularly relevant when testing samples from remote locations, when time to send samples to the lab is prolonged, and demonstrates the practical advantages of the LFD where detection is possible after other techniques have failed.

The pAb was found to be specific to *Xcm*, detecting all strains representing the geographic spread of *Xcm*, however it also cross-reacts with *Xav*. Whilst it would be preferable for the pAb to be fully specific to *Xcm*, *Xav,* the causal agent of a gumming disease of sugarcane, is found in grass species and maize. There are currently no instances of *Xav* being found in banana. Moreover, it has been demonstrated that *Xav* cannot cause disease in banana via artificial inoculation. Therefore the antibody generated in this study represents an important tool, with sufficient specificity that when testing banana will robustly and sensitively detect *Xcm*. Both the ELISA and LFD have been demonstrated to detect *Xcm* in infected plant material and can accordingly be used as an initial screening tool to rule out wilt caused by other bacterial or fungal diseases with similar symptoms. The LFD has a broad detection range and has been demonstrated to readily detect infected plant material.

A number of molecular detection tools have been developed to allow laboratory based detection of *Xcm.* Evaluation of these has lead to conflicting reports regarding assay performance appearing within the literature, with Adriko *et al*. (2012) reporting that the

Adikini *et al.* (2011) assays are not specific for *Xcm* detecting between two and nine non-banana *Xanthomonas* strains. In our independent assessment, only two primer pairs (GspD of Adriko *et al.* 2012 and Xcm35 of Adikini *et al.* 2011) gave specific amplification of *Xcm*, with other published PCR assays cross-reacting with other *Xanthomonas* species. In particular the *Xcm* assay panel of Adikini *et al.* 2011 was found to show significant cross-reactivity, with six of the seven assays found to be non-specific.

The Lewis Ivey *et al.* (2010) assay was designed to amplify all *Xcm* strains and was published as also detecting some strains of *Xav* and *Xvh*, a finding confirmed by Adriko *et al.* 2012. The assay performance in our hands is in contrast to this; however this appears to be due to the non-formalised renaming of *Xav* as *Xvv*. Therefore we found the assay specifically detects *Xcm*, *Xvh* and *Xvv*. Two unidentified *Xanthomonas* species isolates (NCPPB1131 and NCPPB1132) were found to be negative with all *Xcm* specific detection techniques; therefore this supports the finding of Studholme *et al.* (2011) that they fall within the *X. sacchari* clade.

Whilst our study shows that some of the published PCR assays can provide specific detection of *Xcm*, these techniques are still contingent upon a well equipped laboratory with highly trained staff, and unlike LFDs, results are not instantaneous, with samples requiring transportation to the laboratory followed by testing which may take between many hours to days. Therefore the development of simple, rapid detection strategies which can be performed in the field, such as LFDs, are invaluable. LFDs are suited to this setting, being cost-effective, portable, easy to use and providing almost instantaneous results. Crucially, they can then allow prompt action when infected plants are found, vital in the management of diseases such as *Xcm* which utilise multiple modes of transmission, or alternatively used to test planting material at borders to prevent introduction of *Xcm* into countries or regions where it is not yet

present. Based on the results of this study, the LFD can be deployed for in field detection of *Xcm*. If further confirmatory testing is required we have identified two *Xcm* specific PCR assays (GspD of Adriko *et al.* 2012 and Xcm35 of Adikini *et al.* 2011) from the literature which can subsequently be used in a laboratory setting. Large-scale field testing of the LFD is currently underway, and it is hoped that this new detection approach, the first published primary literature report of a LFD for *Xcm*, will become a vital asset to the detection and management of BXW.

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Figure legends

Figure 1. Detection by the BXW LFD**.** From left to right; Pure bacterial culture at 10^8 cells ml^{-1} of *Xcm* NCPPB4378, *Xav* NCPPB796, *Xcc*, NCPPB529, plant material of healthy of *Musa* var. Tropicana, healthy *Saccharum* and *Musa* var. Tropicana experimentally infected with *Xcm* NCPPB4434.

Figure 2. Sensitivity of the LFD, tested with *Xcm* NCPPB2251. Bacterial culture serially diluted in banana sap/buffer A from 10^8 cells ml^{-1} (left) to 10^4 cells ml^{-1} (right).

Table 1. Bacterial isolates used in the study, their plant host and country of origin and results generated by ELISA, LFD and PCR testing. ELISA results using purified pAb at 1:32,000 dilution, average of triplicate wells, scored as; '+' positive with OD>1.7; '-' negative with OD<0.3. LFD results; '+' indicates test and control line positive; '-' indicates only control line positive. PCR results scored as '+' indicates positive; '-' indicates negative; 'weak +' indicates weak positive; 'NS' indicates non-specific amplification.

NCPPB number	Species	Country of origin	Host species	ELISA result	LFD result	PCR result								
						GspDm	BXW	Xcm12	Xcm35	Xcm36	Xcm38	Xcm44	Xcm47	Xcm48
4389	*Xanthomonas campestris pv musacearum*	Rwanda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4390	*Xanthomonas campestris pv musacearum*	Rwanda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4391	*Xanthomonas campestris pv musacearum*	Rwanda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4378	*Xanthomonas campestris pv musacearum*	Uganda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4379	*Xanthomonas campestris pv musacearum*	Uganda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4380	*Xanthomonas campestris pv musacearum*	Uganda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4381	*Xanthomonas campestris pv musacearum*	Uganda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4383	*Xanthomonas campestris pv musacearum*	Uganda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4434	*Xanthomonas campestris pv musacearum*	Kenya	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4433	*Xanthomonas campestris pv*	Burundi	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+

	musacearum													
2005	*Xanthomonas campestris pv musacearum*	Ethiopia	*Ensete ventricosum*	+	+	+	+	+	+	+	+	+	+	+
2251	*Xanthomonas campestris pv musacearum*	Ethiopia	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4387	*Xanthomonas campestris pv musacearum*	D.R.Congo	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4388	*Xanthomonas campestris pv musacearum*	D.R.Congo	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4392	*Xanthomonas campestris pv musacearum*	Tanzania	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4393	*Xanthomonas campestris pv musacearum*	Tanzania	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
4394	*Xanthomonas campestris pv musacearum*	Tanzania	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+
206	*Xanthomonas vasicola pv vasculorum*	South Africa	*Zea mays*	-	-	-	+	+	-	+	+	+	+	+
702	*Xanthomonas vasicola pv vasculorum*	Zimbabwe	*Saccharum officinarum*	-	-	-	+	+	-	+	+	+	+	+
890	*Xanthomonas vasicola pv vasculorum*	South Africa	*S. officinarum*	-	-	-	+	+	-	+	+	+	+	+
989	*Xanthomonas vasicola pv holicola*	USA	*Holcus* sp.	-	-	-	+	+	-	+	+	+	+	+
1060	*Xanthomonas vasicola pv holicola*	Ethiopia	*Sorghum vulgare*	-	-	-	+	+	-	+	+	+	+	+
2417	*Xanthomonas vasicola pv holicola*	New Zealand	*S. vulgare*	-	-	-	+	+	-	+	+	+	+	+
186	*Xanthomonas axonopodis pv vasculorum*	Mauritius	*Thysanolaena maxima*	+	+	-	-	-	-	-	+	+	-	-
796	*Xanthomonas axonopodis pv vasculorum*	Mauritius	*S. officinarum*	+	+	-	-	-	-	-	weak +	+	-	-
899	*Xanthomonas axonopodis pv vasculorum*	Reunion	*S. officinarum*	+	+	-	-	-	-	-	NS	+	-	-
1630	*Xanthomonas arboricola pv celebensis*	New Zealand	*Musa* sp.	-	-	-	-	-	-	-	NS	-	-	-
529	*Xanthomonas campestris pv campestris*	UK	*Brassica oleracea var. capitata*	-	-	-	-	-	-	-	weak +	-	-	-
2985	*Xanthomonas campestris pv perlagonii*	New Zealand	*Pelargonium peltatum*	-	-	-	-	weak	-	-	weak	weak	-	weak

								+			+	+		+
4031	*Xanthomonas campestris pv perlogonii*	UK	*Pelargonium x hortorum*	-	-	-	-	-	-	-	NS	-	-	-
422	*Xanthomonas campestris pv vestcatoria*	New Zealand	*Lycopersicon escutentum*	-	-	-	-	-	-	-	weak +	-	-	-
701	*Xanthomonas campestris pv vestcatoria*	Zimbabwe	*L. esculentum*	-	-	-	-	-	-	-	weak +	-	-	-
1131	*Xanthomonas* sp.	Eastern Samoa	*Musa paradisiaca*	-	-	-	-	-	-	-	weak +	-	-	-
1132	*Xanthomonas* sp.	Western Samoa	*Musa canksii var. samoensis*	-	-	-	-	-	-	-	weak +	-	-	-
2198	*Ralstonia solanacearum*	Trinidad	*Musa* sp.	-	-	-	-	-	-	-	NS	-	-	-
2315	*Ralstonia solanacearum*	Peru	*Musa* sp.	-	-	-	-	-	-	-	-	-	-	-
3205	*Ralstonia solanacearum*	Guyana	*Musa sp. (plantain)*	-	-	-	-	-	-	-	-	-	-	-
3214	*Ralstonia solanacearum*	Colombia	*Plantago sp.*	-	-	-	-	-	-	-	-	-	-	-
4168	*Enterobacter* sp.	Unknown	Unknown	-	-	-	-	-	-	-	NS	-	-	-
2641	*Serratia marcescens* biotype A4a	USA	*Medicago sativa*	-	-	-	-	-	-	-	-	-	-	-
1232	*Pseudomonas marginalis pv marginalis*	Uganda	*Musa* sp.	-	-	-	-	-	-	-	-	-	-	-
n/a	*Melissococcus plutonius*	UK	n/a	-	-	-	-	-	-	-	-	-	-	-
n/a	Healthy *Musa* var. Tropicana	n/a	*Musa* sp.	-	-	-	-	-	-	-	NS	-	-	-
n/a	*Xcm* infected Musa (field sample)	Uganda	*Musa* sp.	+	+	+	+	+	+	+	+	+	+	+

Table 2. Details of the PCR assays evaluated in the study.

Primer name	**Sequence (5' - 3')**	**Target**	**Amplicon size (bp)**	**Reference**
BXW-1	GTCGTTGGCACCATGCTCA	*hrpB* operon	214	Lewis Ivey et al., 2010
BXW-3	TCCGACCGATACGGCT			

GspDm-F2	GCGGTTACAACACCGTTCAAT	GspD gene	265	Adriko et al., 2012
GspDm-R3	AGGTGGAGTTGATCGGAATG			
Xcm 12-F	GCCGGCGTGCGCAACTATCTG	ATP binding hypothetical protein	360	Adikini et al., 2011
Xcm 12-R	GCCATCCGCAAACAATCGCAACCT			
Xcm35-F	GAGCGCGAGGAAACGGGGAAGT	Non-coding region	480	
Xcm35-R	TTGTGTTCGCCCAACCCTCTCAGT			
Xcm36-F	GCTTCGGCGGAGGCGTGCTAAT	Hypothetical protein	420	
Xcm36-R	TCGGCCGGGCGAGAACTTGAA			
Xcm38-F	CCGCCGGTCGCAATGTGGGTAAT	Polymorphic membrane protein	650	
Xcm38-R	CAGCGGCGCCGGTGTATTGAGTG			
Xcm44-F	AATAGCCCGGGTGATTGTCC	Hypothetical protein	350	
Xcm44-R	AGCCGGCAGCTACGATGAG			
Xcm47-F	GCTGCGTAATGGGCGAGATGATGC	Putative membrane protein	370	
Xcm47-R	GCTGCCGCCGGTTTGGTTTGT			
Xcm48-F	CCCGCGATCACTTCCAACAAACAC	Non-coding region	450	
Xcm48-R	GCTCAATCGCCGGAGGGAGAATC