



Comparison Between Serological And Molecular Detection of Citrus Canker Pathogen (*Xanthomonas axonopodis* pv. *citri*)

Pongpan Songwattana¹, Mariena Ketudat-Cairns^{1,2}

1. School of Biotechnology, Institute of Agricultural Technology, 111 University Avenue, Nakhon Ratchasima, 30000, Thailand

2. Embryo and Stem cell Research Center, Suranaree University of Technology (SUT), 111 University Avenue, Nakhon Ratchasima, 30000, Thailand

✉ Corresponding author; ketudat@sut.ac.th; ✉ Authors

Molecular Pathogens 2011, Vol 2 No 3 DOI: 10.5376/mp.2011.02.0003

Received: 25 May, 2011

Accepted: 21 Jun., 2011

Published: 01 Jul., 2011

This is an open access article published under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Preferred citation for this article as:

Songwattana and Ketudat-Cairns, 2011, Comparison between serological and molecular detection of citrus canker pathogen (*Xanthomonas axonopodis* pv. *citri*), Molecular Pathogens, Vol.2 No.3 (doi: 10.5376/mp.2011.02.0003)

Abstract The specificity and sensitivity of serological and molecular tools for the detection of citrus canker pathogen (*Xanthomonas axonopodis* pv. *citri*; *Xac*) were investigated and compared. Virulence *Xac* BP210 was used as antigen for antisera production. The sensitivity of 1:2,000 diluted antisera were at 10⁶ CFU/mL for live cell and 10⁵ CFU/mL for dead cells. The cross-reaction of the antisera was observed only with *X. campestris* pv. *vesicatoria* but not other *Xanthomonas* nor other unrelated bacteria tested. Molecular tool was performed using specific primer to the *rpf* gene on *Xac*. The PCR amplification indicated that, all *Xac* isolates amplified a product of 581 bp which was not seen in other *Xanthomonas* sp. and unrelated bacteria tested. The sensitivity of these specific primers was down to at least 1 cell which was effective to detect the pathogen in both infected symptomatic and asymptomatic lime tissues. The serological tool was able to detect the pathogen only on infected leaves of day 4 post inoculation when the symptoms were already detected by eye. The serological tool can be used to detect and quantify the present of *Xac* to study the disease development on symptomatic tissues.

Keywords Serological tool; Citrus canker; Antiserum; Pathogen detection

Background

Citrus canker is one of the serious diseases in citrus and some citrus relatives. This disease has been caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*) bacteria or *Xac* pathotype A (*Xac*-A) which is the most wide spread pathogen in Asia and other citrus growing areas (Verniere et al., 1998). Most commercial citrus including Mexican lime, grapefruits and commercial lime in Thailand (Pan lime) are susceptible host for *Xac*. This pathogen can infect leaves, twigs and fruits through stomata and wounds (Graham et. al., 2004). Moreover, this disease directly reduce fruit quality and quantity of yield by defoliation, blemished fruit, premature fruit drop, die-back of twigs and general debilitation of the tree (Das, 2003). Normally, the pathogenic bacteria can survive from one crop season to the next as latent infection in propagation organs or epiphytic populations on plant surfaces. The equipments for harvesting are also the cause of disease spread (Graham et al., 2000). Therefore, the method

for their early detection is necessary. Currently, many methods have been developed and are available in laboratories. Polymerase chain reaction (PCR) method is a high sensitive detection method (Cubero et al., 2001) but, it requires laboratory equipments and specialized training. In contrast, immunodiagnostic test kit is an easy method to use at the site where disease is suspected. It does not require neither special equipment nor training to perform the detection. The immunodiagnostic test is based on the ability of an antibody to recognize and bind to a specific antigen, a substance associated with the plant pathogen. This method is known as enzyme-linked immunosorbent assay (ELISA). In this study the specificity and sensitivity of serological and molecular tools for the detection of citrus canker pathogen (*X. axonopodis* pv. *citri*) were investigated and compared.

1 Results and discussions

1.1 Bacteria isolation and pathogenicity test

Xanthomonas axonopodis pv. *citri* (*Xac*) was isolated

from lesion lime leaves. Total of nineteen bacterial isolates were isolated from three different regions in Thailand; Banpaew, Samut Sakhon, Nakhon Ratchasima and Phichit. All bacterial isolates were initially selected on KCD semi-selective medium. The Gram stain indicated that, all isolates were Gram-negative rod bacteria (data not shown). The bacterial isolates were also confirmed the strain by *Xac* specific primers (Coletla-Filho, 2006). The PCR results indicated that, 12 (included 10 isolated from Samut Sakhon (BP102, BP104, BP105, BP107, BP109, BP201, BP202, BP203, BP205 and BP210) and 2 isolates from Phichit (PJ01 and PJ03) contained the 581 bp *Xac* regulation

of pathogenicity factors (*rpf*) gene fragments which encoded pathogenicity effector protein (Coletla-Filho, 2006). But this gene was not detected in samples from Nakhon Ratchasima (K01, K02, SUT02 and SUT06) and unrelated bacteria such as *E. coli*, *Sinorhizobium* and *Agrobacterium* that were used as negative control (Figure 1). These primers were also not able to produce specific PCR product from *X. axonopodis* pv. *vesicatoria*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *glycine*, *X. oryzae* pv. *oryzae* template (Figure 2). Non-target DNA bands were detected in isolated from Nakhon Ratchasima and some from Phichit (Figure 1).

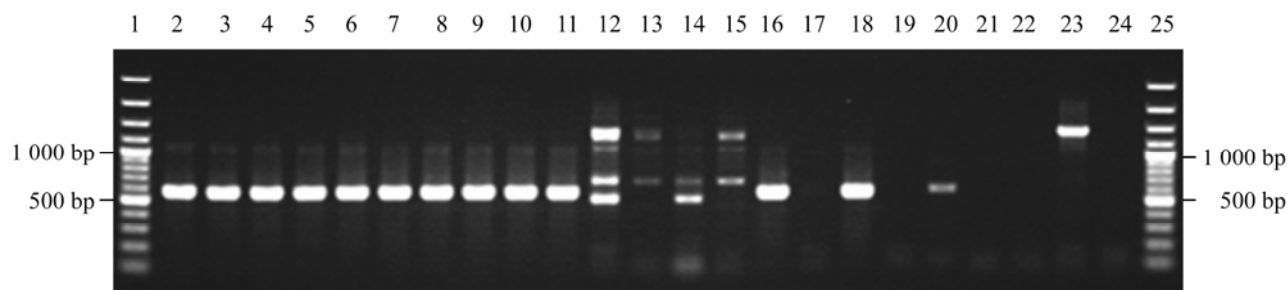


Figure 1 Specific amplification of *Xac* target (581 bp fragment) using XAC01 and XAC02 specific primers
 Note: Lanes 1 and 25: 100 bp DNA ladder (NEB); Lanes 2~11: Bacterial isolated from Samut Sakhon (BP); Lanes 12~15: Nakhon Ratchasima (SUT02, 06 and K01, 02); Lanes 16~20: Phichit (PJ01-05); Lanes 21~23: Unrelated bacteria consist of *E. coli*, *Sinorhizobium* and *Agrobacterium*, respectively; Lane 24: Water control

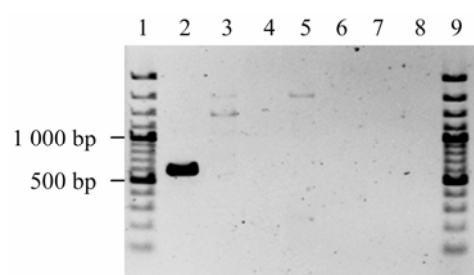


Figure 2 Specific amplification of *Xac* target by PCR. Lane 1 and 9: 100 bp marker (NEB); Lane 2: *X. axonopodis* pv. *citri* (BP210); Lane 3: *X. axonopodis* pv. *vesicatoria*; Lane 4: *X. axonopodis* pv. *phaseoli*; Lane 5: *X. campestris* pv. *campestris*; Lane 6: *X. axonopodis* pv. *glycine*; Lane 7: *X. oryzae* pv. *oryzae*, respectively; Lane 8: negative control.

The bacterial isolates were inoculated to both susceptible (Pan) and resistance limes (Nam Hom and M33) for pathogenicity test. Ten bacterial isolates from Samut Sakhon and 2 isolates from Phichit could infect both susceptible and resistance lime tested.

These 12 pathogenic bacteria should be *Xanthomonas* species since the *Xac* specific genes product were observed and they can infect limes. The lesion caused by isolated BP104 and BP210 on both susceptible (Pan) and resistance limes (Nam Hom and M33) slowly turned brown after 3 week post-infection. The BP104 and BP210 isolates seem to be virulence pathogenic bacteria. These two virulence pathogenic bacteria were identified by 16S rRNA gene sequencing. The accession number HQ875739 and HQ875740 were obtained. The 16S rRNA gene sequences confirmed that both strains were *X. axonopodis* pv. *citri* (*Xac*). In this experiment, *Xac* BP210 isolate was used as bacterial antigen for further antibody production.

1.2 Serological studies

1.2.1 Determination of antiserum titer

Antisera were raised from dead cells of *Xac* BP210 in two New Zealand white rabbits. The titer of *Xac*

Molecular Pathogens

MP2011, Vol.2, No.3 <http://mp.sophiapublisher.com>



BP210 antisera were determined by indirect ELISA. Serial dilutions of the antisera up to 1:16,000 were reacted with dead and live cells of *Xac* BP210 at 10^4 ~ 10^6 CFU/mL. The suspension of *E.coli* at 10^5 CFU/mL and pre-immune sera were used as negative control. The results showed that, 1:4,000 or lower dilutions of both antisera were able to detected dead cells of *Xac* at 10^5 CFU/mL or more. Whereas, 1:2,000 diluted antiserum were reactive against live cells of 10^6 CFU/mL and dead cells at 10^5 CFU/mL or more. The highly concentrate antiserum (1:1,000

dilution) strongly reacted against both live and dead cells (10^5 CFU/mL) of *Xac*. However, antiserum 1 showed stronger reaction than antiserum 2 (Table 1). These results indicated that, at least 1:2,000 dilution of these antisera were able to detected dead cell of *Xac* at 10^5 CFU/mL and live cells at 10^6 CFU/mL. Nevertheless, dead cells of bacteria showed stronger reaction with antisera than live cells. Since these antisera were produced from injection with heat killed cells which might have broken some cells.

Table 1 ELISA for antiserum 1 and antiserum 2 titer test

Antiserum dilution	Cell status	<i>Xac</i> (CFU/mL)	Antiserum 1		Antiserum 2	
			A ₄₀₅	Reaction	A ₄₀₅	Reaction
1:1,000	Live	10^6	0.25	+ ^a	0.20	+
		10^5	0.20	+	0.18	+
		10^4	0.01	- ^b	0.12	-
	Dead	10^6	0.74	+	0.69	+
		10^5	0.24	+	0.22	+
		10^4	0.12	-	0.14	-
1:2,000	Live	10^6	0.20	+	0.18	+
		10^5	0.15	-	0.12	-
		10^4	0.10	-	0.11	-
	Dead	10^6	0.75	+	0.64	+
		10^5	0.21	+	0.18	+
		10^4	0.11	-	0.12	-
1:4,000	Live	10^6	0.16	-	0.12	-
		10^5	0.12	-	0.11	-
		10^4	0.09	-	0.11	-
	Dead	10^6	0.67	+	0.61	+
		10^5	0.19	+	0.16	-
		10^4	0.11	-	0.11	-
1:8,000	Live	10^6	0.12	-	0.10	-
		10^5	0.11	-	0.10	-
		10^4	0.09	-	0.10	-
	Dead	10^6	0.53	+	0.51	+
		10^5	0.17	-	0.14	-
		10^4	0.10	-	0.10	-
1:16,000	Live	10^6	0.10	-	0.09	-
		10^5	0.10	-	0.09	-
		10^4	0.09	-	0.09	-
	Dead	10^6	0.43	+	0.43	+
		10^5	0.16	-	0.13	-
		10^4	0.11	-	0.09	-

Note: Different antisera dilutions were used to detect live and dead cells of different concentrations of *Xac* suspension; Pre-immune serum reaction and *E. coli* at 10^5 CFU/mL was used as negative control; 100 μ L of the cells were used in each well; ^a: The positive result (+) was indicated when A₄₀₅ was greater than twice of the negative control; ^b: The negative reaction (-) was indicated when A₄₀₅ was lower than twice of the negative control



1.2.2 Sensitivity of *X. axonopodis* pv. *citri* detection

Antiserum 1 and antiserum 2 at the dilution of 1:2,000 were tested for the sensitivity with live and dead cells of BP210 at different concentrations (10^3 ~ 10^8 CFU/mL). The positive result is the number which is at least double the reaction intensity of negative control (10^6 CFU/mL of *E. coli* and pre-immune serum reaction). The bacterial suspension of live cells at 10^6 CFU/mL and dead cells at 10^5 CFU/mL showed positive reaction in ELISA (Table 2). Stronger reaction can be observed with the increase of bacterial densities in both antisera tested. Both antisera have the sensitivity of detection at 10^6 CFU/mL for live cell

and 10^5 CFU/mL for dead cells. The sensitivity of ELISA for *X. axonopodis* pv. *citri* (Jin et al. 2001), *Clavibacter michiganensis* subsp. *michiganensis* (de Leon et al., 2008), *C. michiganensis* subsp. *Sepedonicus* and *Erwinia amylovora* (Kokoskova and Mraz, 2008) have also been reported to be at the level of 10^5 ~ 10^6 CFU/mL. The detection efficiency of ELISA is limited by the level of the pathogen population and dependent upon the immunological properties of the antiserum used. The antisera produced in this study have the sensitivity as high as those produced in other reports.

Table 2 Sensitivity of ELISA in detecting live and dead cells of *Xac* BP210

Antiserum	Cell status	<i>Xac</i> BP210 antigen (CFU/mL)						
		10^8	10^7	10^6	10^5	10^4	10^3	<i>E. coli</i> 10^6
Antiserum 1 (1:2,000)	Live	1.30	0.69	0.23	0.15	0.10	0.10	0.09
	Dead	1.99	1.63	0.74	0.21	0.12	0.10	0.09
Antiserum 2 (1:2,000)	Live	0.54	0.47	0.18	0.12	0.10	0.10	0.09
	Dead	0.80	0.76	0.63	0.18	0.10	0.10	0.09

Note: The A_{405} is shown; 100 μ L of the cells were used in each well

1.2.3 Cross-reaction of antiserum to other *Xanthomonas* sp.

Antisera dilutions (1:1,000 and 1:2,000) were tested for the cross-reactivity against live cells of other five *Xanthomonas* sp. and *E. coli* at 10^6 CFU/mL. The results in Table 3 showed that, dilution of 1:1,000 antisera strongly cross-react against *X. axonopodis* pv.

vesicatoria and weakly reacted against *X. axonopodis* pv. *phaseoli* and *X. campestris* pv. *campestris*. While, more diluted antisera (1:2,000) cross-reacted with only *X. axonopodis* pv. *vesicatoria* but not with others *Xanthomonas* (Table 3). These results suggested that, antisera diluted to 1:2,000 are the suitable condition for detection of lived target bacteria of at least

Table 3 Sensitivity of ELISA in detecting live and dead cells of *Xac* BP210

Bacterial strain tested (10^6 CFU/mL)	Diluted antiserum			
	Antiserum 1		Antiserum 2	
	(1:1,000) (%)	(1:2,000) (%)	(1:1,000) (%)	(1:2,000) (%)
<i>X. axonopodis</i> pv. <i>citri</i> (BP210) (Positive control)	100 ^a	100	100	100
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	100	91	80	85
<i>X. axonopodis</i> pv. <i>phaseoli</i>	33	0	39	0
<i>X. campestris</i> pv. <i>campestris</i>	47	0	27	0
<i>X. axonopodis</i> pv. <i>glycine</i>	0	0	0	0
<i>X. oryzae</i> pv. <i>oryzae</i>	0	0	0	0
<i>E. coli</i> (Negative control)	0	0	0	0

Note: a: $\{[A_{405}$ of each *Xanthomonas* species - A_{405} of negative control] / [A_{405} of positive control - A_{405} negative control]} \times 100



10^6 CFU/mL or 10^5 cell/well. Although, these antisera showed cross-reactivity with *X. axonopodis* pv. *vesicatoria* but this bacterium does not infect citrus plant. They only infect tomato and *Capsicum* pathogen (Mirik and Aysan, 2009). Therefore, the cross-reaction detection should not be of alarm. The antigenic molecules from the normal cell surface components are often including in polyclonal antibodies (or antiserum). This mixing of antibodies has multiple specificities and cross-reactivities with unrelated bacteria species. This study attempt to diluted multiple specific antibodies and retain dominant antibodies which showed acceptable specificity for a given pathogen.

Many studies reported that, the ELISA sensitivity 10^5 ~ 10^6 CFU/mL is sufficient for identification of bacteria pathogens from symptomatic plants and colonies on selective media (Jin et al., 2001; Alvarez, 2004; de Leon et al., 2008; Kokoskova and Mraz, 2008). However, boiling the bacteria samples could improve the sensitivity of detection. Several previous reports have mention that using extraction buffer containing EDTA and lysozyme to removed cell surface component could improve the sensitivity of ELISA detection (Jones et al., 1997, Alvarez, 2004). However, EDTA was not used in this study but the sensitivity of detection was still high.

1.2.4 Detach leaf assay

Different concentrations of canker bacteria (10^7 , 10^6 and 10^5 CFU/mL) were inoculated to Pan lime leaves using detach leaf assay. The pathogen on the infected leaves was detected by ELISA method every 2 days post inoculation. The results showed that, infected leaves on the first day of inoculation (day zero) showed negative reactions in ELISA test whether the bacterial cells were killed by heat treatment or not (Table 4). The inoculation of this experiment were 20 μ L of bacterial suspensions dropped on each area. Thus, bacteria populations on the leaves were 10^5 , 10^4 and 10^3 cells on the inoculated leaves area. The sensitivity of the ELISA (10^5 CFU/mL or 10^4 cells/well for dead cells) should be sufficient for detection of dead cells. However, protein or other polysaccharide molecules from the leaf may interfered with the bacteria binding to the plate therefore, low detection of target bacteria

were observed.

Infected leaves on day two showed positive reaction when the leaves were inoculated with 10^5 cells. Consequently, infected leaves 4 days post inoculation showed positive reaction with all bacteria concentration (10^5 , 10^4 and 10^3 cells) whether the cells were boiled or not. The boiled bacteria (dead cell) from infected leaves showed higher reaction in ELISA test than lived cells. These results were in accordance with the results of antibody titer and sensitivity test (Table 1 and Table 2). After day 4 post inoculation, symptoms on the infected areas were observed as slightly raised blister-like lesions. Thus, these results indicated that, ELISA assay is a well-established method for identification of bacterial pathogens from symptomatic plants. However, enrichment techniques can enhance ability of pathogen detection (Jin et al., 2001) on natural samples but more time is needed for the culturing period.

1.2.5 Molecular detection of *Xac* from canker lesions

Canker pathogen on symptom and non-symptom plant materials (leaf and twig) in infected areas were detected by PCR amplification using *Xac* specific primers. The results showed that, the *Xac* specific PCR products were detect in all samples of symptom plant materials and some non-symptom plant materials (Table 5).

These specific primers were also used to detect canker pathogen on infected leaves from the detach leaf experiment. The results showed that, canker pathogen were detected by these specific primers on the first day of inoculation while ELISA was unable (Table 4). The amplification assay using specific primers have been reported to detect at low pathogen population (10^2 ~ 10^3 CFU/mL) (Cubero et al., 2001, Cubero and Graham, 2002, Coletla-Filho 2006, Park et al., 2006, Golmohammadi et al., 2007, de Leon et al., 2008). The sensitivity of PCR amplification of this study showed very high sensitivity at 10^3 CFU/mL or 1 cell in the PCR reaction (Figure 3). Moreover, this methodology showed no cross-reactivity with other *Xanthomonas* species (Figure 2). However, PCR method required laboratory equipment, special reagents and skill to perform.



Table 4 ELISA detection of bacteria on infected leaf using detached leaf assay

Day after inoculation	Initial bacteria density (CFU)	Replication						PCR detection (XAC specific primers)
		Living cell			Dead cell			
		1	2	3	1	2	3	
0	10 ⁵	-	-	- ^a	-	-	-	+
	10 ⁴	-	-	-	-	-	-	+
	10 ³	-	-	-	-	-	-	+
2	10 ⁵	+	+	+	+	+	+	+
	10 ⁴	-	-	-	-	-	-	+
	10 ³	-	-	-	-	-	-	+
4	10 ⁵	+	+	+	+	+	+	+
	10 ⁴	-	+	+	+	+	+	+
	10 ³	-	+	+	+	+	+	+
6	10 ⁵	+	+	+	+	+	+	+
	10 ⁴	+	+	+	+	+	+	+
	10 ³	+	+	+	+	+	+	+
8	10 ⁵	+	+	+	+	+	+	+
	10 ⁴	+	+	+	+	+	+	+
	10 ³	+	+	+	+	+	+	+
10	10 ⁵	+	+	+	+	+	+	+
	10 ⁴	+	+	+	+	+	+	+
	10 ³	+	+	+	+	+	+	+

a: The positive result (+) was indicated when A₄₀₅ was greater than twice of the negative control (-)

Table 5 *Xac* specific amplification of canker pathogen from symptom and non-symptom plant materials

Day after inoculation	No. of plants in plant status		PCR detection(%) ^a	
	Symptom	Non-symptom	Symptom	Non-symptom
Plant materials	10	10	100%	40%
Leaf	6	6	100%	33%

Note: a: [number of sample that can be detect by *Xac* primers/total samples tested] × 100

2 Conclusion

The sensitivity and specificity of serological and molecular tools have been investigated. The molecular tool is more effective than serological tool due to the high sensitivity (at least 1 cell) and specificity. Therefore, molecular detection able to detect the present of pathogen in infected leave even before the appearance of symptom. However, serological test from this study was not inferior to other reports and

sufficient for pathogen detection from symptomatic tissue.

3 Materials and Methods

3.1 Isolation of *Xanthomonas axonopodis* pv. *citri* (*Xac*)

Infected lime leaves from three different regions in Thailand; Samut Sakhon, Nakhon Ratchasima and Phichit which are about 300~500 km apart were used in this study. Different *Xac* isolates were isolated from

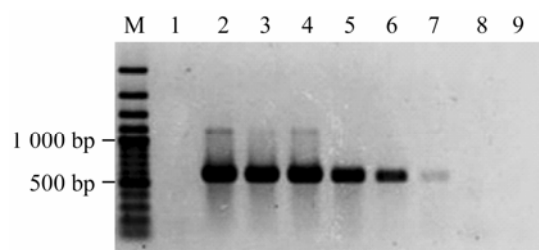


Figure 3 *Xac* specific PCR amplification from a tenfold dilution series of *X. axonopodis* pv. *citri* cultured cells. M: 100 bp marker (NEB); Lane 1: negative control; Lanes 2–7: 10^5 cells, 10^4 cells, 10^3 cells, 10^2 cells, 10^1 cells and 1 cell; lanes 8–9: <1 cell respectively.

infected lime leaves tissue by commonly used methods (OEPP/EPPO, 2005). Briefly, the infected leaves were washed with sterile water and surface sterilize by soaked in 1% sodium hypochlorite for 3 min. Then, the lesions were rinsed in sterile water several times and excised with scalpel. *Xac* isolates were initially selected by streaked the water-soak tissue from the lesion margins on sterile semi-selective media (KCD medium, nutrient agar (NA) supplemented with Kasugamycin (16 $\mu\text{g}/\text{mL}$), Cephalexin (16 $\mu\text{g}/\text{mL}$) and Daconil (Chlorothalonil) (12 $\mu\text{g}/\text{mL}$) prior to enrichment on NA media without antibiotics. The bacteria were grown at 28°C for 24–48 h. The bacterial colonies were collected for Gram staining and further used for pathogenicity tests.

Other *Xanthomonas* strains include *X. axonopodis* pv. *vesicatoria*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *glycine*, *X. oryzae* pv. *Oryzae* obtained from Chulabhorn Research Institute (CRI, Thailand) were used as negative control in strain identification and cross-reaction tests.

3.2 Specific primers identification and 16S rRNA gene sequencing

Single colony of *Xac* isolates and other *Xanthomonas* strains were resuspended in 1 mL DI-water and boiled for 10 min. Then, the suspensions were used as template for PCR reaction, with specific XAC01 and XAC02 primers for *Xac* identification (Coletta-Filho, 2006). The PCR reactions were performed with 50 μL reaction mixture, containing 1 μL of boiled cell suspension, 1X reaction buffer (Promega GoTaq), 1.5 mmol/L MgCl_2 , 0.2 mmol/L dNTPs, 0.2 $\mu\text{mol}/\text{L}$

primers (XAC01: 5'CGC CAT CCC CAC CAC CAC CAC GAC'3, XAC02: 5'AAC CGC TCA ATG CCA TCC ACT TCA'3), 1.25U *Taq* DNA polymerase (Promega). Unrelated bacterial DNA included *Escherichia coli*, *Sinorhizobium* and *Agrobacterium* were also subjected to PCR reaction as negative control. PCR conditions were as followed; An initial cycle of 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, with a final step of 72°C for 5 min. The PCR products were observed under UV light after electrophoresis through 1.0% (w/v) agarose gels and stained with ethidium bromide.

The full length 16S rRNA gene of *Xac* isolates BP104 and BP210 were sequenced by Macrogen (Korea) (<http://dna.macrogen.com/eng/>). The sequence results were applied to BLAST program for gene comparison with data in the GenBank (NCBI).

3.3 Pathogenicity test

3.3.1 Plant samples

M33 hybrid limes (highly resistant to canker) were from Phichit horticulture research center, Samut Sakorn and Prachinburi (Thailand), Nam Hom limes (highly resistant to canker) were from Phichit horticulture research center and Pan limes (susceptible to canker) were from Samut Sakorn. These lime plants were planted in the test field at Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.3.2 *Xac* Inoculation on resistance and susceptible limes

Half of the M33, Nam Hom and Pan limes leaves were wounded with needles and celite, the other halves were not wounded (healthy). 1 mL of the bacterial isolate suspension (10^8 CFU/mL) was sprayed on the leaves. The plants were then covered with plastic bags overnight. In the morning, the bags were removed and the plants were left in natural condition for 2–3 weeks. The virulent canker disease apparent on each leaf was evaluated.

3.4 Serological studies

3.4.1 Preparation of antigen

Antigen was prepared from the cells of *Xac* isolate BP210. The bacterial cells were enriched on NB



(nutrient broth) at 28°C for 48 h. The bacterial cells were collected by low-speed centrifugation (4,200 xg) and washed once in sterile 0.85% NaCl. The bacterial suspension was adjusted spectrophotometrically to A_{600} 0.2 (about 10^8 CFU/mL) and heated to 85°C for 15 min. The killed cell suspension was used as antigen for antiserum production.

3.4.2 Antiserum production

Antiserum against *Xac* (BP210) was prepared using two New Zealand white female rabbits. 1 mL of antigen mixed with Freund's complete adjuvant (1:1 v/v) was immunized to each rabbit by subcutaneous injection on the first day. Then, 1 mL of antigen (mixed with Freund's incomplete adjuvant (1:1 v/v)) was injected subcutaneously 7 day later. After that, 1 mL of bacterial antigen without adjuvant was injected intravenous on day 14 and day 21. One week after the last injection, the blood was drawn from the central ear artery then kept in refrigerator overnight. The antiserum was clarified by centrifugation at 3,000 xg for 15 min at 4°C and stored at -80°C for future use. This antiserum was used for indirect ELISA assay for the antibody titer, the sensitivity and the cross reaction of the antiserum. The pre-immune serum was bleed before immunization to use as negative control in the ELISA assay. Absorbance values of at least double of the control were considered positive (OEPP/EPPO, 2005).

3.4.3 Indirect ELISA assay

The indirect enzyme-linked immunosorbent assay (ELISA) was carried out as described by Alvarez et al. (1991). The bacterial concentrations were adjusted from 10^3 ~ 10^8 CFU/mL in 0.05 mol/L carbonate coating buffer (0.015 mol/L Na_2CO_3 , 0.035 mol/L NaHCO_3 , 0.003 mol/L NaN_3 ; pH 9.6). Half volume of each diluted bacterial suspension were treated at 85°C for 15 min to kill the cells and used as dead cells antigen. 100 μL of each diluted bacterial suspensions (both live and dead cells antigen) were coated in microtiter plate well at 4°C overnight. The plate was washed three times with phosphate buffer saline+0.05% Tween20 (PBST) and 200 μL of 2% skim milk were added and incubated for 1 h at 37°C. Then, the plates were washed as mentioned earlier and 100 μL of diluted antiserum were added to the wells. The ELISA plates were incubated at 37°C for 1 h then,

three times washing were performed. Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (whole molecule, enzymatic activity 1 mg/mL) obtained from Sigma Chem. Co St Louis, Mo (Production#F0382) was diluted to 1:10,000 and 100 μL was added in each well, followed by 1 hour incubation at 37°C. The wells were repeatedly washed and 100 μL of enzyme substrate (1 mg/mL p-Nitrophenyl-phosphate (PNPP) in 10 mmol/L Diethanolamine; Thermo Scientific#34047) were added in each well. The reaction were stopped by adding equal volume of 0.75 mol/L NaOH and the O.D. at 405 nm were measured after 15~30 min incubation in the dark at room temperature. The live and dead cells of *E. coli* (10^6 CFU/mL) and pre-immune serum were used as negative control.

3.4.4 Antibody titer and sensitivity tests

The antiserum was diluted to different dilution (1:1,000, 1:2,000, 1:4,000, 1:8,000 and 1:1,600 v/v) in conjugate buffer (2% polyvinyl pyrrolidone (PVP)+2% oval albumin in PBST) and tested with both live and dead cells of bacterial antigen (10^4 ~ 10^8 CFU/mL) by indirect ELISA assay to test the titer of the antiserum. For sensitivity testing, the 1:2,000 (v/v) diluted antiserum was used to test both live and dead cells of bacterial antigen (10^3 ~ 10^8 CFU/mL *Xac* BP210) by indirect ELISA assay as described above. All experiments were done in duplicated and repeated at least 3 different times.

3.4.5 Cross-reaction test

The antiserum was diluted to 1:2,000 (v/v) and used to test the cross reactivity with other *Xanthomonas* bacteria at the concentration of 10^6 CFU/mL by indirect ELISA assay as described above. The percent of cross-reaction was calculated by; $\{[A_{405}$ of each *Xanthomonas* species- A_{405} of negative control] / $[A_{405}$ of positive control- A_{405} negative control] $\} \times 100$.

3.5 Detached leaf assay

3.5.1 *Xac* inoculation

Full expanded young leaves of lime were washed in running tap water and surface sterilized in 1% sodium hypochlorite for 1~4 min. The leaves were aseptically rinsed thoroughly with sterile distilled water. Each leaf was divided into 4 parts for treatment separation,



included 10^5 , 10^6 and 10^7 CFU/mL infected parts and one uninfected part. Each part of the leaf was wounded by puncturing with a small needle, through the lower surface (5 needle punctured wounds per part). Each wounded leaf was placed on 1% water agar in a Petri dish, with the back of the leaf up. 20 μ L of the different diluted bacterial suspension (10^5 ~ 10^7 CFU/mL *Xac* BP210) were dropped on each part of the wounded leaf. The part of uninfected leaf, 20 μ L of sterile 0.85% NaCl was dropped to use as negative control. The infected leaves were maintained in an incubator at 28°C.

3.5.2 Serological detection of *Xac* in inoculated lime tissues

The canker pathogen on each part of the infected leaf was detected by ELISA method. The leaf was aseptically cut and each part of the infected leaf was ground in 150 μ L coating buffer. 100 μ L of leaf extract was coated in one microtiter plate well and incubated at 4°C overnight before detection with 1:2000 (v/v) diluted antiserum as mention above.

3.6 Molecular studies using specific primers

The sensitivity of PCR amplification was performed with *Xac* isolate BP210 in tenfold dilution series (10^8 ~ 10^1 CFU/mL in 0.85% NaCl). 1 μ L of each dilution was used as template in the PCR reaction. The detection of *Xac* in inoculated lime tissue was also performed in *Xac* specific PCR amplification as described above.

3.7 Canker detection from field samples

Symptom and non-symptom plant materials (leaf and twig) from Pan, Nam Hom and M33 limes were taken from the field and washed in 25 mL 0.85% NaCl and shaken for 30 min. Then, the plant material was removed, and the washing solution was boiled for 10 min. 1 μ L of the boiled washing solution were used as template for specific primers (XAC01 and XAC02) amplification. The PCR condition was performed as described above. The *Xac* BP210 isolate was used as positive control. The detection results of inoculated tissue were demonstrated as percentage (% detection), calculated by; [number of sample that can be detect by *Xac* primers/total samples tested] \times 100.

Author contributions

PS carried out all experiments as her M.Sc. Thesis work. She also drafted the manuscript. MKC was the PI of the lab. She conceived the study, participated in the experimental design, coordinated, drafted and edited the manuscript. Both authors have read and proved the final manuscript.

Acknowledgments

S. Loprasert (CRI) is thanks for the *Xanthomonas* reference strains. PS was supported by SUT scholarship. This research was funded by SUT Research and Development Fund.

References

- Alvarez A.M., 2004, Integrated approaches for detection of plant pathogen bacteria and diagnosis of bacterial diseases, *Annu. Rev. Phytopathol.*, 42: 339-366 doi:10.1146/annurev.phyto.42.040803.140329 PMID:15283670
- Alvarez A.M. Benediet A.A., Mizumoto C.Y., Pollard L.W., and Civerolo E.L., 1991, Analysis of *Xanthomonas campestris* pv. *citri* and *X. c. pv. citrumelo* with monoclonal antibodies, *Phytopathol.*, 81: 857-865 doi:10.1094/Phyto-81-857
- Coletta-Filho H.D., Takita M.A., Souza A.A., de Neto J.R., Destefano S.A.L., Hartung J.S. and Machadoet M.A., 2006, Primers based on the *rpf* gene region provide improved detection of *Xanthomonas axonopodis* pv. *citri* in naturally and artificially infected citrus plants, *J. Appl. Microbiol.*, 100(2): 279-285 doi:10.1111/j.1365-2672.2005.02787.x PMID:16430504
- Cubero J., Graham J.H., and Gottwald T.R., 2001, Quantitative PCR method for diagnosis of citrus bacterial canker, *Appl. Environ. Microbiol.*, 67(6): 2849-2852 doi:10.1128/AEM.67.6.2849-2852.2001 PMID:11375206 PMCid:92950
- Cubero J., and Graham J.H., 2002, Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR, *Appl. Environ. Microbiol.*, 68(3): 1257-1264 doi:10.1128/AEM.68.3.1257-1264.2002 PMID:11872476 PMCid:123760
- Das A.K., 2003, Citrus canker - a review, *J. Appl. Hort.*, 5(1): 52-60
- EPPO/EPP0, 2005, Diagnostic: *Xanthomonas axonopodis* pv. *citri.*, *Bull. OEPP/EPP0 Bull.*, 35: 271-273
- Golmohammadi M., Cubero J., Peñalver J., Quesada J.M., Loópez M.M., and Llop P., 2007, Diagnosis of *Xanthomonas axonopodis* pv. *citri*, causal agent of citrus canker, in commercial fruits by isolation and PCR-based methods, *J. Appl. Microbiol.*, 103(6): 2309-2315 doi:10.1111/j.1365-2672.2007.03484.x PMID:18045415
- Graham J.H., Gottwald T.R., Cubero J. and Achor D.S., 2004, *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker, *Mol. Plant Pathol.*, 5(1): 1-15 doi:10.1046/j.1364-3703.2004.00197.x PMID:20565577
- Graham J.H., Gottwald T.R., Riley T.D., Cubero J. and Drouillard D.L., 2000, Survival of *Xanthomonas campestris* pv. *citri* (*Xcc*) on various surfaces and chemical control of Asiatic Citrus Canker (ACC), *Processing of the International Citrus Canker Research Workshop*, June 20-22, 2000, Ft. Pierce, Florida, pp.7
- Jin K.S., Kang I.B., Ko K.I., Lee E.S., Heo J.Y., Kang Y.K., and Kim B.K., 2001, Detection of *Xanthomonas axonopodis* pv. *citri* on citrus fruits using Enzyme-Linked Immunosorbent assay, *J. Plant Pathol.*, 17(1): 62-66
- Jones J.B., Somodi G.C., and Scott J.W., 1997, Increased ELISA sensitivity using a modified extraction buffer for detection of *Xanthomonas campestris* pv. *vesicatoria* in leaf tissue, *J. Appl. Microbiol.*, 83(4): 397-401 doi:10.1046/j.1365-2672.1997.00251.x PMID:9351221
- Kokoskova B., and Mraz I., 2008, Reliability of diagnostic techniques for



Erwinia amylovora and *Clavibacter michiganensis* subsp. *Sepedonicus*,
Zemdirbyste-Agr., 95(3): 270-276

de Leon L., Rodriguez A., Lopez M.M. and Siverio F., 2008, Evaluation of the efficacy of immunomagnetic separation for the detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds, J. Appl. Microbiol., 104(3): 776-786 doi:10.1111/j.1365-2672.2007.03595.x PMID:17927739

Mirik M. and Aysan Y., 2009, Detection of *Xanthomonas axonopodis* pv. *vesicatoria* in naturally infected pepper seed in Turkey, J. Plant Pathol., 91(2): 433-436

Park D.S., Hyun J.W., Park Y.J., Kim J.S., Kang H.W., Hahn J.H. and Go S.J., 2006, Sensitive and specific detection of *Xanthomonas axonopodis* pv. *citri* by PCR using pathovar specific primers based on *hrpW* gene sequences, Microbiol. Research, 161(2): 145-149 doi:10.1016/j.micres.2005.07.005 PMID:16427518

Verniere C., Hartung J.S., Pruvost O.P., Civerolo E.L., Alvarez A.M., Maestri P. and Luisetti J., 1998, Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia, Eur. J. Plant Pathol., 104(5): 477-487 doi:10.1023/A:1008676508688



Reasons to publish in BioPublisher

A BioScience Publishing Platform

- ★ Peer review quickly and professionally
- ☆ Publish online immediately upon acceptance
- ★ Deposit permanently and track easily
- ☆ Access free and open around the world
- ★ Disseminate multilingual available

Submit your manuscript at: <http://bio.sophiapublisher.com>