Detection of *Xanthomonas oryzae* pv. *oryzae* in Seeds Using a Specific TaqMan Probe

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Abstract

Xanthomonas oryzae pv. *oryzae* is the pathogen that causes bacterial leaf blight in rice. Bacterial leaf blight is the main cause for severe rice underproduction in many countries. However, with conventional methods it is difficult to quickly and reliably distinguish this pathogen from other closely related pathogenic bacteria, especially *X. oryzae* pv. *oryzicola,* the causal organism of bacterial leaf streak in rice. We have developed a novel and highly sensitive real-time method for the identification of this specific bacteria based on a TaqMan probe. This probe is designed to recognize the sequence of a putative siderophore receptor gene cds specific to *X. oryzae* pv. *oryzae*, and can be identified from either a bacterial culture or naturally infected rice seeds and leaves in only 2 h. The sensitivity of the method is 100 times higher than that of the current polymerase chain reaction (PCR) gel electrophoresis method for diagnosis.

Index Entries: *Xanthomonas oryzae* pv. *oryzae;* TaqMan probe; real-time PCR; detection.

1. Introduction

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae,* is a serious disease in rice and a threat to rice production in both temperate and tropic rice-growing regions ***(1)***. The spread of BLB occurs through plant debris, wild rice, and weeds ***(1–3)***. The presence of the pathogen in infected seeds and disease transmission from these infected seeds has been demonstrated ***(1,4)***. *X. oryzae* pv. *oryzae* is considered a quarantine organism and subject to phytosanitary regulations in Europe ***(5)***, China, and many other countries. Conventional methods used for detection of the pathogen include biochemical tests, serological assays, fatty acids, and metabolic profiling. These methods, however, have several limitations including poor sensitivity, nonspecificity, and long waits for results ***(6)***.

The first polymerase chain reaction (PCR)-based assays for detection of plant pathogens in infected plants and seeds were described more than 10 years ago ***(7)***, yet at present few plant disease diagnostic laboratories currently adopt the PCR gel electrophoresis method for routine pathogen identification and disease diagnosis because it is time-consuming and labor intensive ***(8–10)***. Furthermore, the simple presence of a DNA fragment with a particular molecular weight in agarose gel does not prove the identity of the resulting band, and verification of the amplified product must be done by Southern blot hybridization. Also in some cases, PCR product contamination and false-positive results may occur.

The TaqMan probe PCR method is based on the hybridization of a fluorescence oligonucleotide probe with a specific region within the target that is amplified by traditional primers. Several real-

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Table 1
Bacteria Strains Used in Study

Bacteria strains	Origin
X. oryzae pv. *oryzae*	Jiangsu Province
X. oryzae pv. *oryzae*	Zhejiang Province
X. oryzae pv. *oryzae*	Jiangxi Province
X. oryzae pv. *oryzae*	Sichuan Province
X. oryzae pv. *oryzae*	Hubei Province
X. oryzae pv. *oryzae*	Philippines
X. oryzae pv. *oryzae*	Japan
X. oryzae pv. *oryzae*	Indonesia
Xanthomonas oryzae pv. *oryzicola*	CAIQ
Xanthomonas campestris pv. *campestris*	CAIQ
Xanthomonas axonopodis pv. *citri*	CAIQ
Pseudomonas syringae pv. *syringae*	CAIQ
Burkholderia solancearum	CAIQ
Erwinia carotovora pv. *carotovora*	CAIQ
Pantoea stewartii subsp. *stewartii*	CAIQ
Agrobacterium tumefaciens	CAIQ
Clavibacter michiganensis subsp. *sepedonians*	CAIQ
Clavibacter michiganensis subsp. *insidiosus*	CAIQ
Curtobacterium flaccumfaciens pv. *flaccumfaciens*	CAIQ
Paulownia witches-broom phytoplasma	Beijing
Liberobacter asiaticum	Fujian province

CAIQ: Chinese Academy of Inspection and Quarantine.

time PCR assays have been developed to detect plant bacteria including *Clavbacter michiganensis* subsp. *sepdonicus* and *Ralstonia solanacearum* in potato tubes ***(11,12)***, *Xylella fastidiosa* in asymptomatic grape vines, and *Agrobacterium* ***(13,14).*** This technique was also used to identify phytoplasma in fruit trees ***(15)*** and genetically modified organisms (GMOs) in trade ***(16,17)***. In this study our goal is to develop a real-time PCR assay for the rapid, sensitive, and accurate detection of *X. oryzae* pv. *oryzae* in naturally infected rice plants and seeds.

2. Materials and Methods

2.1. Bacteria Strains and DNA Extraction

Bacteria strains involved in this study are listed in **Table 1**. The Paulownia *(Paulownia australis)* plants infected by Paulownia witches-broom phytoplasma were kindly provided by Dr. Guozhong Tian, China Academy of Forestry. The grapefruit infected by *Liberobacter asiaticum* was collected from Fujian province. Healthy rice seeds and plants were collected from a field in Changsha county, Hunan province. Naturally infected rice leaves of *X. oryzae* pv. *oryzae* were obtained from Hunan Agriculture University. The infected seeds were provided by Hunan Academy of Agriculture Science and were collected from five provinces: Fujian, Hubei, Zhejiang, Yunnan, and Jiangxi. The bacteria strains were cultured for 3 d on LB culture medium. Genomic DNA was extracted from bacteria as previously described ***(18)***. Total DNA was extracted from Paulownia naturally infected by witches-broom phytoplasma with the CTAB method ***(19)***. DNA concentration was measured by a Beckman Du series spectrophotometer.

2.2. PCR Primers and Fluorescence Probe

Primers and probe were designed with Primer Express 2.0 software (Applied Biosystems) based on the conservative sequence of putative siderophore receptor gene cds with an expected

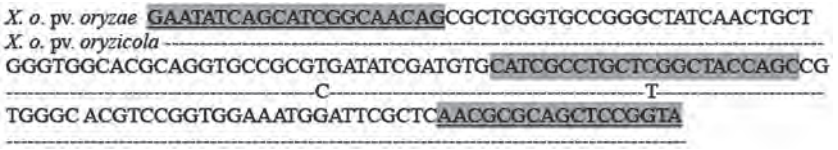

Fig. 1. The PCR fragments of *X. o.* pv. *oryzae* and *X. o.* pv. *oryzicola.* Note: The lengths are both 152 bp. The *X. o.* pv. *oryzae* sequence is on the top line with the *X. o.* pv. *oryzicola* sequence underneath. The middle sequence in shadow is the TaqMan probe. The two ends in shadow are the two primers.

length of 152 bp. The primers were PF: 5'-GAAT ATCAGCATCGGCAACAG-3' and PR: 5'-TAC CGGAGCTGCGCGTT-3'. The probe was 5'-CATCGCCTGCTCGGCTACCAGC-3', whose 5' end was labeled with 6-carboxyfluorescein (FAM) and the 3' end was labeled with tetramethycarboxyrhodamine (TAMRA). The primers and probe were all synthesized by Takara Bio Inc. (Dalian China).

The PCR assays were performed on a PE 7700 DNA sequence detection system (Applied Biosystems) and MJ PTC 200 DNA Engine Cycler. All reactions were carried out in a final volume of 25 μL containing 2.5 μL 10×PCR buffer, 3 m*M* $MgCl_2$, 0.5 m*M* dATP, dCTP, dTTP, dGTP, and dUTP, 0.4 μ*M* forward and reverse primers, 0.8 μ*M* probe, 0.15 U UNG polymerase, 1 U *Taq* polymerase, and 1 μL DNA template. Sterile molecular biology grade water was added to a total volume of 25 μL. Each PCR reaction included a blank control. Reactions were run in 40 cycles as follows: 50°C for 2 min, 95°C for 10 min, 94°C for 15 s, and 68°C for 1 min. Applied Biosystems recommended an annealing temperature of 60°C. However, at this temperature *X. o.* pv. *oryzicola* also gave a fluorescence signal meaning the specificity of the TaqMan probe at 60°C was not high enough to distinguish the two bacteria. By increasing the annealing temperature to 68°C only *X. o.* pv. *oryzae* resulted in a fluorescence signal.

3. Results

3.1. Amplification Sequence Analysis

The PCR fragments amplified from *Xanthomonas* with primers PF and PR were cloned into a PMD18-T vector. The nucleic acid sequence was determined from the positive clone, and compared with the sequence from GenBank. In this assay all four species in the *Xanthomonas* genus had fragments of about 150 bp. By sequence analysis we knew that the lengths of fragments for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are identical except for two base mutations at the sites 69 and 94 (**Fig. 1**). With PCR agarose gel electrophoresis it is impossible to distinguish between these two sequences. *X. oryzae* pv. *oryzicola* , which also occurs in rice seeds, may easily give a false positive for presence of the BLB-causing bacteria. However, using a TaqMan probe with the 94 mutation, the single nucleotide polymorphism can be detected, and the two sequences can be distinguished.

3.2. Specificity of the TaqMan Probe

To determine the specificity of the TaqMan probe one phytoplasma and 12 bacteria samples were used. They represented four strains of *Xanthomonas*, two strains of *Clavibacter*, and single strains of *Pseudomonas*, *Burkholderia*, *Erwinia*, *Pantoea*, *Agrobacterium,* and *Curtobacterium.* With the TaqMan probe, strong fluorescence signals (about 5500) could be collected from only the *X. oryzae* pv. *oryzae* strain. Under optimized reaction conditions the other bacterial samples fluorescence superposed to the baseline (**Fig. 2**). Comparing the different signals we were able to clearly differentiate *X. oryzae* pv. *oryzae* from the other bacterial samples. This included being able to distinguish the pathogenic bacteria *X. oryzae* pv. *oryzicola* whose probe sequence is only one base pair different from our target. These results suggested that our probe had a subspecies level specificity for *Xanthomonas oryzae.* The PCR

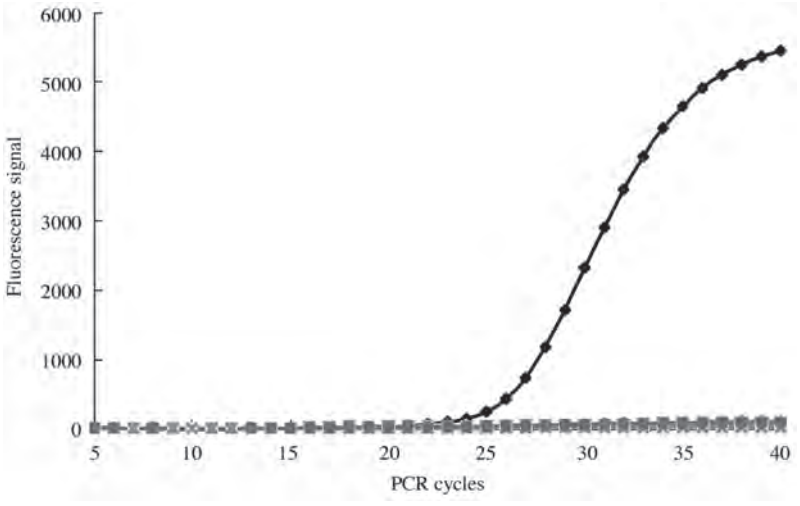

Fig. 2. Results of real-time fluorescence PCR generated using *X. o*. pv. *oryzae* specific probe. Only *X. o. oryzae* got strong signals; signals from the other samples and blank control superposed to the baseline. These samples represented *X. o.* pv. *oryzicola, X. c.* pv. *campestris, X. a.* pv. *citri, P. s.* pv. *syringae*, *B. solancearum*, *E. c.* pv. *carotovora, P. s.* subsp. *stewartii*, *A. tumefaciens, C. m.* subsp. *sepedonians*, *C. m.* subsp. *insidiosus*, *C. f.* pv. *flaccumfaciens*, Paulownia witches-broom phytoplasma, and *L. asiaticum.* This result showed that the probe was specific to *X. o. oryzae*.

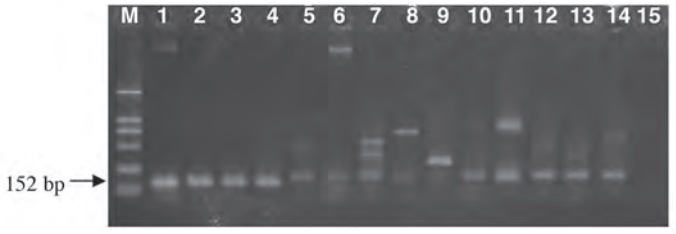

Fig. 3. Agarose gel electrophoresis detection of real-time fluorescence PCR products. Lane 1: *X. o*. pv. *oryzae;* lane 2: *X. o.* pv. *oryzicola;* lane 3*: X. c.* pv. *campestris;* lane 4: *X. a.* pv. *citri;* lane 5*: P. s.* pv. *syringae;* lane 6: *B. solancearum;* lane 7: *E. c.* pv. *carotovora;* lane 8: *P. s.* subsp. *stewartii;* lane 9: *A. tumefaciens;* lane 10: *C. m.* subsp. *sepedonians;* lane 11: *C. m.* subsp. *insidiosus;* lane 12: *C. f.* pv. *flaccumfaciens;* lane 13: Paulownia witches-broom phytoplasma; lane 14: *L. asiaticum;* and lane 15: CK. M: DNA marker DL2000.

products were further analyzed by agarose gel electrophoresis. The four strains of *Xanthomonas* displayed the expected 152-bp band, whereas the other strains did not (**Fig. 3**). This indicated that our pair of primers were specific to the genus *Xanthomonas*, but had no specificity at the species level.

Ten strains of *X. oryzae* pv. *oryzae* and six groups of infected seeds from different regions were used as samples to further validate the specificity. We directly used the pure pathogen culture and soaking solutions of seeds as PCR template. All of the pathogens and infected seeds were successfully detected. These assays proved that the TaqMan probe is feasible and can be used for practical detection in various locations.

3.3. Absolute Sensitivity of the TaqMan Probe

Purified DNA of *X. oryzae* pv. *oryzae* was used as a model to test the sensitivity of this real-time PCR assay. Amplification was performed on 1:10

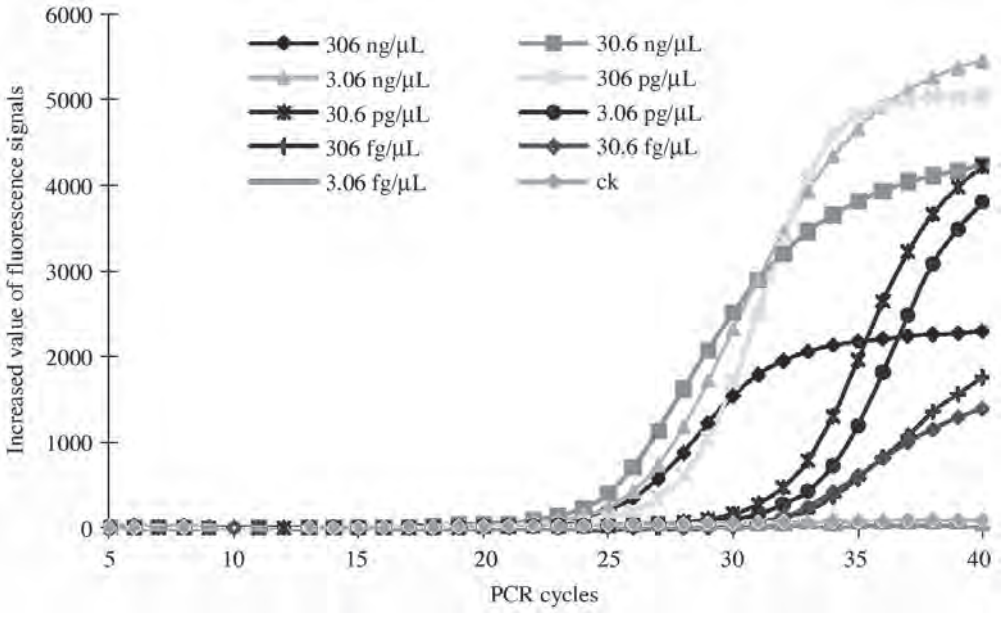

Fig. 4. The absolute sensitivity of the TaqMan probe. In this assay the real-time PCR sensitive threshold was 30.6 fg/μL with a Ct value of 32.1. The dilution of 3.06 fg/μL and blank control got no signal increase.

serial dilutions of the stock. Final dilutions were 306 ng/μL, 30.6 ng/μL, 3.06 ng/μL, 306 pg/μL, 30.6 pg/μL, 3.06 pg/μL, 306 fg/μL, 30.6 fg/μL, and 3.06 fg/μL. In this assay the most sensitive detection concentration was 30.6 fg/μL with Ct value of 32.1 (**Fig. 4**). However, the highest signal value was not from the highest template concentration (306 ng/μL), but from 3.06 ng/μL.This was because a DNA concentration that was too high and decreased the probe hybridizing efficiency. With agarose gel electrophoresis, the sensitivity limit was 3.06 pg/μL. No bands could be observed for concentrations lower than 3.06 pg/μL by naked eye (**Fig. 5**). Thus, the sensitivity of the TaqMan method was 100 times higher than that of the PCR gel electrophoresis.

3.4. Relative Sensitivity of the TaqMan Probe

A pure culture suspension of 10^9 CFU/mL *X. oryzae* pv. *oryzae* was diluted eight times in a 10-fold series. Ten microliters of the final suspension was spread on the LB culture medium plates, cultivated at 28°C for 72 h. Counted clone numbers were: 199 for 10^5 CFU/mL, 31 for 10^4 CFU/mL, and two for 10^3 CFU/mL (**Fig. 6**). This equates to about two bacteria in 10 μL of the 10^3 CFU/mL dilution. Five microliters of each dilution was used as template directly for the probe sensitivity test. For the template of 10^3 CFU/mL dilution, it gave a final fluorescence signal of ΔRn value 300. Because the bacteria are transmitted through rice seeds, we did the following test to determine whether the seed-soaking suspension decreased assay sensitivity. Twenty healthy rice seeds were soaked in 2 mL of each dilution for 12 h at 4°C, and 5 μL of these soaking suspensions were directly used for real-time PCR. The sensitivity limit was approximate 10^5 CFU/mL, 100 times lower than that of pure culture bacteria template. This may be because the PCR reaction was inhibited by some substances from the seeds.

3.5. Detection of Pathogens From Naturally Infected Rice Leaves

Xanthomonas oryzae pv. *oryzae* was detected by real-time PCR in naturally infected leaves. Total DNA was extracted from leaves with the CTAB method. Plasmid containing an amplified fragment was used as a positive control, whereas

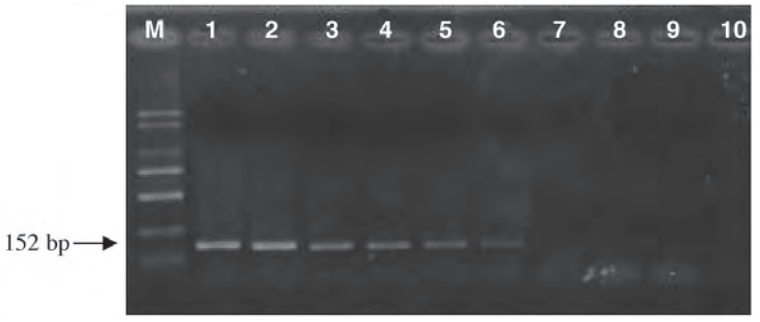

Fig. 5. Sensitivity of agarose gel electrophoresis detection of real-time fluorescence PCR products. Lane 1: 306 ng/μL; lane 2: 30.6 ng/μL; lane 3: 3.06 ng/μL; lane 4: 306 pg/μL; lane 5: 30.6 pg/μL; lane 6: 3.06 pg/μL; lane 7: 306 fg/μL; lane 8: 30.6 fg/μL; lane 9: 3.06 fg/μL; and lane 10: CK. M: DNA marker DL 2000.

healthy leaves and *X. oryzae* pv. *oryzicola* were the negative controls. High signals were observed for positive control and *X. oryzae* pv. *oryzae* infected leaves, but no signal change was recorded for healthy and *X. oryzae* pv. *oryzicola* infected leaves (**Fig. 7**). This result further confirmed the high specificity of the probe in distinguishing *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola* in infected leaves.

3.6. Comparison of the Two Ways of Pathogen Collection From Natural Infected Rice Seeds

Naturally infected rice seeds were soaked in 0.01% Tween-20 overnight at 4°C ***(20)***. One microliter of soaking solution was used as PCR template directly. At the same time, another method was performed, as follows. The soaking solution was centrifuged for 20 min at 16,000 rpm (16,880*g*) and dissolved in 10 μL of sterile water, 1 μL of which was used as the PCR template. Strong fluorescence signals were observed with the two methods when using 50 g of seeds. Ten grams of seeds always gave steady and consistent detection results, whereas the fluorescence signal was not steady if the amount of seeds were less than 5 g. The result from the latter method was better than the former (**Table 2**), although it was more time consuming and prone to the possibility of contamination.

4. Discussion

Xanthomonas oryzae pv. *oryzae* is an important rice seed pathogen responsible for BLB. Previously described phenotypic tests for this bacterial pathogen are limited because they are not only unable to differentiate the bacteria to the subspecies level, but also are time-consuming and require highly skilled technicians ***(6,12,20–22)***. For BLB it is especially important to be able to distinguish the bacteria to a subspecies level because *X. oryzae* pv. *oryzae* has a subspecies relative, *X. oryzae* pv. *oryzicola,* that easily gives a false positive for presence of the BLB-causing bacteria. Our real-time PCR method ensures the clear identification of *X. oryzae* pv. *oryzae* versus *X. oryzae* pv. *oryzicola.*

Furthermore, real-time PCR technology offers several advantages over conventional PCR methods such as less risk of PCR contamination, shorter turnaround time, and quantitative PCR analysis. In this assay we were able to use plant samples directly for PCR tests without extracting DNA and adding reagents to offset endogenous PCR inhibitors. This not only saves time and money, but also reduces the chances of cross-contamination of samples. In this assay all *Xanthomonas* strains could be amplified with the primer pair, and the probe showed high specificity and did not hybridize to any other PCR products.

We found the absolute sensitivity limit to be approx 30.6 fg of DNA. This is 100 times more

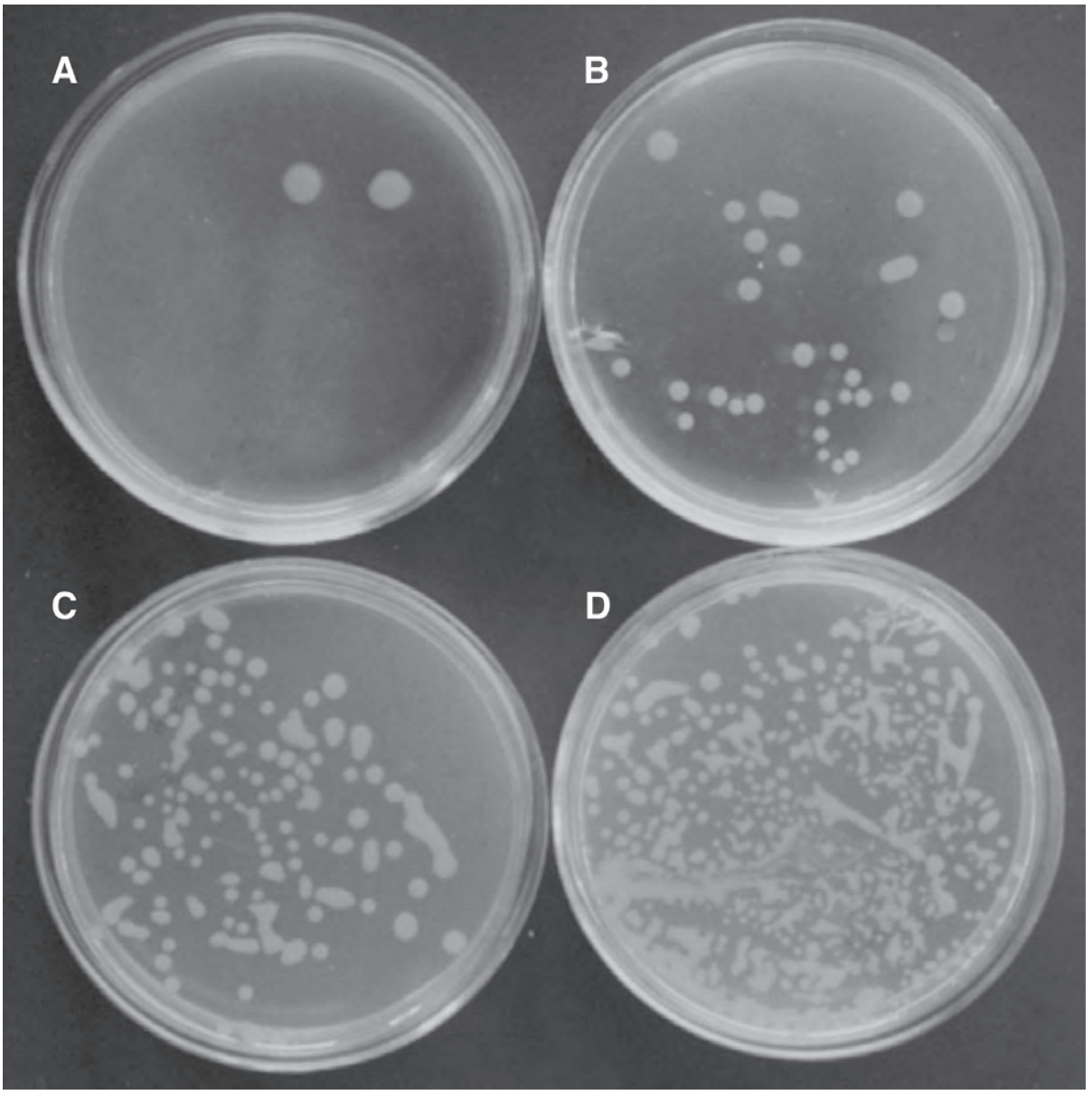

Fig. 6. Different dilutions of *X. o. oryzae* in culture. 1: 10^3 CFU/mL; 2: 10^4 CFU/mL; 3: 10^5 CFU/mL; and 4: 10^6 CFU/mL.

sensitive than the conventional PCR method. In pure bacteria culture about 10^3 CFU/mL can be detected by the probe. Or if assaying directly from plant material, 10 g of infected rice seeds can be detected successfully without DNA extraction.

Because more and more genomes of plant pathogens are sequenced and made available for unique primer and probe design, disease diagnosis using a sensitive real-time PCR system will soon become a standard technique in plant pathology.

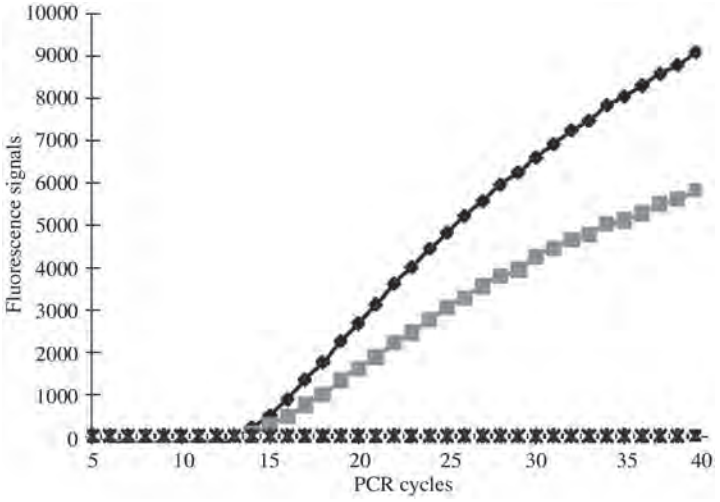

Fig. 7. Detection of pathogens from naturally infected rice leaves. Only positive control (top line) and samples infected by *X. o. oryzae* (middle line) got strong signals; the signals from the healthy leaves and samples infected by *X. o. oryzicola* had no increase and superposed to the baseline.

Table 2
Results of Naturally Infected Rice Seeds Detected by TaqMan Probe

Seeds	Weight (g)	Volume (mL)	Templates collection	Detecting results
Healthy seeds	250	375	D	–
			C	–
	50	75	D	–
			C	–
	10	15	D	–
			C	–
	5	7.5	D	–
			C	–
Seeds infected by *X. o.* pv. *oryzae*	250	375	D	+
			C	+
	50	75	D	+
			C	+
	10	15	D	–
			C	+
	5	7.5	D	–
			C	±

In the fourth column PCR templates collection methods are shown. D: using 1 μL of seed soaking solution directly as reaction templates; C: the soaking solutions were centrifuged for 20 min at 16000 rpm, then dissolved in 10 μL of sterile water, 1 μL of which was used as the PCR template. + : positive; - : negative; ± : difficult to evaluate.

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