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Molecular detection of *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, and *Burkholderia glumae* in infected rice seeds and leaves

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ABSTRACT

The polymerase chain reaction (PCR) is particularly useful for plant pathogen detection. In the present study, multiplex PCR and SYBR Green real-time PCR were developed to facilitate the simultaneous detection of three important rice pathogens, *Xanthomonas oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *Burkholderia glumae*. The unique PCR primer sets were designed from portions of a putative glycosyltransferase gene of *X. oryzae* pv. *oryzae*, an *AvrRxo* gene of *X. oryzae* pv. *oryzicola*, and an internal transcribed spacer (ITS) sequence of *B. glumae*. Using a multiplex PCR assay, *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae* were detected in one PCR reaction that contained the newly developed primer set mix. Using SYBR Green real-time PCR assays, *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae* were detected at 1, 1, and 10 fg μL^{-1}, respectively. These newly designed molecular assays are sensitive and could be reliable tools for pathogen detection and disease forecasting.

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1. Introduction

Rice, one of the most important food crops, is constantly challenged by bacterial pathogens, such as those causing bacterial blight, leaf streak, and bacterial panicle blight. Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae*, is a prevalent and destructive rice disease that causes annual yield losses ranging from 10 to 20% and up to 50% to 70% in severely infected fields [1,2]. This disease also affects grain quality by interfering with the maturation process [3]. Bacterial leaf streak caused by *X. oryzae* pv. *oryzicola*, the pathovar of *X. oryzae* pv. *oryzae*, usually results in the wilting of leaves and

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Table 1 – Sequences, annealing temperature, predicted product size, primers, and primer sources used in this study.

Target pathogen	Primer name	Sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)	Target ID in GenBank
X. oryzae pv. *oryzae*	JLXooF	F: CCTCTATGAGTCGGGAGCTG	58	230	AF169030
	JLXooR	R: ACACCGTGATGCAATGAAGA			
X. oryzae pv. *oryzicola*	JLXocF	F: CAAGACAGACATTGCTGGCA	58	112	AY395713
	JLXocR	R: GGTCTGGAATTTGTACTCCG			
B. glumae	JLBgF	F: TGGGTAGTCTCTGTAGGGAA	58	164	D87080
	JLBgR	R: TCATCCTCTGACTGGCTCAA			

losses as high as 32% in 1000-grain weight [4]. It is important to note that hybrid rice varieties are more susceptible to this bacterial pathogen than non-hybrid varieties [5]. Rice bacterial panicle blight (bacterial grain rot), caused by *Burkholderia glumae* was first reported in Japan in 1956 [6]. Yield losses due to *B. glumae* can reach as high as 40% in the southern U.S. [7]. Given that the optimal temperature for the growth of *B. glumae* ranges from 30 to 50 °C [7], warmer temperatures during the rice-growing season increase the severity of the disease [8]. The presence of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, *B. glumae* in infected seeds may cause disease transmission, so that many countries have listed the three bacteria as quarantined

Table 2 – Bacterial and fungal strains used for specificity tests.

Species	Strain [a]	Host or source	Amplication with primer sets		
			JLXooF/JLXooR	JLXocF/JLXocR	JLBgF/JLBgR
X. oryzae pv. *oryzae*	OS225	Rice	+	–	–
X. oryzae pv. *oryzae*	OS198	Rice	+	–	–
X. oryzae pv. *oryzae*	OS86	Rice	+	–	–
X. oryzae pv. *oryzae*	Z173	Rice	+	–	–
X. oryzae pv. *oryzae*	JS158-2	Rice	+	–	–
X. oryzae pv. *oryzae*	CJO13-1	Rice	+	–	–
X. oryzae pv. *oryzicola*	AHB4-75	Rice	–	+	–
X. oryzae pv. *oryzicola*	JSB3-22	Rice	–	+	–
X. oryzae pv. *oryzicola*	YNB10-32	Rice	–	+	–
X. oryzae pv. *oryzicola*	GXB3-14	Rice	–	+	–
X. oryzae pv. *oryzicola*	SCB4-1	Rice	–	+	–
X. oryzae pv. *oryzicola*	CJOC13-1	Rice	–	+	–
X. maltophilia	90056	unknown	–	–	–
X. campestris	CJXC-131	Broccoli	–	–	–
X. campestris	CJXC-132	Broccoli	–	–	–
X. campestris	96024	Wild cabbage	–	–	–
X. axonopodis	ZJUR22578		–	–	–
X. axonopodis	ZJUR22579		–	–	–
X. asonopodis	LMG5401		–	–	–
X. asonopodis	LMG5402		–	–	–
B. gladioli	BC20157	Gladiolus	–	–	–
B. cepacia	LMG1222	Onion	–	–	–
B. andropogonis	R22578		–	–	–
B. unamae	CJBU	Maize	–	–	–
B. sacchari	CJBS		–	–	–
B. glumae	CU-1	Rice	–	–	+
B. glumae	CU-2	Rice	–	–	+
B. glumae	CU-3	Rice	–	–	+
B. glumae	LMG2196	Rice	–	–	+
Acidovorax avenae	RS-1	Rice	–	–	–
Ralstonia solanacearum	ZAAS-1	Tomato	–	–	–
Ralstonia solanacearum	GT-1	Tobacco	–	–	–
Agrobacterium tumefaciens	EHA105		–	–	–
Pellicularia sasakii	CJPS-1	Rice	–	–	–
Fusarium oxysporum	CJF-1	Watermelon	–	–	–
Magnaporthe oryzae	C30	Rice	–	–	–
Magnaporthe oryzae	CHL441	Rice	–	–	–
Ustilaginoidea oryzae	LN	Rice	–	–	–
Ustilaginoidea oryzae	SX0201	Rice	–	–	–

[a] Name of the strain.

organisms. Both conventional and real-time PCR have been widely used to detect or verify the presence of *X. oryzae* pv. *oryzae* [9–13], *X. oryzae* pv. *oryzicola* [14–16], and *B. glumae* [17–20] in recent decades. These molecular-based methods are rapid, accurate and sensitive for detecting pathogens. However, they can detect only one pathogen each. Several methods have been developed to distinguish highly similar pathovars of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* using multiplex or real-time PCR [21,22].

In the present study, we used genome sequence information available in public databases to develop PCR primers for accurate identification of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae*. The objective of this study was to develop multiplex PCR and SYBR Green real-time PCR methods for simultaneous detection of the presence of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae*.

2. Materials and methods

2.1. Bacterial and fungal strains and culture conditions

Strains of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae*; the other closely related pathogens *Xanthomonas campestris*, *Xanthomonas maltophilia*, *Burkholderia gladioli* pv. *alliicola*, and *Burkholderia cepacia*; and the rice fungal pathogens *Magnaporthe oryzae* and *Ustilaginoidea oryzae* were used to develop specific primer sets. Bacterial strains were cultured on a Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar) at 28 °C for two days. Fungal isolates were cultured on corn meal medium (3% corn meal and 1.5% agar) at room temperature for four to five days [23].

2.2. DNA preparation

Genomic DNA of bacterial strains was extracted with a Genomic DNA Prep Kit (Sangon, Shanghai, China) following the manufacturer's protocol, except that DNA was eluted in 30 μL double-distilled water (ddH_2O). Genomic DNA of fungal and leaf tissue was prepared using the CTAB method [24,25]. DNA concentrations were measured with a Nanodrop 2000 instrument (Thermo Fisher Scientific, Wilmington, DE). The OD260: OD280 ratios of all samples were approximately 1.8. All samples were diluted to 1 ng μL^{-1} in ddH_2O.

2.3. Development of specific DNA primers

The sequence of the putative *glycosyltransferase* gene of *X. oryzae* pv. *oryzae* (AF169030.1) was identified in GenBank, and then aligned with the putative *glycosyltransferase* genes of *X. oryzae* pv. *oryzicola* (CP003057.1), *X. campestris* pv. *campestris* (AF204145.1), *X. campestris* pv. *vesicatoria* (AM039952.1), *Xanthomonas axonopodis* pv. *citrumelo* (CP002914.1), and *Xanthomonas albilineans* (FP565176) using BioEdit [26]. Specific primers for *X. oryzae* pv. *oryzae* were designed from non-conserved regions (Table 2, Fig. S1). Using the same strategy, the *AvrRxo* gene of *X. oryzae* pv. *oryzicola* (AY395713.1) was used as a template for designing specific primers for *X. oryzae* pv. *oryzicola* (Fig. S2). Ribosomal internal transcribed spacers (ITSs) of *B. glumae* (D87080), *B. plantarii* (AB183680.1), *B. gladioli* (EF552066.1), *B. gladioli* pv. *alliicola* (D87082.1), *B. gladioli* pv. *agricicola* (EF552068.1), and *B. cepalia* (FJ870551.2) were aligned, after which the nonconserved regions were used to design specific primers (Fig. S3). The PCR product lengths ranged from 100 to 250 bp for both conventional and real-time PCR assays.

2.4. Polymerase chain reaction (PCR)

Conventional PCR assays were used to test the specificity and sensitivity of primers using a T100 Thermal Cycler (Bio-Rad, California, USA). The concentration of the sample used for testing the specificity of the primers was 1 ng μL^{-1}. The pathogens *X. oryzae* pv. *oryzae* OS198, *X. oryzae* pv. *oryzicola* AHB4-75, and *B. glumae* LMG2196 were diluted to 5×10^{-1}, 1×10^{-1}, 5×10^{-2}, 1×10^{-2}, 5×10^{-3}, 1×10^{-3}, 5×10^{-4}, and 1×10^{-4} ng μL^{-1} with ddH_2O to test primer sensitivity. PCR reactions were performed in a final volume of 20 μL containing 10 μL of 2 × *Taq* master mix (Sangon, Shanghai, China), 0.4 μL of each 10 μmol L^{-1} primer, 1 μL of genomic DNA, and 8.6 μL ddH_2O, vortexed thoroughly. PCR amplification was as follows: initial denaturation for 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, and final extension for 10 min at 72 °C. PCR products were separated on a 1% agarose gel (1 × TAE buffer) by electrophoresis at 100 V for 30 min and visualized with a Gene Genius Bio Imaging System (Syngene, Cambridge, UK). DNA templates were replaced with ddH_2O as a negative control.

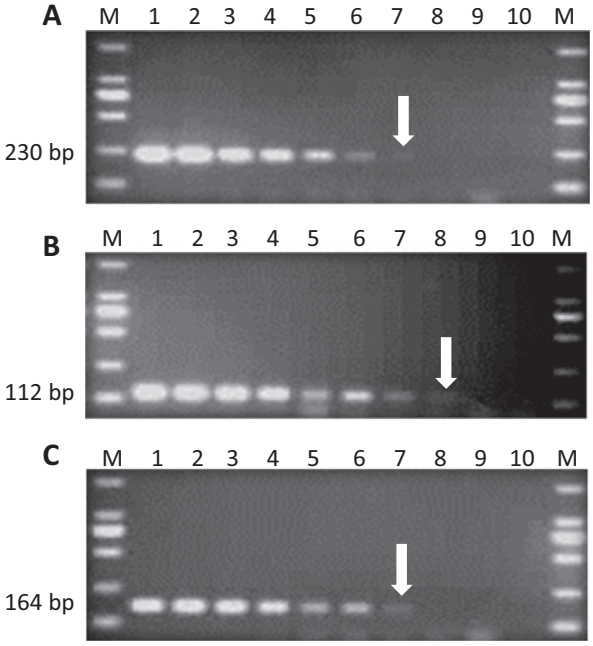


Fig. 1 – Sensitivity tests of primer sets using conventional PCR. A: sensitivity test of JLXooF/R with the template OS198; B: sensitivity test of JLXocF/R with the template AHB4-75; C: sensitivity test of JLBgF/R with the template LMG2196. Lane M, DNA ladder (DL 2000, Takara, Shiga, Japan); lanes 1–9: 1, 5×10^{-1}, 1×10^{-1}, 5×10^{-2}, 1×10^{-2}, 5×10^{-3}, 1×10^{-3}, 5×10^{-4}, and 1×10^{-4} ng μL^{-1}; lane 10: negative control. The arrows point to the limiting detection concentrations of the primer sets.

2.5. SYBR Green real-time PCR

The SYBR Green real-time PCR assay was used to test the sensitivity of the primers with an IQ5 Multicolor real-time PCR Detection System (Bio-Rad, Hercules, CA). DNA of OS198, AHB4-75, and LMG2196 was 10-fold serially diluted from 1 to 1×10^{-6} ng μL^{-1}. Each PCR reaction contained 10 μL of 2 × SYBR Premix Ex *Taq* (TaKaRa, Shiga, Japan) and 0.4 μL of each 10 μmol L^{-1} primer, 1 μL template, and 8.6 μL ddH_2O. Real-time PCR was performed with the following program: 45 s at 95 °C; 40 cycles of 5 s at 95 °C, 30 s at 61 °C for 30 s; and melting curve at 65 to 95 °C with increases of 0.5 °C. DNA templates were replaced by ddH_2O as a negative control.

2.6. Multiplex PCR

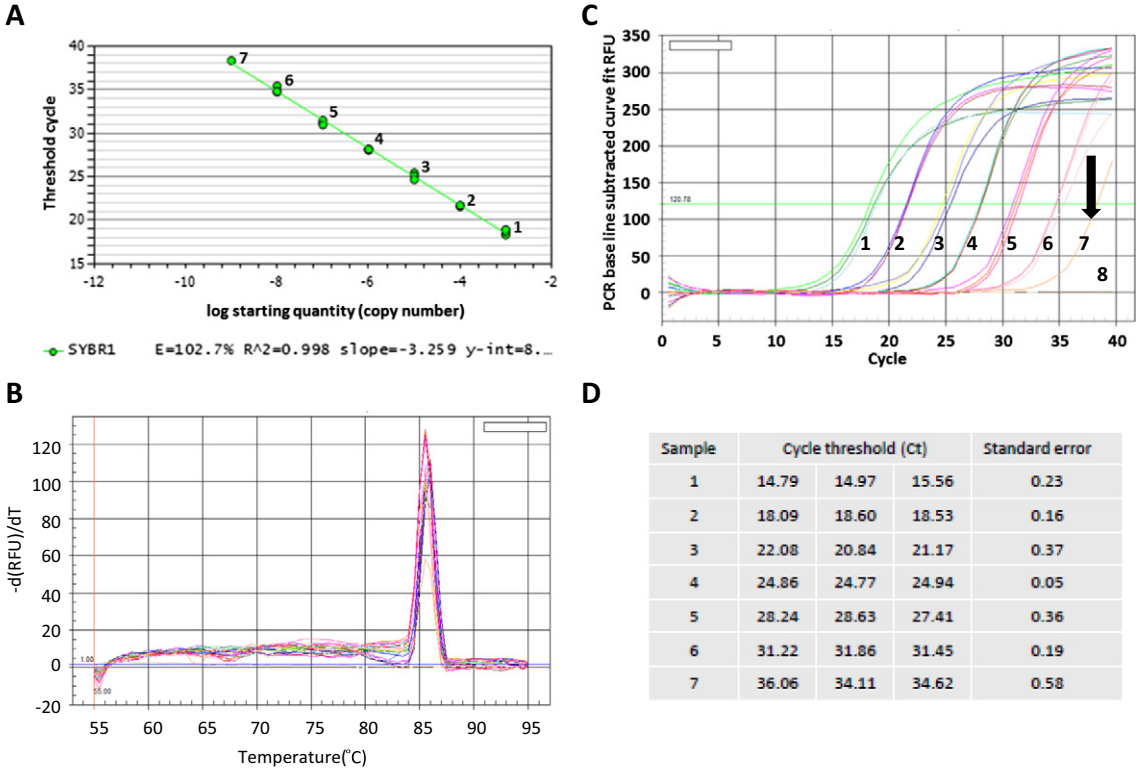
To perform multiplex PCR, 1 ng μL^{-1} genomic DNA of OS198, AHB4-75 and LMG2196 was used as positive templates in three PCR tubes, respectively. The three genomes were mixed with different concentrations and proportions of DNA to test the primers' sensitivity in a multiplex PCR reaction. The total volume of multiplex PCR was 20 μL (10 μL of 2 × *Taq* master mix, 0.4 μL of 10 μmol L^{-1} of each primer, and 1 μL DNA mix). PCR products were separated on a 1.5% agarose gel (1 × TAE buffer) by electrophoresis at 90 V for 50 min and visualized with the Gene Genius Bio Imaging System. DNA templates were replaced by ddH_2O as a negative control.

2.7. Artificial inoculation of seeds with *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae*

Five grams (approximately 150 seeds) of rice cultivar Nipponbare were surface-disinfected in 75% ethanol for 10 min, incubated in approximately 0.5% chlorine solution for 30 min, and rinsed three times with sterilized distilled water. After disinfection, the seeds were transferred to Petri dishes containing sterilized filter paper and allowed to air-dry for 3 h in a laminar-flow chamber. The surface-disinfected seeds were inoculated with 5 mL g^{-1} of bacterial suspensions of OS198 or AHB4-75 or LMG2196 or a mixture of OS198, AHB4-75, and LMG2196 with OD_{600} equal to 0.01 ($\times 10^8$ CFU mL^{-1}), respectively. OD_{600} values were measured using a Nanodrop (ND 100 spectrophotometer, NanoDrop Technologies, Inc.). The inoculation was vacuum infiltrated for 60 min. After inoculation, the artificially infected seeds were allowed to air-dry in the laminar air flow chamber and stored at 4 ºC until use.

2.8. The detection of pathogens on rice seeds

Detection of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae* in rice seed lots was performed by washing 1 g healthy and 1 g infected seeds infected by *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, *B. glumae*, or a mixture of the three bacteria in 5 mL sterile dH_2O, shaking at 100 r min^{-1} for 2 h at 4 °C. One microliter of suspension was used as the template for the multiplex PCR described above for detection of



Sample	Cycle threshold (Ct)			Standard error
1	14.79	14.97	15.56	0.23
2	18.09	18.60	18.53	0.16
3	22.08	20.84	21.17	0.37
4	24.86	24.77	24.94	0.05
5	28.24	28.63	27.41	0.36
6	31.22	31.86	31.45	0.19
7	36.06	34.11	34.62	0.58

Fig. 2 – Sensitivity tests of JLXooF/R primer set using SYBR Green RT-PCR. A: Standard curve. For each assay, templates (1–7) were diluted 10-fold to concentrations ranging from 1.0 to 1.0 × 10^{-6} ng μL^{-1}. B: Melting-peak analysis. C: Fluorescence intensity; 1.0 to 1.0 × 10^{-6} ng μL^{-1}; 1–7: samples; 8: negative control. The arrow points to the limiting detection concentration of the primer set; D: CT (cycle threshold) and SE (standard error).

X. oryzae pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae*. All experiments were repeated twice.

3. Results

3.1. Primer design and specificity

The specific primers JLXooF/R for *X. oryzae* pv. *oryzae*, JLXocF/R for *X. oryzae* pv. *oryzicola*, and JLBgF/R for *B. glumae* were developed based on the polymorphic regions of the corresponding putative *glycosyltransferase* gene, *AvrRxo* gene and ITS sequence, respectively (Table 1, Figs. S1, S2, and S3). The 230 bp DNA fragments were amplified from all *X. oryzae* pv. *oryzae* strains using the JLXooF/R. However, the expected fragments were not amplified either from closely related bacterial strains, including *X. oryzae* pv. *oryzicola* and *X. campestris*, or from other bacterial or fungal strains (Table 2, Fig. S4). An expected 112 bp DNA product was amplified only from *X. oryzae* pv. *oryzicola* strains using the primer set JLXocF/R (Table 2, Fig. S5), and a product of 164 bp was amplified only from *B. glumae* using JLBgF/R (Table 2, Fig. S6). The results suggest that these primer sets were specific to the target pathogens tested.

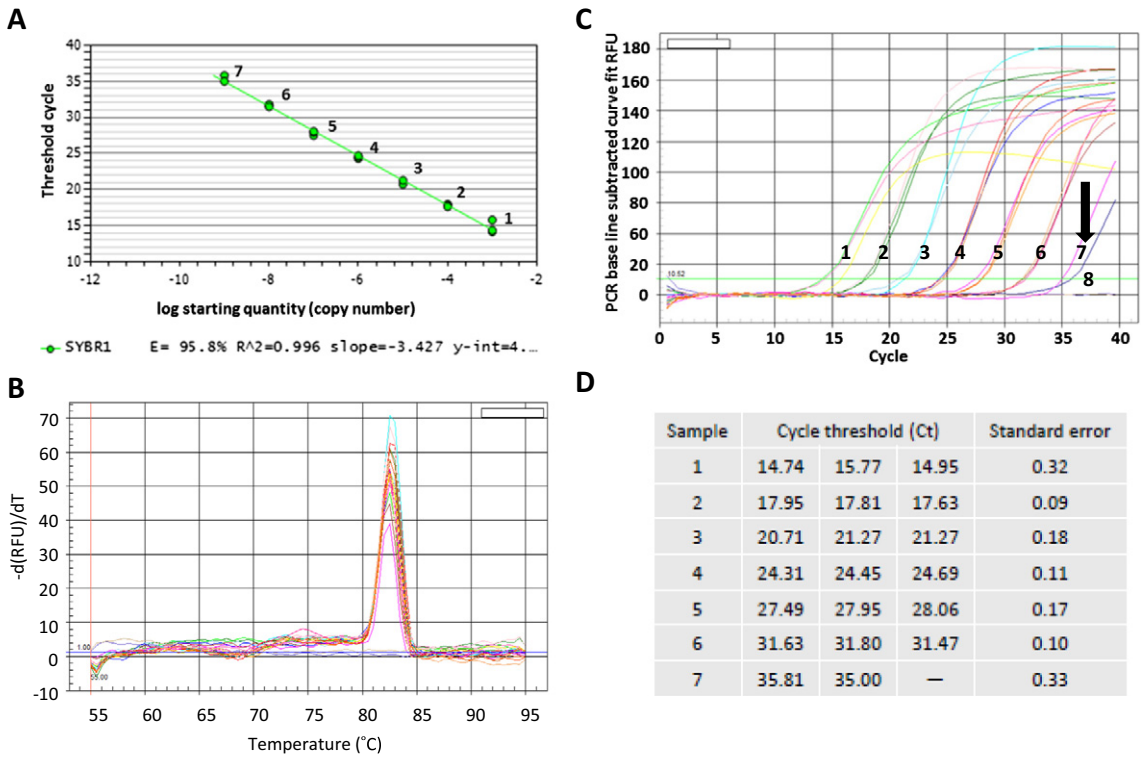
3.2. Sensitivity of PCR amplification

The purified DNA was used to test the primers' sensitivity in both conventional PCR and real-time PCR assays. The primer sets JLXooF/R, JLXocF/R, and JLBgF/R detected as little as 1 pg μL^{-1} DNA of OS198, 0.5 pg μL^{-1} DNA of AHB4-75, and 1 pg μL^{-1} DNA of LMG2196 in the 20 μL PCR reactions (Fig. 1).

SYBR Green real-time PCR was also used to test the sensitivity of the primer sets. The amplification profiles of OS198, AHB4-75, and LMG2196 dilutions are shown in Figs. 2, 3, and 4, respectively. The R^2 values of JLXooF/R, JLXocF/R, and JLBgF/R were equal to 0.998, 0.996, and 0.992, respectively, indicating a good linear response of each primer set. The linear regression slope gave coefficients of −3.359 for JLXooF/R, −3.426 for JLXocF/R, and −3.245 for JLBgF/R, corresponding to PCR efficiencies of 102.7%, 95.8%, and 107.9%, respectively (Figs. 2-A, 3-A, 4-A). Melting curve analysis showed a single peak for each primer at around 85 °C (Figs. 2-B, 3-B, 4-B) suggesting the absence of primer dimers. The cycle threshold (Ct) in a real-time PCR assay is defined as the number of cycles required for the fluorescent signal to pass the threshold. The sample is considered to be negative or to represent environmental contamination when the Ct value is above 38.5. The detection limits of the genomic DNAs by SYBR Green PCR were 1 fg μL^{-1} for OS198 (Fig. 2-C), 1 fg μL^{-1} for AHB4-75 (Fig. 3-C), and 10 fg μL^{-1} for LMG2196 (Fig. 4-C). The primer sets developed in this study can be used to detect the presence of the target pathogens by both conventional and real-time PCR.

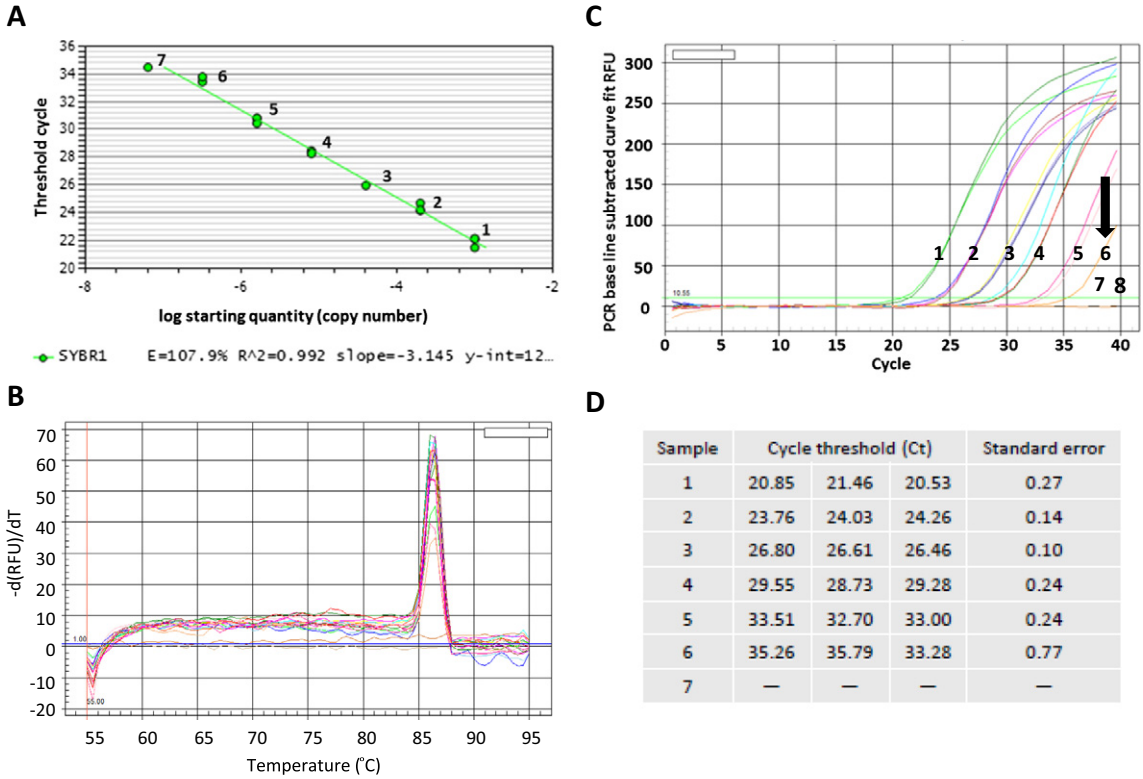
3.3. Multiplex PCR for detection of three pathogens and its sensitivity

To test further whether the primer sets could be used to detect the three target bacterial organisms simultaneously, artificial



Sample	Cycle threshold (Ct)			Standard error
1	14.74	15.77	14.95	0.32
2	17.95	17.81	17.63	0.09
3	20.71	21.27	21.27	0.18
4	24.31	24.45	24.69	0.11
5	27.49	27.95	28.06	0.17
6	31.63	31.80	31.47	0.10
7	35.81	35.00	—	0.33

Fig. 3 – Sensitivity assay of JLXocF/R primer set for *X. oryzae* pv. *oryzicola* using SYBR Green RT-PCR. A: Standard curve. For each assay, templates (1–7) were diluted 10-fold to concentrations ranging from 1.0 to 1.0 × 10^{-6} ng μL^{-1}. B: Melting-peak analysis. C: Fluorescence intensity; 1.0 to 1.0 × 10^{-6} ng μL^{-1}; 1–7: samples; 8: negative control. The arrow points to the limiting detection concentration of the primer set; D: CT (cycle threshold) and SE (standard error).



Sample	Cycle threshold (Ct)			Standard error
1	20.85	21.46	20.53	0.27
2	23.76	24.03	24.26	0.14
3	26.80	26.61	26.46	0.10
4	29.55	28.73	29.28	0.24
5	33.51	32.70	33.00	0.24
6	35.26	35.79	33.28	0.77
7	—	—	—	—

Fig. 4 – Sensitivity assay of JLBgF/R primer set for *B. glumae* using SYBR Green RT-PCR. A: Standard curve. For each assay, templates (1–7) were diluted 10-fold to concentrations ranging from 1.0 to 1.0 × 10^{-6} ng μL^{-1}. B: Melting-peak analysis. C: Fluorescence intensity; 1.0 to 1.0 × 10^{-6} ng μL^{-1}; 1–7: samples; 8: negative control. The arrow points to the limiting detection concentration of the primer set; D: CT (cycle threshold) and SE (standard error).

genomic DNA mixtures of OS198, AHB4-75, and LMG2196 were prepared based on different concentrations displayed in Table 3. When mix 1–4 was used as template in multiplex PCRs, all of the products specific to the three pathogens were visible on the 1.5% agarose gel (Table 3 and Fig. 5). However, the specific amplicon of *B. glumae* was not detectable when mix 5 was used as template. Only the amplicon of *X. oryzae* pv. *oryzicola* was detected when mix 6 was used as template in multiplex PCR. The detection limits for the multiplex PCR assay were 0.3 pg μL^{-1} for *X. oryzae* pv. *oryzae*, 0.167 pg μL^{-1} for *X. oryzae* pv. *oryzicola*, and 16.7 pg μL^{-1} for *B. glumae* in the 20 μL reaction. The detection limits of each pathogen in multiplex PCR were highly similar to those of the single pathogen in conventional PCR.

3.4. *Pathogen detection in the artificial inoculated rice seeds*

To determine whether multiplex PCR could detect the target pathogens in infected rice seeds, rice seeds were artificially infected by *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, or *B. glumae* and the mixture of the these three pathogens, respectively. If the seeds were infected by one pathogen, only the corresponding PCR product appeared on the gel using multiplex PCR assays. As a negative control, no amplification was observed from sterile distilled water-treated seeds. When the seeds were infected with a mixture of the three pathogens, the 230, 164, and 112 bp fragments for *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae*, respectively, were detected (Fig. 6).

Table 3 – Sample mixtures for multiplex PCR.

Sample	Mix 1 [a] (Conc. [b]/final conc. [c]) 1/0.3	Mix 2 0.5/0.167	Mix 3 0.1/0.03	Mix 4 0.05/0.0167	Mix 5 1 × 10^{-3}/0.3 × 10^{-3}	Mix 6 0.5 × 10^{-3}/0.167 × 10^{-3}
OS198	+ [d]	+	+	+	+	–
LMG2196	+	+	+	+	–	–
AHB4-75	+	+	+	+	+	+

[a] Genomic DNA mixture of three samples.
[b] The concentration of DNA (ng μL^{-1}) used to prepare the DNA mixtures; equal volume DNA of each sample was used.
[c] The concentration (ng μL^{-1}) of each sample in the mixture.
[d] The specific PCR products generated in the multiplex PCR. "+" means existence of specific products; "–" means absence.

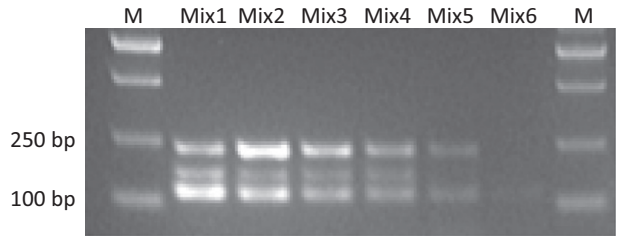


Fig. 5 – One-tube multiplex PCR for diagnosing three pathogens and its sensitivity. Lane M, DNA ladder (DL2000; TaKaRa); lanes 1–6 mixture of *X. oryzae* pv. *oryzae* strain OS225, *X. oryzae* pv. *oryzicola* AHB4-75, and *B. glumae* strain LMG2196, in concentrations 1 ng μL^{-1}, 5×10^{-1} ng μL^{-1}, 10×10^{-1} ng μL^{-1}, 5×10^{-2} ng μL^{-1}, 1×10^{-2} ng μL^{-1}, 5×10^{-3} ng μL^{-1}.

4. Discussion

Conventionally, identification or detection of a plant pathogen requires pathogen isolation, cultivation, and verification based on bacteriological characteristics, colony morphology, electron microscopic observation, and other means—a time-consuming process. In addition, the detection process requires much equipment and chemicals, increasing the cost. In the present study, an efficient multiplex PCR method was used to rapidly and accurately detect the rice bacterial pathogens *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae* simultaneously in infected rice seeds, using new specific primer sets developed from specific sequence comparisons of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, and *B. glumae* against their closely related species.

The bottleneck for PCR-based diagnostic or detection tools has been the availability of pathogen-specific primers. Sequence polymorphisms of 16S–23S ITS are often observed in strains of different species. In previous studies, specific DNA primers and probes have been designed based from 16S–23S ITS sequences for identification, separation and classification of some species of pathogens [6,9,17,27–32]. 16S–23S ITS of different species of *Burkholderia* were used to separate *B. glumae* from other *Burkholderia* species. However, it is difficult to separate pathovars using 16S–23S ITS [9]. With advances in sequencing techniques, more and more bacterial genomic DNA sequences have been deposited in the GenBank database, allowing the development of specific primers using genomic comparisons [21]. By genomic comparison among the *X. oryzae* pv. *oryzae* strains (PXO99A, MAFF311018, and KACC 10331), *X. oryzae* pv. *oryzicola* strains (BLS256), we identified the putative *glycosyltransferase* gene specific to *X. oryzae* pv. *oryzae*, and the *AvrRxo* gene specific to *X. oryzae* pv. *Oryzicola* (X. Wang, unpublished data). We then designed specific primers from the polymorphic DNA regions of these specific genes (Figs. S1, S2, S3). Although we used a limited number of strains of each pathogen, the primer sets we developed were specific. We amplified no sequences from the closely related bacterial pathogens *X. campestris*, *X. maltophilia*, *B. gladioli* pv. *alliicola*, or *B. cepacia*, or from the fungal pathogens, *M. oryzae* and *U. oryzae*.

For pathogen quarantine and inspection, primer sets are often required to be not only specific to the templates, but also sensitive to small quantities of the pathogens. Given that the amplified PCR fragments ranged from 112 to 230 bp in length, these primer sets can be used for both conventional and SYBR Green PCR. This knowledge will allow users to select the desired PCR platform to detect the pathogens.

Multiplex PCR has been applied to detect several pathogens in one PCR tube. Given that the lengths of the amplicons were very different, they were clearly visible on the 1.5% agarose gel after 50 min of separation. When complex templates consisting of three mixed samples were used, the detection limits of each sample were highly similar to those when single samples was used as the PCR template, suggesting that the multiplex PCR developed in the study can be used for simultaneous detection of the three rice bacterial pathogens. One common problem is that the detection sensitivity of multiplex PCR is lower than that of real-time PCR. To determine whether each primer set could amplify the corresponding DNA fragment from mixed samples with multiple pathogens using SYBR Green real time PCR, we made the following DNA mixtures: 1. DNA of OS198, AHB4-75 and LMG2196 with 1 ng μL^{-1} at equal volume; and 2. detection limits of OS198, AHB4-75, and LMG2196 at equal volume. We observed specific real-time PCR products using the complex genomic DNA as templates and with even tiny amounts of DNA (Fig. S7). These findings suggest that our primers are specific and sensitive for simultaneous use in both multiplex and real-time PCR.

Sowing rice seeds containing the organisms of *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*, or *B. glumae* can cause severe yield and economic losses in rice production. Rice leaves naturally infected by *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* were collected from rice fields in Hangzhou in 2013 and infections were verified by phenotypic examination. The mixture of primer sets was used to detect different pathogens in these diseased leaves using multiplex PCR. The PCR products expected from positive controls were amplified using DNA from diseased leaf tissue infected by *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (Fig. S8), suggesting that these primer sets are highly effective and specific.

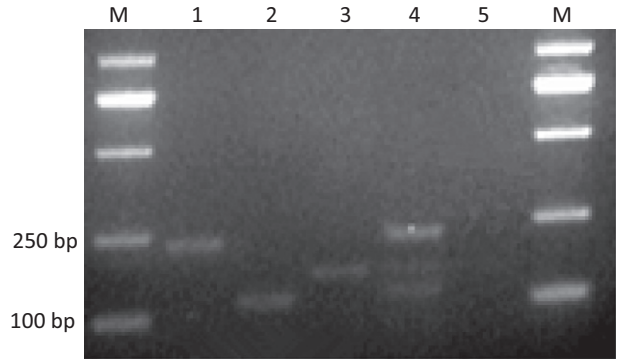


Fig. 6 – Pathogen detection in artificial inoculated rice seeds. One-tube multiplex PCR for diagnosing three pathogens. Lane M, DNA ladder (DL 2000; TaKaRa); lane 1: seeds infected by *X. oryzae* pv. *oryzae* strain OS198; lane 2: seeds infected by *X. oryzae* pv. *oryzicola* strain AHB4-75; lane 3:seeds infected by *B. glumae* strain LMG; lane 4: mixture of seeds infected by OS198, AHB4-75, and LMG; lane 5: negative control.

In conclusion, we have developed a user-friendly PCR based method to detect pathogens at extremely low levels in infected rice seeds and leaves. This method should be tested using diseased rice seeds from commercial fields before world-wide adoption for rapid pathogen inspection and quarantine.

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Supplementary material

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.cj.2014.06.005.

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