Rapid and Specific Detection of *Burkholderia glumae* in Rice Seed by Real-Time Bio-PCR Using Species-Specific Primers Based on an *rhs* Family Gene

Byoung Kyu Kim, Min Seok Cho, Myeong Ho Kim, Hyeon Jin Choi, Man Jung Kang, and Hong Sik Shim, National Academy of Agricultural Science, Rural Development Administration, 441-707 Suwon, Republic of Korea; Tae-Young Ahn, Department of Microbiology, Dankook University, 330-71, Cheonan, Republic of Korea; Jaisoo Kim, Department of Life Science, Kyonggi University, Suwon 443-760, Republic of Korea; and Dong Suk Park, National Academy of Agricultural Science, Rural Development Administration, 441-707 Suwon, Republic of Korea

Abstract

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In this study, we developed a reliable, quick, and accurate quantitative polymerase chain reaction (qPCR) assay to detect grain rot caused by *Burkholderia glumae* in rice seed. The control of bacterial grain rot is difficult, and the only practical methods for disease management rely on the use of pathogen-free seed, appropriate culture practices, and resistant cultivars. Therefore, the specific detection of this pathogen in seed is essential for effective control of the disease. However, other *Burkholderia* spp. are also detected by currently available molecular and serological methods. In this study, we exploited the available genome sequence information in public databases to develop specific

PCR primers for accurate diagnosis of *B. glumae*. An SYBR Green real-time PCR primer set was designed based on the *rhs* family gene (YD repeat protein) of *B. glumae* BGR1 because these genes are structurally diverse. The specificity of the primers was evaluated using purified DNA from 5 isolates of *B. glumae*, 6 different species of *Burkholderia*, and 18 other reference pathogenic bacteria. The assay was able to detect at least one genome equivalent of cloned amplified target DNA using purified DNA or 1 CFU per reaction when using calibrated cell suspension. This method is rapid and reliable and has great potential for analyzing large numbers of samples.

Bacterial grain rot of rice caused by the bacterium *Burkholderia glumae* is a frequent cause of yield loss on certain cultivars in Korea, Japan, the United States, and other rice-growing areas around the world. The pathogen also causes seedling rot in nursery boxes and is one of the most important bacterial rice pathogens (2,16). Yield losses of 15% are common, and losses of 80% have been recorded in severely infected fields (4). This disease is highly dependent on weather conditions at the flowering stage (19). In recent years, its incidence has increased in rice-growing regions of many Southeast Asian countries, the Americas, and Africa. Meanwhile, it was revealed that *B. gladioli* also causes gain rot and seedling rot, and appeared to cause 20% of bacterial panicle blight in the United States (7,14,20). In addition, it was reported that *B. glumae* is also an important opportunistic human pathogen for chronic granulomatous disease patient (21).

Unfortunately, some rice cultivars currently grown in Korea are susceptible to *B. glumae* to varying degrees. In addition, the control of bacterial grain rot is difficult, and the only practical methods for disease management rely on the use of pathogen-free seed, appropriate culture practices, and resistant cultivars. Therefore, the specific detection of this pathogen in seed is essential for effective control of the disease. However, there have been few efficient detection methods for *B. glumae* from rice thus far. In order to effectively manage the disease and prevent the pathogen from spreading, it is urgent to establish an accurate, rapid, and practical method for detection of *B. glumae* from rice.

Currently, molecular assays based on the 16S-23S rDNA spacer region, *gyrB*, and *rpoD* are used for the detection of *B. glumae* (12,14,17) but there have been critical defects in the diagnosis and identification of *B. glumae* isolates, in that these assays also detect

other *Burkholderia* spp. such as *Ralstonia* spp. because 16S-23S rDNA internal transcribed spacer regions are highly conserved in genomes at a level of species or genus. Therefore, we have investigated *rhs* family genes (YD repeat proteins) to develop more specific primers for identifying *B. glumae*. The nucleotide sequences of the gene were assessed for specificity and variety among *Burkholderia* spp. through BLASTN searches. In this study, we designed species-specific primers based on the *rhs* family gene (YD repeat protein) of *B. glumae* BGR1 and found that the primer set showed high specificity for detecting the pathogen in rice seed. It is reported that *Rhs* repertoires (YD repeat proteins) are ubiquitous and comprise six structurally distinct lineages within the family Enterobacteriaceae, and there is considerable intergenomic variation in *Rhs* repertoire (8). These results revealed that this real-time polymerase chain reaction (PCR)-based method can be used for rapid detection of *B. glumae*, simplifying diagnosis, monitoring of this pathogen, and guiding of plant disease management.

Materials and Methods

Bacterial strains, culture condition, and DNA preparation.

Bacterial strains were obtained from the Korean Agricultural Culture Collection in Republic of Korea, the Belgian Coordinated Collections of Micro-organisms, the National Collection of Plant Pathogenic Bacteria in the United Kingdom, and the American Type Culture Collection (ATCC) in the United States. All microorganisms used in this study are listed in Table 1. The conditions for culturing of all bacterial species were as recommended in the Handbook of Microbiological Media (1). Total DNA from microorganisms was extracted using the bacterial genomic DNA extraction kit supplied by Qiagen (Qiagen, Hilden, Germany). All purified DNAs were quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Primer design and conventional PCR. A primer set was designed for an *rhs* family gene (YD repeat protein) of *B. glumae* BGR1 (GenBank accession number 237878396), with predicted PCR products of 138 bp (Table 2). Conventional PCR assays were performed with a PTC-225 thermocycler (MJ Research, Water-

Corresponding author: D. S. Park, E-mail: dspark@rda.go.kr

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town, MA). All amplifications were carried out in a final volume of 50 µl containing 20 mM Tris-HCl, 100 mM KCl, 50% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5% Tween 20, 0.5% Nonidet P-40, 1 mM PMSF, 0.2 mM each dNTP, 20 pM each primer, 2 units of *Taq* polymerase (SolGent, Daejeon, Republic of Korea), and approximately 25 ng of genomic DNA from a given microorganism. Reactions were run for 35 cycles, each consisting of 60 s at 94°C, 30 s at 63°C, and 60 s at 72°C, with an initial denaturation of 5 min at 94°C and a final extension of 10 min at 72°C. A 10-µl aliquot of each amplified PCR product was electrophoresed on a 1.5% agarose gel, stained with LoadingStar (DYNEBIO, Republic of Korea), and visualized with a UV transilluminator.

SYBR Green real-time PCR assay. The SYBR Green real-time PCR assay was performed in a 20-µl reaction volume containing KAPA SYBR FAST qPCR Master Mix (2×) Universal (KAPA Biosystem, Inc.). Each PCR reaction contained 10 µl of KAPA SYBR qPCR Master MIX Universal, 5 pM each BG1F/R, and 5 ng of cloned DNA from each sample. Amplification was carried out using the CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA), and the thermal cycling parameters were as follows: 95°C for 3 min, 45 cycles of 95°C for 10 s and 63°C for 20 s, and a melting curve of 65 to 95°C with an increment of 0.5°C. A copy number of the cloned DNA was calculated with the following equation: copies/µl = $[6.022 \times 10^{23} \text{ (copy/mol)} \times \text{amount (g)}] / [\text{length (bp)} \times 660 \text{ (g/mol/bp)}] \text{ (5)}.$

Detection of pathogen by Bio-PCR. The rice (*Oryza sativa* ‘Odae’) seed affected with bacterial grain rot were collected from Icheon, Gyeonggi province, Republic of Korea in August 2011. Depending on the degree of grain rot severity in rice seed, five grains from naturally infected seed lots sampled in rice fields were selected and each grain was soaked in 500 µl of sterile distilled water for 30 min. Then, 2 µl from the water treated with each single grain was used in SYBR Green real-time and conventional PCR assays as described above.

Results

Sequence specificity of designed primers. Specificity of the primer set designed from an *rhs* family gene (GenBank accession number 237878396) was tested in silico by similarity search against the sequence database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). It showed some significant matches with previously determined sequences (BLASTN ver. 2.2.25+). BLASTN searches showed similarity to *rhs* family protein (identity = 79%, score = 3,065 bits [3,398], and expect = 0.0) from *B. multivorans* ATCC 17616. BLAST searches with the predicted protein sequence (BLASTX) revealed similarity to YD repeat-containing protein (identity = 81%, score = 1,988 bits [5,150], and expect = 0.0) from *B. multivorans* ATCC 17616. However, the C-terminal sequence region to be amplified with the designed primer set (Table 2)

Table 1. Bacterial strains used in this study and results by conventional polymerase chain reaction assays

Scientific name ^b	Collection ^c	Origin	Primer sets ^a		
			BG1F/R	Glu-FW/Glu-RW (12)	Forward/reverse (17)
<i>Burkholderia glumae</i> ^T	LMG 2196	Japan	+	+	+
<i>B. glumae</i>	LMG 6952	Japan	+	+	+
<i>B. glumae</i>	LMG 10905	Japan	+	+	+
<i>B. glumae</i>	LMG 10906	Japan	+	+	+
<i>B. glumae</i>	LMG 1277	Japan	+	+	+
<i>B. gladioli</i> ^T	LMG 2216	United States	–	–	–
<i>B. plantarii</i> ^T	LMG 9035	Japan	–	+	–
<i>B. cepacia</i> ^T	LMG 1222	United States	–	–	–
<i>B. caryophylli</i> ^T	LMG 2155	United States	–	–	+
<i>B. andropogonis</i> ^T	LMG 2129	United States	–	–	+
<i>B. cenocepacia</i> ^T	KACC 12021	United Kingdom	–	–	+
<i>Acidovorax avenae</i> subsp. <i>avenae</i> ^T	NCPPB 1011	Unknown	–	–	–
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> ^T	LMG 5047	India	–	–	–
<i>X. oryzae</i> pv. <i>oryzicola</i> ^T	LMG 797	Malaysia	–	–	–
<i>X. campestris</i> pv. <i>campestris</i> ^T	LMG 568	United Kingdom	–	–	–
<i>Pseudomonas syringae</i> pv. <i>oryzae</i> ^T	KACC 10133	Unknown	–	–	+
<i>P. syringae</i> pv. <i>japonica</i> ^T	LMG 5068	Japan	–	–	–
<i>P. jesseni</i> ^T	ATCC 700870	France	–	–	–
<i>P. libanensis</i> ^T	KACC 10809	Lebanon	–	–	–
<i>P. mucidolens</i> ^T	LMG 2223	United States	–	–	–
<i>P. oryzae</i> pv. <i>oryzicola</i> ^T	LMG 7040	Japan	–	–	–
<i>P. fuscovaginae</i> ^T	LMG 2158	Japan	–	–	–
<i>P. ludensis</i> ^T	LMG 13517	Unknown	–	–	–
<i>Ralstonia solanasearum</i> ^T	LMG 2299	US	–	–	–
<i>Erwinia persicina</i> ^T	LMG 11254	United Kingdom	–	–	–
<i>E. tracheiphila</i> ^T	LMG 2707	Japan	–	–	–
<i>Pectobacterium chrysanthemi</i> ^T	LMG 2804	United States	–	–	–
<i>P. carotovora</i> subsp. <i>carotovorum</i> ^T	LMG 2404	Denmark	–	–	–
<i>Escherichia coli</i> ^T	LMG 2092	Unknown	–	–	–

^a BG1F/R, this study; + and – indicates species was detected or not detected, respectively.

^b T = type strain.

^c Collection number: LMG, The Belgian Coordinated Collections of Micro-organisms, Belgium; KACC, Korean Agricultural Culture Collection, Republic of Korea; NCPPB, The National Collection of Plant Pathogenic Bacteria, United Kingdom; ATCC, The American Type Culture Collection, United States.

Table 2. Primers used in this study

Primer	Sequences (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	References
BG1F	CCGCGCTGTTTCATGAGGGATAA	63	138	This study
BG1R	CGGGCGGAACGACGGTAAGT	...	...	This study
Forward	ACGTTTCAGGGATCTGAGCAG	60	282	17
Reverse	AGTCTGTCTCGCTCTCCCGA	...	...	...
Glu-FW	GAAGTGTGCGCGATGGAG	63	529	12
Glu-RW	CCTTCACCGACAGCACGCAT	...	...	...

revealed no significant matches in both BLASTN and BLASTX searches.

Specificity test of primers. The primer set BG1F/R was tested by conventional PCR against five isolates of *B. glumae*. As expected, a 138-bp DNA fragment was amplified (Fig. 1). To check the specificity of the primers, a large collection of other microorganisms, including other *Burkholderia* spp., was tested by SYBR Green real-time PCR with the BG1F/R primer set. All *B. glumae* strains gave a consistent positive result regardless of the existence of other species of the pathogen; only *B. glumae* strains showed a single amplified DNA fragment (Table 1).

Sensitivity test by SYBR Green real-time PCR. The SYBR Green real-time PCR standard curve constructed by plotting the mean threshold cycle (C_T) ($n = 3$) versus logarithmic concentrations of (ranging from 5×10^6 to 5×10^0 fg/ μ l; cloned DNA) displayed a good linearity of response ($R^2 = 0.994$). The detection limit of the reaction was less than 5×10^0 plasmid copies of *B. glumae*. Standard regression analyses of the linear part of the slope gave a coefficient of -2.972 and the PCR efficiency calculated from the slope of the standard curve was 117.0%. Analysis of

melting temperature (T_m) and melting peak on SYBR Green real-time PCR of *B. glumae* showed a reproducible T_m of 81.50°C specific peaks. A sensitivity test was performed using a 10-fold dilution series of genomic DNA and cell suspension of the pathogen. The minimum detection level of genomic DNA and cell suspension by SYBR green real-time PCR assay was

Table 3. Mean threshold cycle (C_T), end-point fluorescence, and 10-fold serially diluted *Burkholderia glumae* genomic DNA and cell suspensions by SYBR Green real-time polymerase chain reaction

Genomic DNA		Cell suspensions	
fg/ μ l	C_T values	CFU/ml	C_T values
5×10^6	16.24 ± 0.25	1.0×10^8	18.65 ± 0.41
5×10^5	18.75 ± 0.03	1.0×10^7	21.33 ± 0.26
5×10^4	22.22 ± 0.08	1.0×10^6	24.43 ± 0.08
5×10^3	25.85 ± 0.08	1.0×10^5	27.98 ± 0.31
5×10^2	28.91 ± 0.2	1.0×10^4	31.56 ± 0.40
5×10^1	32.41 ± 0.2	1.0×10^3	34.98 ± 0.59
5×10^0	35.00 ± 0.3	1.0×10^2	37.25 ± 0.87

Fig. 1. Specific detection of *Burkholderia glumae* by conventional polymerase chain reaction. Lane M, size marker (1 kb plus DNA ladder); lanes 1–5, *B. glumae*; lanes 6–29, listed in Table 2; lane 30, negative control (distilled water).

Fig. 2. Naturally infected rice grains and specific detection of *Burkholderia glumae* by SYBR Green real-time polymerase chain reaction (PCR). **A**, Severity of disease symptoms and nonsymptoms in rice grain. **B**, Fluorescence intensity as function of templates. For each assay, a series of rice grains were used as the template for PCR. **C**, Melting curve analysis showed a reproducible melting temperature of 81.50°C and specific peaks. RFU = relative fluorescence units. (P, *Burkholderia glumae* genomic DNA; 1, nonsymptomatic sample; 2–5, symptomatic samples; N, no template).

approximately 5×10^0 fg/ul copies and 1×10^2 CFU/ml of *B. glumae*, respectively. Standard curves showed that there was a linear correlation between the C_T values and the concentrations of input DNA or cell suspension, which ranged from genomic DNA ($R^2 = 0.997$, slope = -3.225) to cell suspension ($R^2 = 0.993$, slope = -3.222) of the pathogen (Table 3).

Detection of the pathogen by Bio-PCR in infected rice seed. The primer set developed in this study was tested against *B. glumae*. The detection results of *B. glumae* from each grain sampled from naturally infected seed lots were presented (Fig. 2). As expected, a 138-bp DNA fragment from the infected grain samples was amplified by real-time PCR. On the other hand, no single DNA fragment was amplified from the control.

Discussion

The results presented in this study describe the development of effective assays for the reliable detection of *B. glumae*. Although the classical microbiological techniques currently in use for *B. glumae* detection and identification are satisfactory in most situations, more rapid and reliable tests may be useful in some specific situations (10,13). Detection methodologies for pathogenic bacteria have benefited greatly from modern molecular technology involving specific amplification of target DNA fragments (3,9,17).

Recently, development of specific primers from the 16S-23S rDNA spacer region for identification and detection has been reported for *B. glumae* (4,17,18). However, the molecular identification of *Burkholderia* spp. is often difficult and controversial (3,15). The sequence analyses of the 16S rRNA gene, *gyrB*, and *rpoD* are widely employed for the identification of bacteria (6,7,11,12). However, these regions are insufficient to discriminate between the species of *Burkholderia* (12,17).

In fact, detection of pathogen in nonsymptomatic seedlings is of importance in certification programs, for both domestic and international quarantine, because a latent population can lead to serious epidemics under favorable conditions. Early detection of diseases is important for assessing the health status of a rice nursery before the transplantation of seedlings to fields.

Therefore, in this study, species-specific primers from the whole-genome sequences of *B. glumae* BGR1 (GenBank accession number CP001504.1), deposited in GenBank (<ftp://ftp.ncbi.nih.gov/genomes/Bacteria/>), were designed. The *rhs* family gene (YD repeat protein) (GenBank accession number 237878396) was analyzed by BLAST searches and found to be highly variable in different bacteria at the species level.

It is reported that *Rhs* repertoires (YD repeat protein) are ubiquitous and comprise six structurally distinct lineages within the family Enterobacteriaceae, and there is considerable intergenomic variation in *Rhs* repertoire (8). Successful detection of different strains of *B. glumae* but not other phytopathogenic bacteria further suggests that the specificity of primer set BG1F/R to this pathogen may be due to its unique sequence.

There is a common problem in the appearance of unspecific amplification bands or curves when cell suspensions or tissue extracts are used directly for PCR, hampering interpretation of the results. However, no spurious band (curve) was observed in any of the samples analyzed in this investigation using this primer set. This result indicates that the primer set developed through the bioinformatics method is highly specific to *B. glumae* and reliable for the detection and diagnosis of this pathogen.

Acknowledgments

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