Species-Specific PCR as a Tool for the Identification of *Burkholderia gladioli*

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Burkholderia gladioli colonizes the respiratory tracts of patients with cystic fibrosis and chronic granulomatous disease. However, due to the high degree of phenotypic similarity between this species and closely related species in the *Burkholderia cepacia* complex, accurate identification is difficult. Incorrect identification of these species may have serious repercussions for the management of patients with cystic fibrosis. To develop an accurate procedure for the identification of *B. gladioli*, a molecular method to discriminate between this species and other species commonly isolated from the sputa of patients with cystic fibrosis was investigated. The 23S ribosomal DNA was cloned from several clinical isolates of *B. gladioli*, and the nucleotide sequence was determined. Computer-assisted sequence comparisons indicated four regions of the 23S rRNA specific for this species; these regions were used to design three primer pairs for species-specific PCR. Two of the primer pairs showed 100% sensitivity and specificity for *B. gladioli* when tested against a panel of 47 isolates comprising 19 *B. gladioli* isolates and 28 isolates of 16 other bacterial species. One of the primer pairs was further assessed for species specificity by using a panel of 102 isolates obtained from the *Burkholderia cepacia* Research Laboratory and Repository. The species-specific PCR was positive for 70 of 74 isolates of *B. gladioli* and was negative for all other bacterial species examined. Overall, this primer pair displayed a sensitivity and specificity of 96% (89 of 93) and 100%, respectively. These data demonstrate the potential of species-specific PCR for the identification of *B. gladioli*.

Burkholderia gladioli (basonym, *Pseudomonas gladioli* or *Pseudomonas marginata*) was originally described in 1921 as a phytopathogen of gladioli and other flowers (11). Initially considered a harmless commensal colonizing the respiratory tracts of cystic fibrosis (CF) patients, *B. gladioli* has recently been associated with a number of pulmonary infections in both CF (4) and immunocompromised patients (12, 13). However, the extent to which this organism contributes to disease in CF patients is unclear. Recently Khan and colleagues (7) reported empyema and bacteremia caused by *B. gladioli*; however, the infection was subsequent to lung transplantation, during which the pleural cavity was inadvertently seeded. Evidence of pathogenicity is more forthcoming in patients with chronic granulomatous disease; several reports describe *B. gladioli* pneumonia (12) and severe sepsis (5).

Currently, there is great interest in the taxonomy of the genus *Burkholderia*, primarily focused on the characterization of the species of the *Burkholderia cepacia* complex. Since the designation of five distinct genomovars within this group (14), it is becoming increasingly apparent that the members of genomovar III are responsible for the majority of deaths of patients with CF ascribed to “*cepacia* syndrome” (14). Therefore, it is important that clinical microbiology laboratories be able to correctly distinguish members of the *B. cepacia* complex from other closely related species. To facilitate this identification, the *Burkholderia cepacia* Research Laboratory and Repository (BcRLR) tests all forwarded strains; a high incidence of misidentified isolates (10%) has been detected (9). Of the isolates

misidentified as *B. cepacia*, approximately 50% are confirmed by the BcRLR as *B. gladioli* (data not shown).

Misidentification of closely related bacteria creates significant clinical dilemmas. Identification may be inaccurate due to the high degree of similarity between the *B. cepacia* complex and *B. gladioli*, as well as the existence of strains which appear to be hybrids of the two species (2, 13) and an inability of commercial tests to provide definitive identifications of the related species (8). Some CF centers treat *B. cepacia*-positive patients as a cohort, and incorrect identification of isolates could result in exposure of non-*B. cepacia*-colonized individuals to patients colonized with *B. cepacia*. Misidentification may also have a negative impact on treatment, transplant eligibility, and perceived disease progression. To facilitate the accurate and rapid identification of *B. gladioli*, we developed a *B. gladioli* species-specific PCR (SS-PCR) method.

MATERIALS AND METHODS

Bacterial strains. The specificity of this method was tested by using bacterial species known to colonize CF patients and other *Pseudomonas* species of medical importance. The following microbial species were tested: *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas stutzeri* ATCC 17588, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 29906, *Stenotrophomonas maltophilia* ATCC 13637, *Moraxella catarrhalis* ATCC 25238, *B. gladioli* ATCC 10854, ATCC 19302, and ATCC 10248, *Ralstonia solanacearum* ATCC 10692 and ATCC 11696, *Ralstonia pickettii* ATCC 27511, *Burkholderia caryophylli* ATCC 25418 and ATCC 11441, a clinical isolate of *Pseudomonas aeruginosa*, and a clinical isolate of *Acinetobacter anitratus*. In addition, a total of 16 clinical *B. gladioli* isolates, 13 *B. cepacia* complex isolates, and 2 *R. pickettii* isolates were used to establish sensitivity and specificity (for simplicity, the five genomovars of the *B. cepacia* complex are treated as separate species within this article). The identities of the clinical isolates of *B. cepacia* complex were confirmed at the BcRLR (9). The identities of the clinical *B. gladioli* isolates were confirmed by using *Burkholderia*/*Ralstonia*-specific PCR (9) in conjunction with biochemical reactions: oxidase negative, *o*-nitrophenyl- β -D-galactopyranoside (ONPG) positive, lysine decarboxylase negative, and no oxidation of lactose or sucrose. When the identity was

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TABLE 1. Oligonucleotide primers selected for PCR

Primer	Sequence ^a	Target site ^b
LP1	GGGGGGTCCATTGCG	872→886
LP2	TAGCAAGCTTAACCGA	595→610
LP3	CTGCGGATGCACATTTATG	1151→1170
LP4	AGAAGCTCGCGCCACG	1523→1508
23RNA11	GGGACTTCAATCAAGAGCTTGCAC	1869→1846
23RNA5	GGTCAAGCGAACAAGTGC	1→18
23RNA3	AGGATCAAGCCTTACGGGC	2865→2847

^a Underlined nucleotides are putatively specific for *B. gladioli*.

^b Numbering corresponds to the *B. gladioli* 23S rRNA nucleotide sequence (EMBL accession no. Y17182). The arrow indicates the direction of the oligonucleotide (5' to 3').

still questionable, it was confirmed by whole-cell protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by LiPuma et al. (9).

Isolation of genomic DNA. Genomic DNA was purified from cultures of each individual strain with the QIAamp Tissue Kit (QIAGEN, Valencia, Calif.). DNAs were resuspended in sterile high-pressure liquid chromatography-grade water at a final concentration of 1 µg/ml.

Cloning of the 23S ribosomal DNA and determination of the nucleotide sequence. PCR primers were designed based on published *B. cepacia* 23S rRNA gene sequence data (6) (Table 1). These primers, 23RNA3 and 23RNA5, were used in separate PCRs to amplify an approximately 2,900-bp product from three *B. gladioli* clinical isolates designated BG2, BG3, and BG4. The products were cloned into the pCR-2.1 TOPO vector (Topo TA Cloning Kit; Invitrogen, Carlsbad, Calif.) to produce pBG2A, pBG3A, and pBG4A. The insert of each clone was sequenced with an ABI 373 automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.). DNA sequences were assembled and analyzed with the GCG Wisconsin Package (Genetics Computer Group Inc., Madison, Wis.).

Development of *B. gladioli*-specific PCR. The *B. gladioli* 23S rRNA consensus nucleotide sequence was determined from the inserts of pBG2A, pBG3A, and pBG4A. Differences between this consensus and the published *B. cepacia* 23S rRNA gene sequence (6) were determined. These regions were further analyzed with the BLASTN nucleotide sequence alignment algorithm to determine if the putatively specific *B. gladioli* regions had homology with other 23S rRNA genes. Four regions putatively specific for *B. gladioli* were determined. Oligonucleotide PCR primers were designed at these regions, incorporating the *B. gladioli*-specific nucleotides at the 3' end; these were designated LP1, LP2, LP3, and LP4 (Table 1). An additional primer, 23RNA11, was used. This primer is complementary to a conserved region of the 23S rRNA gene. LP1, LP2, and LP3 initiate extension in the same direction, while LP4 and 23RNA11 initiate extension in the opposite direction. Individual PCRs were optimized for the primer pairs LP1-23RNA11, LP2-23RNA11, and LP3-23RNA11 by using genomic DNA derived from BG2 and BG4. PCRs were performed with the Rapid Cycler thermocycler (Idaho Technologies, Idaho Falls, Idaho). All PCRs had an initial denaturation of 95°C for 5 min with a subsequent 30-cycle amplification, and the PCR mixture contained 1 µM each primer, 10 ng of genomic DNA, 200 µM each deoxynucleoside triphosphate, and 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.) in a 3 mM MgCl₂ PCR buffer (Idaho Technologies), in a total volume of 50 µl. For LP1-23RNA11, the cycle parameters consisted of annealing at 69°C for 10 s, extension at 72°C for 60 s, and denaturation at 95°C for 10 s. For the primers LP2-23RNA11 and LP3-23RNA11, the PCR parameters consisted of annealing at 60°C for 10 s, extension at 72°C for 60 s, and denaturation at 95°C for 10 s. Similarly, reactions were optimized for the LP1-LP4, LP2-LP4, and LP3-LP4 primer pairs. For these primer pairs the PCR parameters were identical: annealing at 60°C for 10 s, denaturation at 95°C for 10 s, and extension at 72°C for 60 s. Following amplification, 20 µl of each reaction mixture was subjected to electrophoresis in a 0.8% agarose gel in 0.5× Tris-borate-EDTA buffer (pH 8.0) alongside a 100-bp molecular ladder. The PCR products were visualized and photographed after ethidium bromide staining. Positive results were assessed by the amplification of a band of the correct size for the primer pair used. For LP1-LP4, LP2-LP4, and LP3-LP4, the amplified bands were 700, 900, and 400 bp, respectively. Samples that failed to amplify a product with these primer pairs were further analyzed with the PSL and PSR primers (3) as a control.

RESULTS

Cloning and sequencing of the 23S rRNA gene. The 2.9-kb 23S rRNA genes from three independent clinical isolates of *B. gladioli*, were amplified, and the products were cloned into the TOPO TA vector (Invitrogen) and sequenced in both directions. The resulting nucleotide sequences were aligned by

using the GCG PILEUP algorithm, yielding a consensus sequence for the *B. gladioli* 23S rRNA gene.

Development of a *B. gladioli*-specific PCR. The consensus sequence for the *B. gladioli* 23S rRNA gene was compared with the published sequence for the *B. cepacia* 23S rRNA gene described by Hopfl et al. (6). Four regions of the 23S rRNA were putatively specific for *B. gladioli*. Oligonucleotides were designed at these regions to encompass the variable nucleotide(s) at the 3' end for use in SS-PCR. These primers were named LP1, LP2, LP3, and LP4 (Table 1).

The specificities of LP1, LP2, and LP3 were tested by performing individual PCRs with a second oligonucleotide primer, 23RNA11, designed to hybridize to a conserved region of *B. gladioli* and *B. cepacia*. Initially, template DNA for the PCR was purified genomic DNA from two clinical isolates of *B. gladioli* and two clinical isolates of *B. cepacia*. Following optimization of the PCRs, each primer pair was subsequently tested, blindly, against a panel of 47 different isolates representing 17 species; at least one isolate of each of the five genomovars of the *B. cepacia* complex was included in this study. PCRs with the primer pair LP1-23RNA11 identified all the *B. gladioli* isolates; however, this primer pair also gave positive results for seven *B. cepacia* isolates. LP2-23RNA11 also gave positive results for all the *B. gladioli* isolates; however, this primer pair gave a positive result with one of the *B. caryophylli* isolates, two *B. cepacia* isolates, and three *R. pickettii* isolates. LP3-23RNA11 was the only primer pair that did not identify all the *B. gladioli* isolates, missing 2 of 19. This primer pair also gave a positive result with one isolate of *B. cepacia*. Interestingly, this was the *B. cepacia* isolate also identified by primer pairs LP1-23RNA11 and LP2-23RNA11.

To improve the sensitivity, the PCRs were repeated, again blindly, with the putatively *B. gladioli*-specific primer LP4 substituted for the universal primer 23RNA11. Both primer pairs LP1-LP4 and LP2-LP4 yielded identical results, identifying all of the 19 *B. gladioli* isolates and 1 of the *B. cepacia* isolates. LP3-LP4 gave negative results for the same two *B. gladioli* isolates that were negative with the LP3-23RNA11 primer pair and gave false-positive results for isolates (one each) of *P. aeruginosa*, *R. solanacearum*, *R. pickettii*, and *B. cepacia*. The latter isolate was PCR positive with all primer pairs tested. The results of the PCRs are shown in Table 2.

To further investigate this isolate, 10 single colonies were isolated and each was subjected to PCR using both *B. gladioli*- and *B. cepacia*-specific primers (10, 15). Of these, eight were positive with the *B. cepacia* primers while two were positive with both the LP1-LP4 and the LP2-LP4 primer pair. No isolate was positive with both *B. gladioli*- and *B. cepacia*-specific PCR. Thus, we conclude that the original isolate was a mixed culture of these two species.

The performance of the *B. gladioli* SS-PCR was further assessed by the BcRLR, which blindly analyzed isolates from its collection. A total of 102 clinical isolates, consisting of 74 *B. gladioli* isolates and 28 other isolates from CF patients, were examined. The SS-PCR with LP1-LP4 correctly identified 70 of the *B. gladioli* isolates and was uniformly negative for the other isolates.

DISCUSSION

Previous attempts to distinguish *B. gladioli* from the closely related species *B. cepacia* used biochemical tests. However, these tests are inaccurate in differentiating the species but have shown how closely they resemble each other. (2, 13). The failure of such an approach, coupled with the success of molecular approaches to distinguish the five genomovars of the

TABLE 2. Identification of *B. gladioli* by PCR

Species ^a	No. of isolates with the indicated PCR result	Identification as <i>B. gladioli</i> by PCR with the indicated primer pair					
		23RNA11 and:			LP4 and:		
		LP1	LP2	LP3	LP1	LP2	LP3
<i>B. gladioli</i>	15	+	+	+	+	+	+
	2	+	+	—	+	+	+
	2	+	+	—	+	+	—
<i>B. cepacia</i> complex	6	—	—	—	—	—	—
	5	+	—	—	—	—	—
	1	+	+	—	—	—	—
	1	+	+	+	+	+	+
<i>B. caryophylli</i>	1	—	+	—	—	—	—
<i>R. solanacearum</i>	1	—	—	—	—	—	+
<i>R. pickettii</i>	2	—	+	—	—	—	—
	1	—	+	—	—	—	+
<i>P. aeruginosa</i>	1	—	—	—	—	—	+

^a The identity of each isolate was confirmed by the BcRLR. Other isolates tested include the following: *R. solanacearum*, *B. caryophylli* ATCC 25418, *P. stutzeri* ATCC 17588, *P. mirabilis* ATCC 29906, *K. pneumoniae* ATCC 13883, *S. maltophilia* ATCC 13637, *P. fluorescens* ATCC 13525, *A. anitratus*, and *M. carthagenensis* ATCC 25238. All were negative in all PCRs.

B. cepacia complex (10, 15), led us to examine the possibility of a molecular analysis for the identification of *B. gladioli*.

During the development of the protocols described in this report, Bauernfeind et al. published a molecular method for distinguishing *B. gladioli* from other *Burkholderia* spp. by SS-PCR of the 16S and 23S rRNA genes (1). This report identifies a single site showing interspecies variation in the 23S rRNA, at the 45a/b variable region. Our study identified three additional variable regions. The site identified by Bauernfeind et al. corresponds to the region of variability that primer LP3 targets. While this region has the largest number of nucleotide differences between *B. gladioli* and *B. cepacia*, in our study it turned out to be the least sensitive when analyzed in PCRs using the LP3 primer and the universal primer 23RNA11. This may be due, in part, to the high degree of variability at this site. Primers LP1 and LP2 were designed to be complementary to regions of lower variability; LP1 has two nucleotide positions specific for *B. gladioli*, while LP2 has only the terminal nucleotide specific for *B. gladioli*. Use of these primers in combination with the universal primer 23RNA11 identified species other than *B. gladioli*. However, the combination of the specific primers with another specific primer, LP4, improved both the sensitivity and specificity. Thus, the use of two species-specific primers may be advantageous in SS-PCR.

Strains of *B. gladioli* reported in previous publications were also analyzed. Nine isolates reported by Wilsher et al. in their study on the nosocomial acquisition of *B. gladioli* (17) were tested in PCRs with the primer pairs described above. Of the nine isolates, two were confirmed by the BcRLR to be *B. gladioli*. The other isolates were *B. cepacia* and, in one instance, *P. aeruginosa*. In concordance with the BcRLR findings, all the PCRs detected the two confirmed *B. gladioli* isolates; with the exception of one other isolate, all were uniformly negative for the other species examined. One isolate was determined by the BcRLR to be *B. cepacia* but appeared to be *B. gladioli* by the PCRs. This sample was further analyzed by *B. cepacia*-specific PCR as described by LiPuma et al. and Whitby et al. (10, 16). This isolate was positive with *B. cepacia* complex-specific PCR and was putatively ascribed to genomovar I or IV (data not shown). This was the only sample for which the PCR protocols gave a result that conflicted with the isolate identity. This sample was further analyzed by isolating

10 colonies from the parent culture (10). The results indicated that the stock culture was a mixture of both *B. cepacia* and *B. gladioli*. One isolate that was PCR positive with the *B. gladioli* primers and two that were positive with the *B. cepacia* primers were forwarded to the BcRLR. The reported identities of these isolates correlated with the PCR results, confirming an original mixed culture.

The previous misidentification of some of the organisms used in this study emphasizes the requirement for a highly discriminatory protocol for the accurate identification of bacterial isolates. Without accurate determination of an isolate's identity, it is impossible to determine the epidemiology of infection. In this instance Wilsher et al. (17) may have inadvertently reported person-to-person transmission of *B. cepacia* as *B. gladioli*. This study also demonstrates that the PCR protocols described here are an effective tool for the identification of *B. gladioli*.

A review of the strains forwarded to the BcRLR has indicated a high incidence of misidentified isolates originally designated *B. cepacia* by the clinical laboratory. Of these, approximately 50% have been shown by phenotypic analysis to be *B. gladioli*. By using the SS-PCR with primer pair LP1–LP4, 102 isolates were examined. The SS-PCR accurately identified 70 of 74 *B. gladioli* isolates and was negative for 13 *R. pickettii* isolates, 1 *S. maltophilia* isolate, 1 *Alcaligenes xylosoxidans* isolate, 2 *Pseudomonas* sp. isolates, 8 *B. cepacia* complex isolates, and 3 isolates of unknown identity (data not shown). Overall, the sensitivity and specificity of the LP1–LP4 SS-PCR for *B. gladioli* are 96 and 100%, respectively. In addition, this experiment indicates that the protocol developed for the SS-PCR can be readily performed in different laboratories.

In summary, an SS-PCR for *B. gladioli* has been developed. Use of this technique in combination with phenotypic analysis will facilitate the accurate identification of *B. gladioli*.

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