Papers

Evaluation of three oligonucleotide primer sets in PCR for the identification of *Burkholderia cepacia* and their differentiation from *Burkholderia gladioli*

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Accepted for publication
11 November 1998

Abstract
***Aims*—To evaluate three oligonucleotide primer pairs—two specific for 16S and 23S rRNA sequences of *Burkholderia cepacia*, and the third specific for internal transcribed spacer region of 16S–23S sequences of *B gladioli*—for the identification and differentiation of reference and clinical strains of these and other species.**
***Methods*—The three primers sets were applied in polymerase chain reaction (PCR) to a collection of 177 clinical isolates submitted for identification from diagnostic laboratories as presumed *B cepacia*.**
***Results*—At an annealing temperature of 63°C, all eight *B cepacia* and four *B gladioli* reference strains reacted with their specific primers. *B vandii* was the only other species that was positive with both *B cepacia* primers but five *Burkholderia* or *Ralstonia* species reacted with one of these primers. Seventy eight isolates were typical of *B cepacia* in biochemical tests and 75 of these reacted with specific primers; three, however, were positive with the *B gladioli* primers. Fifteen asaccharolytic isolates were confirmed as *B cepacia* by PCR but other non-fermenting Gram negative species were negative with each of the primers.**
***Conclusions*—PCR using 16S rRNA sequences is recommended for identification of *B cepacia* that give atypical results in biochemical tests.**
(*J Clin Pathol* 1999;52:173–176)

Keywords: PCR; *B cepacia*; cystic fibrosis.

Table 1 Primer sets used for confirmation of B cepacia and B gladioli

Primer set	Ref	Sequence	Target	Amplicon size (bp)
P1	PC1	5'GCTGC GGATG CGTGC TTTGC 3'	23S rRNA	323
	PC2	5'GCCTT CTCCA ATGCA GCGAC3'	*B cepacia*	
P2	PSR1	5'TTTCG AGCAC TCCCG CCTCT CAG3'	16S rRNA	209
	PSL1	5'AACTA GTTGT TGGGG ATTCA TTTC3'	*B cepacia*	
P3	PG1	5'TTCAAT GACAA ACGTT CGGG 3'	ITS rRNA	274
	PG2	5'GCTTT CGCTT GACAG GCC 3'	*B gladioli*	

ITS, internal transcribed spacer.

Since the mid-1980s, *Burkholderia (Pseudomonas) cepacia* has been increasingly associated with respiratory infection in a minority of patients with cystic fibrosis.[1] Some patients show little or no clinical decline following acquisition of the organism but others may succumb to a severe necrotising pneumonia, sometimes with septicaemia which is often rapidly fatal. This clinical problem is further compounded by the innate resistance of the species to many antibiotics and the demonstration of transfer of some strains from patient to patient.[2,3] As a consequence, many cystic fibrosis centres in Europe and North America segregate patients for treatment on the basis of their sputum bacteriology and advise patients with *B cepacia* against social interaction with non-colonised patients.

The clinical and social implications of colonisation with *B cepacia* make rapid and accurate identification of paramount importance. Most clinical laboratories use colistin containing selective media for the isolation and presumptive identification of *B cepacia* but several colistin resistant environmental Gram negative species may grow on these media.[4] Furthermore, traditional methods of identification may wrongly classify closely related spe-

Table 2 Reactions of reference strains of Burkholderia and Ralstonia with primer sets

Species	Strain No	P1	P2	P3
B cepacia	NCTC 10661	+	+	–
B cepacia	NCTC 10744	+	+	–
B cepacia	ATCC 29424	+	+	–
B cepacia	ATCC 25608	+	+	–
B cepacia	ATCC 27515	+	+	–
B cepacia	ATCC 17460	+	+	–
B cepacia	ATCC 25610	+	+	–
B cepacia	ATCC 17765	+	+	–
B gladioli	NCTC 12378	–	–	+
B gladioli	ATCC 25417	–	–	+
B gladioli	ATCC 10247	–	–	–
B gladioli	ATCC 10248	–	–	+
B glumae	LMG 1277	–	+	–
B plantarii	LMG 10908	–	–	–
B vietnamiensis	LMG 6998	+	–	–
B vandii	LMG 16020	+	+	–
R solanacearum	LMG 2295	+	–	–
B andropogonis	LMG 2126	–	–	–
B caryophylli	LMG 2155	–	+	–
B cocovenenans	LMG 11626	+	–	+
R pickettii	NCTC 11149	–	–	–
Ps aeruginosa	NCTC 10332	–	–	–

Primers as in table 1.

*Table 3 PCR reactions of presumptively identified "B cepacia" with primer sets**

Biochemical identification	Number of isolates	P1	P2	P3	No reaction
Typical *B cepacia*	78	75	75	3	0
Asaccharolytic *B cepacia*	15	15	15	0	0
"*B cepacia*"	5	0	0	0	5
B gladioli	11	10	10	0	1
Ps aeruginosa	28	0	0	0	28
Other glucose non-fermenters	24	0	0	0	24
Glucose fermenters	3	0	0	0	3
Not identified	13	0	0	0	13
Total	177	100	100	3	74

*Primer sets as in table 1.
Isolates submitted for identification from diagnostic laboratories.

cies (for example, *B gladioli*), as *B cepacia* and many presumed *B cepacia* cystic fibrosis isolates may give atypical biochemical reactions, making unequivocal identification difficult. Indeed, the Edinburgh epidemic strain of *B cepacia* has been reported to have phenotypic features indicative of both *B cepacia* and *B gladioli*.[5 6]

Tyler *et al* targeted 23S rRNA sequences to generate oligonucleotide primers for amplification of a region specific for *B cepacia* and used sequences from the internal transcribed spacer (ITS) region of 16S–23S rRNA for the identification of *B gladioli*.[7] *B cepacia* specific 16S rRNA sequences were also exploited by Campbell *et al* for rapid polymerase chain reaction (PCR) identification of the species.[8] We have evaluated sets of primers from the above studies on a number of biochemically typical and atypical isolates to determine their value for specific identification and differentiation of *B cepacia* and *B gladioli* from patients with cystic fibrosis.

Methods

BACTERIAL ISOLATES

The respective type strains of *B cepacia*, *B gladioli*, and *Pseudomonas aeruginosa* were obtained from the national collection of type cultures (NCTC) and the American type culture collection (ATCC). Other *Burkholderia* and *Ralstonia* species were provided by the LMG culture collection, Belgium. One hundred and seventy seven clinical isolates from cystic fibrosis patients were collected over 18 months and included those sent to the Laboratory of Hospital Infection for identification or typing. Environmental isolates of *B cepacia* and some representative isolates of the "Edinburgh" epidemic strain of *B cepacia* prevalent in the cystic fibrosis population, were kindly provided by Dr J R W Govan, University of Edinburgh Medical School, Edinburgh, UK.

BIOCHEMICAL IDENTIFICATION

All isolates were tested in the API 20NE gallery (BioMerieux) and examined for Gram stain reaction, motility, hydrolysis of casein, gelatin, starch, tyrosine, Tween 20 and Tween 80, production of DNase, catalase, oxidase, lecithinase, and growth on MacConkey, *B cepacia* selective media (Mast), and polyhydroxybutyrate agars.

PRIMER SETS

Oligonucleotide primer pairs were synthesised by Cruachem (Glasgow, UK) and the sequences are shown in table 1.

POLYMERASE CHAIN REACTION

Crude bacterial DNA was prepared by emulsifying five colonies of 48 hours growth on nutrient agar in 100 µl of sterile distilled water. After vortexing and centrifuging at 13 000 *g* for five minutes, 3 µl of the supernatant were added to 12 µl of water. A water blank (15 µl) was also prepared. The PCR mix contained 100 pmol of each primer, 50 pmol of $MgCl_2$, 2.5 pmol of each of the deoxynucleotide triphosphates, 1.25 units of Taq DNA polymerase, and 2.5 µl of 10× PCR buffer (Life Technologies). In the multiplex PCR, each extra primer used replaced 1 µl of water.

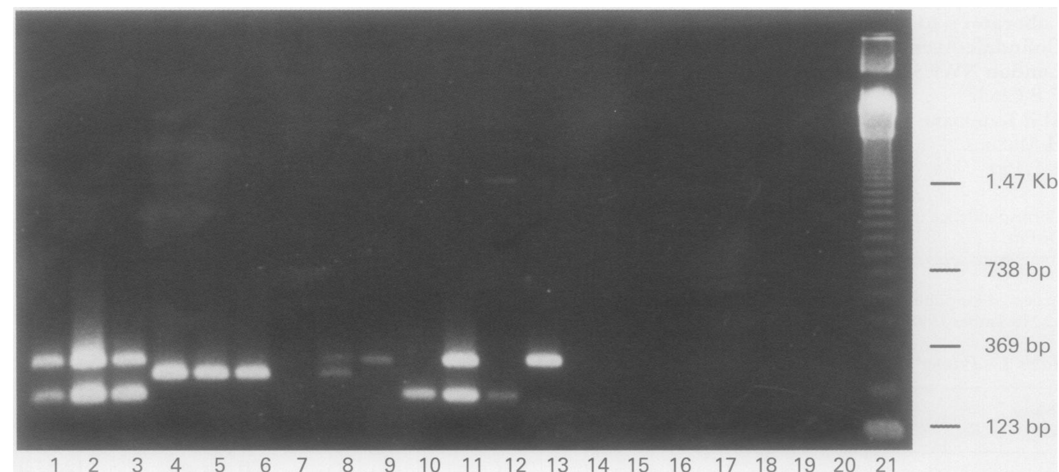

Figure 1 Multiplex PCR with three primers (see table 1) and reference strains of Burkholderia spp and other species. Lane 1: B cepacia NCTC10661; lane 2: B cepacia NCTC10744; lane 3: B cepacia ATCC25610; lane 4: B gladioli NCTC12378; lane 5: B gladioli ATCC10248; lane 6: B gladioli ATCC25417; lane 7: R pickettii NCTC11149; lane 8: B cocovenenans LMG11626; lane 9: R solanacearum LMG2295; lane 10: B caryophylli LMG2155; lane 11: B vandii LMG16020; lane 12: B glumae LMG1277; lane 13: B vietnamiensis LMG6998; lane 14: B plantarii LMG10908; lane 15: B andropogonis LMG2126; lane 16: Ps aeruginosa NCTC10332; lane 17: Ps aeruginosa NCTC10662; lane 18: Acinetobacter baumannii; lane 19: S maltophilia; lane 20: water blank; lane 21: 123 bp size markers.

Amplification was carried out in a Thermal cycler (Hybaid) for 25 cycles. DNA was denatured at 96°C for five minutes for one cycle, and at 96°C for 15 seconds for cycles 2 to 25. The optimum annealing conditions were determined between temperatures of 58°C and 63°C for 30 seconds and primer extension was at 72°C for 90 seconds. An additional primer extension was carried out at 70°C for five minutes. PCR products were separated in a 1.5% Nusieve agarose gel (Flowgen) at 100 V for 1.5 hours and molecular weights were determined by comparison with a 123 bp ladder (Life Technologies).

Results

The reactions of the *Burkholderia* and *Ralstonia* reference strains and *Ps aeruginosa*, with the three sets of primers, are shown in table 2 and illustrated in fig 1 as a multiplex PCR. The optimal annealing temperature for each of the primers was 63°C and all *B cepacia* reference strains produced amplicons with both sets of homologous primers, but not with the *B gladioli* primers and vice versa. However, one reference strain of *B gladioli* failed to react with primer P3. Four other *Burkholderia spp* and the *R solanacearum* reference strain were positive in the PCR with either primer P1 or P2 but *B vandii* reacted with both. *B andropogonis*, *B plantarii*, and *R pickettii* were negative with each of the three primer sets.

Over 18 months, 177 isolates were submitted for species confirmation by clinical diagnostic laboratories as presumed *B cepacia* from cystic fibrosis patients and most isolates grew on the selective medium. Table 3 summarises the results of the PCR with the three primer pairs and shows that 100 isolates reacted with primers P1 and P2. Of these, 75 were typical of *B cepacia* in biochemical tests, 15 were asaccharolytic, and 10 gave biochemical reactions most consistent with an identification of *B gladioli*. None of the latter isolates formed products with primer P3. However, this primer reacted with three biochemically typical *B cepacia* isolates. Five clinical isolates which were negative in the PCR, grew on the selective medium, and were classified by colonial morphology, pigment, and other phenotypic tests as closest to "*B cepacia*" although the API 20NE classified them as "doubtful profile" for the species. The 24 other glucose non-fermenters included alkaline forming pseudomonads and *Stenotrophomonas maltophilia*; 28 strains proved to be *Ps aeruginosa* and three were found to ferment glucose. None of these reacted in the PCR. Ten environmental isolates of *B cepacia* from the Edinburgh Botanical Garden were confirmed by PCR as was the Edinburgh epidemic strain.

Discussion

Yabuuchi *et al* proposed that seven species formerly of pseudomonas RNA group II should be transferred to a new genus, *Burkholderia*, with *B cepacia* as the type species.[9] The genus included *B caryophylli, B gladioli, B mallei, B pseudomallei, R pickettii*, and *R solanacearum*. Recently, the latter two species have been transferred to the genus *Ralstonia*.[10] Two species, *B plantarii* and *B glumae*, were added to the genus on the basis of phenotypic and chemotaxonomic characteristics[11] and they were later joined by *B vandii* and *B cocovenenans*.[12] Gillis *et al* described nitrogen fixing strains of the same rRNA complex as *B cepacia* and named them *B vietnamiensis* because of their isolation from macerates of rice cultured in Vietnam.[13] Two further pseudomonads (*Ps glathei* and *Ps pyrrocinia*) have been proposed for reclassification as *Burkholderia*.[14]

A selective and differential medium[15] is widely used in clinical laboratories for the culture of *B cepacia* from cystic fibrosis sputum, but identification of the species remains problematic. Holmes[16] found that 4% of Gram negative non-glucose fermenting strains sent to the NCTC for identification were *B cepacia*, which suggested that the species occurs regularly in clinical material and that diagnostic laboratories have difficulty in identifying it. Indeed, the five genomic species recently recognised by Vandamme *et al*[14] include strains with asaccharolytic or atypical characteristics that API 20NE fails to identify to confident levels. API 20NE also fails to distinguish between *B cepacia* and *B gladioli*. The latter has been isolated from the cystic fibrosis lung and is generally considered to be more of a hindrance to the identification of *B cepacia* than a pathogen in its own right.[5,17] However, a fatal empyema and bloodstream infection was reported in a patient with cystic fibrosis following lung transplantation.[18]

The three primer pairs tested here were equally sensitive and specific, but for *B cepacia* P2 was preferred for routine use as the product band was invariably clearer than with P1. The absence of reaction by any of the primers with the *R pickettii* type strain or *S maltophilia* is noteworthy as these organisms may on occasion colonise the cystic fibrosis lung. Indeed, Burdge *et al* described three instances of the latter species being misidentified as *B cepacia* in sputum from cystic fibrosis patients.[19] In each case the organisms grew well on selective medium and were initially incorrectly characterised as oxidase positive and DNase negative. Of the strains examined here, fewer than 50% were biochemically typical *B cepacia* and so the confirmation by PCR was essential. It was unexpected to receive *Ps aeruginosa* isolates as presumed *B cepacia* as the former are invariably sensitive to colistin in the selective medium. This may have been because of incorrect colony picks from non-selective media or because colistin resistant *Ps aeruginosa*, albeit rare, occur in cystic fibrosis patients. The five "*B cepacia*" strains may represent genomic groups of *B cepacia* different from those detected with the primers used here and further investigation in this area is warranted.

In summary, the perception and consequences of *B cepacia* colonisation by cystic fibrosis patients and their carers makes it of paramount importance to have unequivocal identification of the species. The PCR method described here is rapid, sensitive and specific in

the light of current taxonomic classification. It is therefore recommended for the confirmation of the identity of isolates presumptively classified as *B cepacia* by biochemical tests.

We thank Dr Wendy Johnson and Shaun Tyler for providing us with sequences and advice before publication of their paper.

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