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Characterisation of *Burkholderia cepacia* from cystic fibrosis patients living in Wales by PCR ribotyping

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Summary. Polymerase chain reaction (PCR) ribotyping detects differences in the intergenic spacer region between the 16S and 23S rRNA genes. This method was applied to *Burkholderia cepacia* isolates from 16 Welsh cystic fibrosis (CF) patients attending three different clinics. Amplification of the intergenic spacer followed by an additional digestion step with *TaqI* restriction endonuclease identified seven distinct electrophoretic patterns among the patient isolates. Each of the seven patterns was distinct from that of the so called “epidemic strain” commonly isolated from patients attending clinics elsewhere in the UK. Two environmental isolates from the hospital clinics and four NCTC reference strains gave different patterns. The simplicity of the method lends itself to use in a general microbiological laboratory.

Introduction

Burkholderia cepacia has emerged recently as an important opportunist respiratory pathogen of cystic fibrosis (CF) patients whose acquisition of *B. cepacia* is associated with a poor prognosis.^{1,2} In Wales, the incidence of *B. cepacia* isolates from CF patients is generally lower than that reported in other UK regions^{3,4} but it has risen in recent years. Consequently, clinical management of the infection has become an increasing priority and an understanding of the epidemiology within this patient population is essential. A number of typing methods—including bacteriocin typing, ribotyping and pulsed field gel electrophoresis (PFGE)—have been applied to *B. cepacia*,^{5–8} but most methods are unsuitable for the routine clinical laboratory because of the complexity of the methods or the need to obtain substantial control and indicator organisms.

In 1992, Kostman *et al.*⁹ showed that epidemiologically unrelated isolates of *B. cepacia* could be differentiated by the amplification patterns of the intergenic spacer regions between the 16S rRNA and 23S rRNA genes. Most bacteria have multiple copies of the ribosomal RNA operon and the length and sequence of the 16S–23S intergenic spacer regions within the operons can differ both between operons on the same genome and between operons within a bacterial species. Therefore, amplification of these

spacer regions yields multiple products which, after electrophoretic separation, yields patterns that may be strain specific.

This method was evaluated for *B. cepacia* isolates from a Welsh CF patient population and the findings are reported here, together with methodological modifications to improve speed and specificity.

Methods and materials

Patients and bacterial isolates

Since 1989, *B. cepacia* has been isolated from nine patients attending the paediatric CF clinic at the University Hospital of Wales, Cardiff, five patients attending the CF adult clinic at Llandough Hospital, Cardiff and two further patients attending clinics in South Wales (Swansea) and West Wales (Carmarthen). One of these patients (patient R, see the table) had shared care with the adult CF clinic in Cardiff and attended that clinic on a bi-annual basis. The organism was first isolated from a sputum specimen collected at the Cardiff adult clinic. The two CF clinics in Cardiff are sited in hospitals on opposite sides of the city and there is no known patient contact between the attendees of each clinic.

B. cepacia was isolated from sputum specimens by culture on *Pseudomonas cepacia* Selective Medium containing ticarcillin and polymyxin B (Mast Diagnostic Ltd, Merseyside) and identified with the API 20 NE test system (bioMérieux). Environmental isolates were obtained from swabs taken from sinks in

Table. Comparison of the modified PCR ribotype with bacteriocin and PFGE typing results

Patient	Clinic	PCR ribotype	TaqI PCR ribotype	Bacteriocin type	PFGE type
A	Cardiff paediatric CF clinic	I	P1	S13/PO mel	1
B	Cardiff paediatric CF clinic	I	P1	S13/PO mel	1
C	Cardiff paediatric CF clinic	I	P1	S13/PO mel	1
D	Cardiff paediatric CF clinic	I	P1	S13/PO mel	1
E	Cardiff paediatric CF clinic	I	P1	S13/PO mel	1
F	Cardiff paediatric CF clinic	I	P1	ND	1
G	Cardiff paediatric CF clinic	II	P2	S22/PO	3
H	Cardiff paediatric CF clinic	III	P3	ND	4
J	Cardiff paediatric CF clinic	I	P1	S13/PO mel	1
K	Swansea paediatric clinic	IV	P4	ND	5
L	Cardiff adult CF clinic	V	A1	S22/PO	2
M	Cardiff adult CF clinic	V	A1	S22/PO	2
N	Cardiff adult CF clinic	II	A2	ND	ND
O	Cardiff adult CF clinic	V	A1	ND	2
P	Cardiff adult CF clinic	V	A1	S22/PO	2
R	Carmarthen with shared care with Cardiff adult CF clinic	VI	A3	ND	6

ND, not determined.

adjacent rooms in the paediatric CF clinic, a sink in the adult CF clinic and from a bacteriological blood culture bottle believed to have been contaminated with *B. cepacia* at point of manufacture and prior to use. In addition, NCTC strains 10661, 10734, 10743 and 10744 were obtained from the National Collection of Type Cultures, 61 Colindale Avenue, London. Also included was a clinical isolate representing the "epidemic strain" described by Govan *et al.*⁵ as the predominant *B. cepacia* strain in patients attending other CF clinics in the UK. All isolates were stored at -70°C in Microbank vials (Pro-Lab Diagnostics, Canada) and subcultured on to the *B. cepacia* selective medium for further study.

Extraction of *B. cepacia* DNA

Genomic DNA was extracted by the Chelex 100 resin method.¹⁰ Briefly, one 5- μl loopful of organisms was harvested from a culture plate and suspended in phosphate-buffered saline, pH 7.3, in a 1.5-ml Eppendorf tube, centrifuged, and the pellet was resuspended in 100 μl of water; 100 μl of a suspension of Chelex 100 (BioRad Laboratories Ltd) 10% w/v in distilled water was added. The mixture was vortex mixed for 15 s, heated in a boiling water bath for 5 min, cooled and then centrifuged at 11 600 *g* for 2 min. The supernate was transferred to a fresh tube and the DNA content was estimated with the aid of a Pharmacia GeneQuant RNA/DNA Calculator (Pharmacia LKB Biochrom Ltd).

During the development of this study, two other methods of DNA template preparation were tried. These methods were the phenol-chloroform extraction method as described by Kostman *et al.*⁹ and a celite-guanidinium thiocyanate absorption method described by Boom *et al.*¹¹

PCR amplification of 16S–23S rRNA intergenic spacer regions

Amplifications were performed in a total volume of 100 μl containing 1.25 U of GeneAmp Taq polymerase (Perkin-Elmer Cetus), a 1 in 10 dilution of the buffer provided with the enzyme, a final magnesium chloride concentration of 1.5 mM, 200 μM of each deoxy-nucleoside triphosphate (100 mM stock solutions from Pharmacia Biotech), 100 pmols each of the primers (1:5'-TTGTACACACCGCCCGTCA-3' and 2:5'-GGTACCTTAGATGTTTCAGTTC-3')⁹ and c. 1 μg of DNA template. Amplification conditions were as described by Kostman *et al.*⁹ and the cycles were performed in a Grant Autogene II Programmable Cycling Water Bath (Grant Instruments (Cambridge) Ltd, Cambridge). Other sources of Taq polymerase were used, including Taq polymerase (Promega) and Dynazyme (Flowgen Instrument Ltd,) with similar results.

Purification of PCR products

To optimise the conditions for the subsequent restriction endonuclease digestion step, it was necessary to both concentrate and transfer the PCR amplification products into water and this was achieved by a modified silica extraction method.¹¹ A suspension of silica particles (Silicon Dioxide; Sigma) 50% w/v in 10 mM HCl was prepared after particles had been washed and fractionated.¹¹ The 100 μl of PCR amplified solution were placed in a 1.5-ml centrifuge tube and 900 μl of guanidinium thiocyanate lysis buffer (4.6 M guanidinium thiocyanate, 20 mM EDTA and Triton X100 1.2% in 50 mM TrisCl buffer, pH 6.4) were added, followed by 10 μl of silica suspension. After a brief vortex mix, the mixture was allowed to stand for 10 min, mixed, and centrifuged at 11 600 *g* for 2 min

and the supernate was carefully discarded. The pellet was resuspended in 1 ml of guanidinium thiocyanate washing buffer (5.6 M guanidinium thiocyanate in 50 mM TrisCl, pH 6.4) centrifuged at 11 600 *g* for 2 min, washed twice with ethanol 70% and any residual ethanol wash was removed. The silica-DNA pellet was resuspended in 30 μ l of distilled water, heated at 56°C for 10 min, centrifuged at 11 600 *g* for 2 min and the supernate containing extracted DNA was transferred to a fresh tube. The pellet was resuspended in 20 μ l of distilled water, mixed, centrifuged as before and the resulting supernate was combined with the other. The DNA content of the combined extract was estimated by the GeneQuant DNA/RNA calculator. Three- μ l amounts were separated by electrophoresis in agarose gel 1.5% in 100 mM Tris, 83 mM boric acid, 1 mM sodium EDTA electrophoresis buffer (TBE buffer) and stained with ethidium bromide 0.05%.

Restriction endonuclease digestion of PCR products

Amplification products were digested with each of eight restriction endonucleases to determine which gave the best discrimination between isolates. Seven endonucleases had a four-base recognition site (*AluI*, *HaeIII*, *HinfI*, *HpaII*, *RsaI*, *Sau3A* and *TaqI*) and one had a five-base recognition site (*DdeI*). All enzymes were obtained from Appligene and used with a 10-fold dilution of appropriate buffer supplied by the manufacturer. In each case, 400 ng of PCR products were digested for 2 h with 5 units of enzyme at 37°C with the exception of *TaqI*, in which case digestion was performed at 65°C. The restriction fragments were analysed by electrophoresis in an agarose gel (Metaphor Agarose; FMC Bioproducts) 2.5% in the TBE electrophoresis buffer, stained with ethidium bromide 0.05% and photographed with UV transillumination.

Alternative methods of typing B. cepacia

The isolates in this study were also typed independently by bacteriocin production and sensitivity⁵ (Professor J. R. W. Govan, University of Edinburgh) and by PFGE under the conditions described by Smith *et al.*⁴ (Dr T. L. Pitt, Central Public Health Laboratory, 61 Colindale Avenue, London).

Results and discussion

In their original method, Kostman *et al.*⁹ extracted DNA from *B. cepacia* grown in liquid medium overnight by classical phenol-chloroform extraction procedures. The extraction method used in the present study, based on the use of a suspension of Chelex 100 and heating in a boiling water bath for 5 min, involves no potentially harmful chemicals and can be performed with small amounts of bacteria (two-to-five colonies) taken from solid media where contaminating organisms can be detected readily. In this study, DNA extracted by phenol-chloroform, celite-guanidinium

thiocyanate absorption or Chelex 100 method gave identical results when used in the subsequent PCR but the latter method was by far the most rapid.

From the typing of the Welsh *B. cepacia* isolates done by other laboratories, it was known that there were at least six strain types present in the isolates from the 16 CF patients (table). However, in contrast with the original report of Kostman *et al.*⁹ and a subsequent report from Dasen *et al.*,¹² little or no differences were found between the PCR ribotyping patterns from the *B. cepacia* strains analysed here. It was not possible to differentiate the types subsequently identified as P3, A2 and the "epidemic strain" (fig. 1) with this method. Increasing the time of separation or concentration of agarose, or both, gave no improvement. The original method is based on the detection of differences in molecular size of the 16S–23S intergenic spacer regions. However, restriction endonuclease digestion of the amplification products with subsequent electrophoretic analysis could detect possible nucleotide base sequence differences and this approach was investigated in the present study. Of the eight restriction endonucleases used to digest the 16S–23S intergenic spacer amplification products from the 16 isolates, three, *TaqI*, *RsaI* and *HpaII*, gave seven different electrophoretic patterns. Pattern differentiation was based on the number of bands and the relative position and intensity of each band within the electrophoretic pattern. *TaqI* digestion produced the largest electrophoretic pattern variation between the seven Welsh strain types and was, therefore, considered the most suitable for epidemiological studies. Three electrophoretic patterns were detected with *TaqI* digestion of PCR products from the nine isolates from the Cardiff paediatric clinic (types P1, P2, P3) and the isolate from the paediatric patient attending the Swansea clinic gave a different electrophoretic pattern (type P4) (fig. 2). Three further distinct electrophoretic patterns (types A1, A2, A3) were obtained from isolates from the patients attending the Cardiff adult clinic. Each of these electrophoretic patterns was clearly distinct from that obtained with the "epidemic strain" of Govan *et al.*⁵ (fig. 2). Within the Cardiff paediatric clinic, seven patients carried the P1 type, and types P2 and P3 were isolated from single patients. Within the Cardiff adult clinic, four patients carried the type A1 strain and types A2 and A3 were isolated from single patients. Because epidemiologically unrelated isolates from the three clinics gave different patterns, the sharing of a similar type such as P1, by patients attending the same clinic is probably indicative of cross-infection between patients. The opportunity for such cross-infection, including concurrent admission to the same ward, has been shown for the paediatric patients carrying the P1 type¹³ and adult patients carrying A1 type (Millar-Jones, unpublished data). Patients carrying a unique type had probably acquired the organism from outside the clinic.

Of the four environmental isolates examined, two from sinks in adjacent rooms in the paediatric out-

Fig. 1. Amplification products of the 16S–23S rRNA intergenic spacer region of the four *B. cepacia* isolate types from paediatric CF patients (P1–4), three *B. cepacia* isolate types from the adult CF patients (A1–3) and the epidemic strain (ES). M, BioRad ampliSize DNA markers.

Fig. 2. *TaqI* digests of the amplification products of the 16S–23S rRNA intergenic spacer region from the four *B. cepacia* isolate types from paediatric CF patients (P1–4), three *B. cepacia* isolate types from the adult CF patients (A1–3) and the epidemic strain (ES). M, BioRad ampliSize DNA markers.

patients clinic (type E1) and one from a sink in the adult out-patients (type E1') were indistinguishable (fig. 3). A fourth organism isolated from a contaminated bacteriological blood culture bottle (type E2) was a different type from the others. The non-clinical isolates were clearly differentiated from the clinical isolates and the four reference strains (fig. 3).

The reproducibility of the method was examined with DNA extracts from five isolates with different

pattern types. In all cases, the profiles obtained after *TaqI* digestion of the PCR products from different target preparations of the same isolate were very similar. However, there was variation in the concentration, although not in the size, of the initial PCR products, despite apparently identical processing. This variation in concentration appeared to be related to the quality of the extracted DNA template. The most consistent results were obtained when fresh cultures

Fig. 3. *TaqI* digests of the amplification products of the 16S–23S rRNA intergenic spacer region from the three environmental *B. cepacia* isolate types from hospital clinics (E1, E1' and E2) and four *B. cepacia* reference isolates (N1, NCTC 10734; N2, NCTC 10743; N3, NCTC 10744; N4, NCTC 10661). M, BioRad ampliSize DNA markers.

were extracted and the target region was amplified within hours of extraction. Generally, PCR amplification with DNA from certain types (P2, A2) gave little or no product 24–48 h after extraction when stored at 4°C. Various extraction methods were used, including the methods described by Kostman *et al.*,⁹ without improving this apparent instability of the extract. However, other types (P4 and A3) could be stored at 4°C for up to 5 days with little effect on PCR amplification. Poor PCR amplification was found frequently from all strains when DNA was extracted from cultures older than 24 h. The reasons for this “inactivation” of the DNA template are unclear but the effect may have implications in the proposed extension of the method to identify *B. cepacia* directly in sputum specimens with simultaneous typing by use of specific primers to the ribosomal RNA gene

complex of *B. cepacia*.¹² A negative result from sputum could indicate the absence of *B. cepacia* or failure to amplify for reasons other than primer mismatch.

The present study showed that PCR ribotyping followed by *TaqI* digestion is simple to perform and provides rapid, reproducible results. In comparison with the other typing methods applied to *B. cepacia* isolates from the Welsh CF patients, the results from this technique were as discriminatory as those obtained by PFGE and more discriminatory than those from bacteriocin typing (table). Currently it is the method of choice in our study of the epidemiology of *B. cepacia*.

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