Pandoraea apista isolated from a patient with cystic fibrosis: problems associated with laboratory identification

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A 17-year-old male patient with cystic fibrosis (CF), with CF mutation homozygous ΔF508, was admitted to the Royal Belfast Hospital for Sick Children in August 2001 for a course of intravenous antibiotics to treat a chronic pulmonary infection. He had an 11-year history of pulmonary infection with both a mucoid and a non-mucoid *Pseudomonas aeruginosa*, which had remained relatively sensitive to several antibiotics used routinely in the treatment of *P. aeruginosa* CF chest infections, including gentamicin, tobramycin, aztreonam, ceftazidime, ciprofloxacin, piperacillin/tazobactam, imipenim and meropenem. During that time he was not reported to have been colonised/infected with any other bacterial organism in his respiratory tract.

On admission, an unidentified Gram-negative rod was isolated in addition to his chronic strains of mucoid and non-mucoid *P. aeruginosa*. This new organism was isolated from freshly expectorated sputum as the sole organism type on *Burkholderia cepacia* selective agar (BCSA; MAST Diagnostics Ltd, Merseyside, UK) following incubation at 37˚C for 48 h. As this was a new isolate growing on BCSA, it was sent for molecular identification and characterisation.

Subsequently, this organism was isolated from the patient's sputum on five occasions over a two-month period, and presently remains a part of the established resident bacterial flora. Semi-quantitative determination of organism numbers demonstrated that it was consistently present at the ++ level, approximating to 10^4 - 10^5 colony forming units(cfu)/g sputum.

Clinically, since the first isolation of this organism from the patient's sputum, there has been deterioration in his clinical status, including increased cough, chest tightness, wheeze, shortness of breath, production of purulent sputum, fatigue and weight loss of 1.8 kg over this two-month period. In addition, his forced vital capacity (FVC) fell from 89% to 77% predicted. Drug therapy during this period included a two-week course of intravenous (iv) antibiotics (ceftazidime and tobramycin), as well as an oral course of ciprofloxacin and nebulised colomycin. He also received a course of oral steroids and itraconazole during this period, as there was some evidence of allergic bronchopulmonary aspergillosis (ABPA).

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Microbiologically, this organism was poorly identified (48% identification) phenotypically by the API 20NE scheme as *Alcaligenes faecalis*, with the profile 0000457. The organism was resistant *in vitro* by standard NCCLS disc diffusion assay to gentamicin, temocillin, ceftazidime, azlocillin, meropenem, aztreonam and colistin, and was sensitive to tobramycin, piperacillin/tazobactam, imipenem and ciprofloxacin.

Subsequently, in order to aid identification, the organism was examined using molecular techniques. All DNA isolation procedures were carried out in a Class II biological safety cabinet in a room geographically separate from that used to set up reaction mixes and also from the 'post PCR' room, in order to minimise false-positive results and in accordance with good molecular diagnostic practice (GMDP), as detailed in the guidelines of Millar *et al*.[1] DNA was extracted from a single colony using the Roche High Purity PCR Template kit (Roche Diagnostics Ltd, UK), following the manufacturer's instructions. All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA, and from the amplification and post-PCR room, in order to minimise contamination.

Reaction mixes (50 μL) were set up as follows: 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 μmol/L (each) dATP, dCTP, dGTP and dTTP, 1.25 units *Thermus aquaticus* (Taq) DNA polymerase (Amplitaq; Perkin Elmer), 0.2 μmol/L (each) of the 16S rRNA primers P11P (forward) 5′ - GAG GAA GGT GGG GAT GAC GT -3′ and P13P (reverse) 5′ - AGG CCC GGG AAC GTA TTC AC -3′, as previously described,[2] and 4 μL DNA template.

Following a hot start, the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96˚C for 3 min, followed by 40 cycles of 96˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min, and a final extension at 72˚C for 10 min. During each run, molecular-grade water was included randomly as a negative control and appropriate DNA template from *Staphylococcus aureus* was included as a positive control. Following amplification, samples (15 μL) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]), stained with ethidium bromide (5 μg/100 mL). Gels were visualised under ultraviolet illumination, using a gel image analysis system (UVP Products, England), and all images archived as digital (*.bmp) graphic files.

Subsequently, amplicons were purified (particularly to remove dNTPs, polymerases, salts and primers) using a QIAquick PCR purification kit (Qiagen Ltd., UK) and eluted in Tris-HCl (10 mmol/L [pH 8.5]) prior to sequencing. Cy-5′-labelled primer (P11P) was prepared and used for sequencing in the forward direction with the ALF Express II (Amersham-Pharmacia Ltd., Bucks, UK) employing the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK; cat no: RPN 2438). Thermal cycling parameters were: 96˚C for 1 min, followed by 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec, 60˚C for 5 sec, followed by a 4˚C hold. Sequences obtained were compared with those stored in the GenBank data system, using BLAST alignment software

Table 1. Evaluation of three sets of *Burkholderia cepacia*-specific primers (PSL1/PSR1, G1/G2 and RHGF/RHGR) to co-amplify *Pandoraea* spp.

	Number of similar bases in BCC primer (% similarity)					
Organism (GenBank accession number)	PSL1[a]	PSR1[a]	G1[b]	G2[b,c]	RHGF[d]	RHGR[c]
P. sputorum (AF139176)	25/25 (100%)	21/23 (91%)	19/20 (95%)	ND	21/21 (100%)	21/21 (100%)
P. pulmonicola (AF139175)	25/25 (100%)	22/23 (96%)	18/20 (90%)	ND	21/21 (100%)	21/21 (100%)
P. pnomenusa (AF139174)	25/25 (100%)	22/23 (96%)	18/20 (90%)	ND	21/21 (100%)	21/21 (100%)
P. apista (AF139173)	25/25 (100%)	22/23 (96%)	18/20 (90%)	ND	21/21 (100%)	21/21 (100%)
P. norimbergensis (AF139171)	25/25 (100%)	20/23 (87%)	18/20 (90%)	ND	20/21 (95%)	21/21 (100%)

a: Campbell et al;[8] b: Whitby et al;[11] c: primer based on 23S rRNA; d: LiPuma et al.[10]

(www.blast.genome.ad.jp). Direct sequencing of the PCR amplicon identified the organisms as *Pandoraea apista*, with 183/183 bases called (100% homology with *P. apista* AF139173 and AF139172 – no other species matched), and subsequently this sequence was deposited in GenBank (accession number AF419312).

The genus *Pandoraea* was described recently by Coenye *et al*.[3] and presently consists of five species. These organisms have been shown to form part of the microbial community in the lungs of CF patients,[3] and diagnostically present several challenges to identification. The majority of isolates described to date have been cultured from BCSA. In the case described here, particular attention was given to the correct identification of the isolate, as the patient was negative for the *B. cepacia* complex (BCC) and it was considered important to rule out BCC, particularly for infection control purposes.

Extended phenotyping schemes, such as the API 20NE system, are unable to identify this organism, as presently the genus is not included in the profile listing, leading to potential misidentification as *A. faecalis*, or alternatively as *A. denitrificans*, CDC IV C2 and *Acinetobacter* spp., as recently described.[4] More recently, this grouping has been renamed as three newly described *Pandoraea* genomospecies.[5]

One solution to aid absolute identification of such organisms would be to include molecular analyses in the diagnostic algorithm. As such organisms are capable of growth on BCSA, the primary consideration is to exclude BCC organisms. Thus, we performed several species-specific PCR assays for BCC, including those associated with the *recA* locus for BCC organisms, as previously described.[6,7] All were negative, demonstrating the presence of a pure culture of *Pandoraea* spp.

Further consideration was given to the possibility of cross-reactivity of other BCC PCR-specific assays, especially those based on 16S ribosomal RNA (rRNA), leading to the potential for molecular misidentification of *Pandoraea* spp. as BCC. Previously, we showed that the PSL1/PSR1 primers, described by Campbell *et al*,[8] also amplify *P. norimbergensis*.[9] As a result, we further investigated other 16S rRNA BCC-specific primer pairs and noted that the RHGF/RHGR primer set described by LiPuma *et al*.[10] would also amplify *Pandoraea* spp. (Table 1).

At this stage, it is unclear whether or not the G1/G2 primer pair described by Whitby *et al*.[11] would amplify *Pandoraea* spp., as 23S rRNA of *Pandoraea* spp. has yet to be described, nor was this primer set initially challenged with *Pandoraea* spp. Thus, we suggest that when a suspect colony resembling *Pandoraea* spp. is isolated in respiratory secretions from a CF patient, a molecular sequence-based approach be adopted, employing broad-range or universal 16S rRNA sequencing, as described here and previously.[12]

Where no (or limited) molecular facilities exist in the primary diagnostic laboratory, such isolates should be forwarded to a reference or specialty laboratory for further examination and confirmation. Recently, a specific PCR technique based on 16S rRNA was described for *Pandoraea* spp.,[13] as well as species-specific primers for the respective species within this genus. Routine employment of such primer sets in diagnostic algorithms may help to avoid potential misidentification of *Pandoraea* spp. as BCC, and the various complications for infection control associated with Gram-negative organisms in CF.

Clinically, there is limited data in the literature describing disease progression associated with the presence of this organism in the airway of CF patients, probably resulting from problems associated with its laboratory identification. In the case described here, it is difficult to separate disease progression resulting from chronic *Pseudomonas aeruginosa* infection with that associated with the *Pandoraea* sp.

In view of the relatively resistant nature of this organism's antibiogram, the patient's progressive clinical deterioration and the ecological establishment of this organism as part of the bacterial flora within the patient's respiratory tract, careful clinical consideration should be given to such cases until more information about disease progression and optimised management are known. However, the opportunity to perform such studies will depend on microbiologists employing extended identification schemes to confirm the presence of these species in patients' sputa. □

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Cloning and sequence analysis of the *recA* gene in urease-positive thermophilic campylobacter (UPTC)

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The *recA* gene is essential for the homologous genetic recombination and for the post-replicative repair of DNA damage, and in responses induced by DNA-damaging agents.[1] Genetic analysis of *recA* in campylobacters has been performed,[2-4] but little work has been done on thermophilic campylobacters.[5]

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Fig. 1. *recA* gene fragments of UPTC amplified using a primer pair *recA* FL-f and *recA* FL-r. Lane KL, 1kb DNA ladder; lane L, 100 bp DNA ladder. Lane 1, UPTC NCTC 12894; lane 2, UPTC CF89-12; lane 3, UPTC A1; lane 4, *C. lari* JCM2530T; lane 5, *C. jejuni* 2013; lane 6, no template DNA (negative-control).

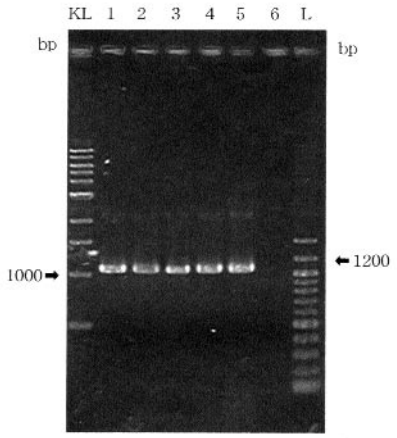

Table I. Origins of campylobacter isolates used in the present study

Isolate No.	Campylobacter	Source	Country
NCTC12894	UPTC	Sea water	England
CF89-I2[12]	UPTC	River water	Japan
AI[13]	UPTC	Seagull	N. Ireland
JCM2530[13]	*C. lari*	Seagull	Japan
JCM20I3	*C. jejuni*	Human	Japan

Urease-positive thermophilic campylobacter (UPTC), a microaerophilic and Gram-negative bacterium, is an organism only relatively recently identified in England.[6,7] After the original description, UPTC isolates were reported in France, Northern Ireland and The Netherlands, and, recently, strains were also found in Japan, where they were characterised both phenotypically and genotypically.[8-13] The aim of the present study is to clone and characterise the *recA* gene in UPTC and *Campylobacter lari*.

Strains of thermophilic campylobacters used in the present study are shown in Table 1. Genomic DNA for polymerase chain reaction (PCR) amplification was prepared by proteinase K treatment, phenol-chloroform extraction and ethanol precipitation.[14]

In the present study, a degenerate primer pair (*recA*FL-f and *recA*FL-r) used for PCR amplification of almost the full-length of the *recA* gene was designed from sequences of the gene in *C. jejuni* 81-176 (U03121)[5] and *C. fetus* 23D (AF020677),[15] taken from EMBL and GenBank. Primer sequences were as follows: *recA*FL-f 5′-GGAAA[A,C,G,T]T[A,C,G,T] ATGGATGATAAT-3′ and *recA*FL-r 5′-[A,C,G,T]A[A,C,G,T]CATTA[A,C,G,T]- TC[A,C, G,T]TCTCCTTC-3′.

PCR mixture contained 10 mmol/L Tris-HCl [pH 9.0], 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.01% gelatine, 0.1% Triton