Inquilinus limosus isolated from a cystic fibrosis patient: first UK report

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A variety of unusual Gram-negative, glucose non-fermentative organisms have been isolated from the respiratory secretions of cystic fibrosis (CF) patients, where their pathogenic role is often considered uncertain.[1] Accurate speciation is therefore important as erroneous identification, particularly for *Burkholderia cepacia* complex (BCC) organisms, can have a significant medical and psychosocial impact for the infected patient, as well as for infection control practices for healthcare workers, hospitals and care centres.[2] *Inquilinus limosus* is such an organism and is a recently described genus and species (2002), previously only isolated from eight CF patients in the USA, two from Germany and five from France.[3–5] To date, *I. limosus* has not been reported in CF patients from either the UK or Ireland.

A Gram-negative mucoid, non-fermenting rod was cultured from the sputum of a nine- year-old girl with CF. This colonial morphotype had been cultured on 12 occasions over a two-year period, during which time the patient had remained clinically well. The child had been treated with three monthly maintenance courses of intravenous antipseudomonal antibiotics (usually ceftazidime and gentamicin) and with bi-monthly courses of nebulised tobramycin.

The isolate had been cultured from routine sputum specimens obtained at presentation to her CF clinic on *B. cepacia* selective agar (BCSA; Mast Diagnostics, Bootle, UK) containing ticarcillin, polymyxin and phenol red. The organism produced a characteristic and very mucoid red colony on BCSA, not typical of *B. cenocepacia*. It was identified as a catalase-positive, oxidase-positive aerobic Gram-negative rod, which grew at 37°C and 42°C. All isolates failed to ferment glucose.

Routine biochemical identification was attempted on the isolates using the API 20NE system (bioMérieux, France). However, unacceptable biochemical profiles were obtained, which included the highest score for *Agrobacterium radiobacter* (API 20NE biochemical profile 1467744 and 1227744, with percentage identifications of 99.8% and 90.2%, respectively). Further phenotypic analysis employing fatty acid analysis showed a very low similarity of the isolate to *Ochrobactrum anthropi*.

Using antibiotic disc sensitivity tests, isolates were consistently colistin-resistant, but were initially sensitive to ceftazidime, ticarcillin, ciprofloxacin, carbenicillin and aminoglycosides when tested on Iso-Sensitest agar. However, antibiotic-resistant variants were noted when a single colony type was retested on blood Iso-Sensitest agar. Subsequently, definite tobramycin resistance emerged on repeat disc testing.

Given the relative difficulties experienced with the phenotypic identification of the organism, the isolate was forwarded for molecular identification by polymerase chain reaction (PCR) amplification and direct sequencing of a large but partial region of the 16S ribosomal RNA (rRNA) gene, corresponding to base positions of approximately 257–1304 of *Stenotrophomonas maltophilia* 16S rRNA (GenBank accession number AY169434).

All DNA isolation procedures were carried out in accordance with the DNA contamination management guidelines of Millar *et al*.[6] and in a class II biological safety cabinet (MicroFlow, England) in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room, in order to minimise contamination and hence the possibility of false-positive results.

Bacterial DNA was extracted from the isolate using the Roche High Purity PCR Template Preparation Kit (Roche, England) in accordance with the manufacturer's instructions. Extracted DNA was transferred to a clean tube and stored at –80°C prior to PCR. For each batch of extractions, a negative extraction control containing all reagents but no isolate was performed. All reaction mixes were set up in a PCR hood.

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 µM (each) dATP, dCTP, dGTP and dTTP, 1.25 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer), 0.2 µmol/L of the appropriate broad-range primers XB1 (forward: 5'–CAG ACT CCT ACG GGA GGC AGC AGT -3') and XB4 (reverse: 5'–GTG TGT ACA AGG CCC GGG AAC -3') and 4 µL DNA template. Prior to PCR cycling, sealed tubes containing DNA template and all PCR reagents were introduced to the thermal cycler at 96°C to avoid non-specific annealing during the initial ramp stage. The reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer Cetus 2400 thermocycler: 96°C for 3 min followed by 40 cycles of 96°C for 1 min, 55°C for 1min, 72°C for 1 min, followed by a final extension at 72°C for 10 min.

During each run, molecular grade water was included randomly as negative controls and DNA template from *Staphylococcus aureus* was included as a positive control, as appropriate. For each batch of extractions, an extraction control containing all reagents minus bacterial organism, was performed. Following amplification, 15-µL samples 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 (UV) illumination using a gel image analysis system (UVP Products, England), and all images were archived as digital graphic (*.bmp) files.

Amplicons chosen for automated sequencing were purified using a QIAquick PCR purification kit (Qiagen, UK) and eluted in Tris–HCl (10 mmol/L; pH 8.5) prior to sequencing to remove dNTPS, polymerases, salts and

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primers. The amplicon was sequenced on the ALF II Express automated sequencer using the primer XB1 and PSL,[5] which was labelled with Cy-5 fluorescent dye (Clarke Stevenson, Oligosynthesis Unit, The Queen's University of Belfast, UK) and used in conjunction with the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit (Amersham, UK). The resulting sequence obtained (1006 bp) was compared with those stored in GenBank using BLAST alignment software (www.ebi.ac.uk), and was deposited in GenBank (accession number AY360342).

On BLAST analysis, in combination with previously reported criteria used for interpretation of partial 16S rRNA gene sequences,[8] the sequence gave a 99% (1003/1006 bases) identification for *I. limosus* (GenBank accession number AY043775).

Phenotypic identification was again inconclusive and gave an API 20NE profile for *Agrobacterium* (*Rhizobium*) *radiobacter*. This organism typically is a plant pathogen and has been isolated only rarely from human specimens, with just two previous descriptions of this organism's association with CF[3,9] Further analysis using molecular methods of identification, as described above, combined with the phenotypic data obtained, allowed definitive identification of the isolate as *I. limosus*.

Although this genus was first described by Coenye *et al*.,[3] the first clinical description was probably by Pitulle *et al*. in 1999.[10] This Nevada/Californian group described a case of severe infection with an unidentifiable "very mucoid Gram-negative rod" isolate classified as an α-Proteobacteria in a 22-year-old female CF patient undergoing lung transplantation. Given the phylogenetic diversity of this isolate, these workers described the isolate as an organism that represented a new genus-level divergence within the bacterial subdivision α-Proteobacteria. It is this isolate that gave the closest phylogenetic match to the isolate in the current study (1004/1006 bases; 99% identity; AF085496).

The increasing incidence of unusual and atypical Gram-negative resistant organisms in patients with CF creates diagnostic, treatment and infection control dilemmas. Several criteria have been proposed to help estimate the clinical significance of such organisms in CF patients.[11] In the present case, a child who was relatively well grew an unusual and atypical Gram-negative organism repeatedly from her sputum, which was identified by molecular techniques.

The primary concern in such cases is that the isolate originated from a selective medium designed to grow the BCC organisms. Phenotypically, however, the isolate on this medium did not resemble *B. cenocepacia* (formerly *B. cepacia* genomovar III), although the colonial appearance on BCSA was typical for *I. limosus*.[4] Hence, it was the local laboratory's primary aim to rule out *B. cenocepacia* by molecular diagnostic methods.

Experienced biomedical scientists in CF microbiology play an invaluable role in helping to give provisional identifications of unusual Gram-negative organisms, especially *B. cenocepacia.* However, misdiagnosis can result in the adoption of a series of inappropriate infection control measures. In parallel with the laboratory identification of such isolates, immediate consideration must be given to appropriate infection control practices involving such patients, prior to a definitive molecular laboratory identification. This can be relatively simple in the CF out-patient setting, where such patients should attend clinics at the end of a session, so that their potential contact with other CF patients is minimised. Where CF centres do operate separate clinics for *Pseudomonas aeruginosa*, *B. cenocepacia* and 'other organisms', such patients should attend the 'other organisms' clinic, or have a separate arrangement made for clinic attendance.

Infection control guidance for in-patients is more problematic, especially when they are not known to be previously colonised with either *P. aeruginosa* or *B. cenocepacia*. In such circumstances, if the possibility of *B. cenocepacia* carriage is low and there is no evidence of clinical deterioration in the patient, it would be reasonable to continue to manage these patients in a non-*B. cepacia* unit, but with increased stringency. This may include ensuring such patients are in a self-contained, single-bedded room with *en suite* facilities, and minimising social contact with other CF patients.

Therefore, the authors suggest that any phenotypically unusual or atypical organism isolated on BCSA medium is identified using the described polyphasic approach, which combines phenotypic and molecular techniques at both the local and specialist reference laboratory level. As the superiority of molecular techniques for the identification of unusual Gram-negative, oxidase-positive rods in CF patients is now well recognised,[12] this point needs to be emphasised when UK microbiology CF guidelines are next revised.

This report highlights the rarity of *I. limosus* isolation in the UK and of the importance of close collaboration between district and specialised reference microbiology laboratories, if misidentification of the increasing number of unusual Gram-negative organisms isolated from CF sputa is to be avoided. This is particularly important when oxidase-positive, colistin-resistant isolates are cultured from BCSA, due to the potential confusion with BCC organisms.

Although clinical experience of *I. limosus* is very limited, the organism does not appear to have caused persistent infection in the case presented. This is consistent with observations from previous case studies.[13] □

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Environmental persistence of *Pseudomonas aeruginosa* and *Burkholderia multivorans* in sea water: preliminary evidence of a viable but non-culturable state

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Cystic fibrosis (CF) is the most common inherited fatal disease in persons of a white and European background, and currently affects approximately 30,000 adults and children in the USA.[1] The defective gene carrying the mutation responsible is carried by one in every 31 Americans (one in 28 Caucasians), which equates to more than 10 million symptomless carriers of the defective gene.[1] It is an autosomal recessive condition whereby two alleles carrying a polymorphism in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene phenotypically manifest the disease state through a variety of multi-organ problems associated with a pharmacological dysfunction to regulate anion (chloride) secretion across cell membranes.

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The most common complication of CF is the recurrence of chronic chest infection usually caused by bacterial pathogens.[2] Cystic fibrosis patients continue to suffer from recurrent and chronic respiratory tract infection and most of their morbidity and mortality is due to such infections throughout their life.[3] These infections are usually dominated by Gram-negative organisms, especially pseudomonads such as *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*. However, modern antibiotic management using improved antimicrobial agents, such as the aminoglycosides and carbapenems, means that CF patients have an improved survival, resulting in more adults in employment.

Water has been documented as an important environmental source of *P. aeruginosa*[4] and has been associated with various clinical episodes of infection, including mainly dermatological infections[4] and otitis externa.[5] Acquisition of *P. aeruginosa* is particularly important for patients with respiratory disorders such as CF and bronchiectasis, as chronic colonisation with this organism has been shown to lead to a poor prognosis.[6] Hence, it is important to establish environmental and clinical reservoirs of this organism, as well as the survival dynamics of Gram-negative pathogens in such environments.

As many CF patients question their healthcare professionals about where they might acquire *P. aeruginosa* and respiratory pathogens in the *Burkholderia cepacia* complex, and how such pathogens survive in these environments, it is the aim of this study to examine the survival of three important Gram-negative bacterial pathogens (*P. aeruginosa*, *B. multivorans* and *B. cenocepacia*) in sea water over a one-year period.

Three Gram-negative organisms were employed in this study, namely *P. aeruginosa* (NCTC 10662), *B. multivorans* (formerly *B. cepacia* genomovar II) and *B. cenocepacia* (formerly *B. cepacia* genomovar III). The *Pseudomonas* isolate was a reference strain (NCTC 10662) and was obtained from the National Collection of Type Cultures, Health Protection Agency, Colindale, London, and the *Burkholderia* isolates were obtained from the sputum of adult CF patients. The identity of all isolates was confirmed initially using the phenotypic API 20NE identification scheme (bioMérieux, France), as well as by molecular techniques including 16S ribosomal DNA (rDNA) polymerase chain reaction (PCR) and automated sequencing, as described previously.[7] All isolates were subcultured on Columbia agar base (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood (Oxoid SR0048), and incubated at 37°C for 48 h.

Fresh natural sea water was obtained from Strangford Lough, Co. Down, Northern Ireland (54.591° N 5.68° W), courtesy of Dr. Niall McDonough, Marine Biology Research Institute, Queen's University of Belfast, Portaferry, Co. Down. The sea water was sterilised by filtration through a 0.2 µm cellulose nitrate membrane filter and aliquoted aseptically into 3 x 25 mL volumes in plastic sterile universal containers (Sterilin, UK), for individual inoculation with the three organisms. Each sea water microcosm was inoculated with approximately 10^4 colony-forming units of each organism and was incubated at approximately 18°C in natural sunlight for 12 months. After this period, 20 µL of