

Case Report

Primary bacteraemia caused by *Rhizobium radiobacter* in a patient with solid tumours

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A case of *Rhizobium radiobacter* primary bacteraemia in a patient with solid tumours is reported. Corticosteroid therapy and diabetes mellitus were the predisposing factors. The patient was treated successfully with amikacin and piperacillin/tazobactam. The clinical isolate was identified as *R. radiobacter* by 16S rRNA gene sequencing. Phytopathogenicity tests and a PCR assay demonstrated that the organism was not a plant pathogen.

Introduction

The genus *Agrobacterium* contains five species. *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium vitis* and *Agrobacterium rubi* all cause tumorigenic plant disease (crown gall, cane gall and hairy-root). In contrast, the fifth species, *Agrobacterium radiobacter*, which is indistinguishable phenotypically from *A. tumefaciens*, does not contain the Ti plasmid associated with tumour induction in plants (Sawada *et al.*, 1993).

Agrobacterium species, together with *Allorhizobium undicola*, have recently been reclassified in the genus *Rhizobium*, based on comparative 16S rRNA gene analyses. The proposed new combinations are *Rhizobium radiobacter*, *Rhizobium rhizogenes*, *Rhizobium vitis*, *Rhizobium rubi* and *Rhizobium undicola* (Young *et al.*, 2001). Given that this reclassification is not universally accepted (Farrand *et al.*, 2003), we use the terms '*R. radiobacter*' and '*Agrobacterium* species' for human and plant isolates, respectively. *R. radiobacter* is the only species of the genus known to cause human disease (Mastroianni *et al.*, 1996; Lai *et al.*, 2004).

Definitive identification of clinical isolates by molecular methods, such as rRNA gene analysis, has been described in only one study (Giammanco *et al.*, 2004). In addition, information regarding the phytopathogenicity of clinical strains is very limited (Freny *et al.*, 1985; Harrison *et al.*, 1990; Hammerberg *et al.*, 1991; Dunne *et al.*, 1993; Alnor *et al.*, 1994). Here, we describe a case of primary bacteraemia caused by *R. radiobacter*, which was identified by conventional and

molecular methods. In addition, the phytopathogenicity of the clinical isolate was analysed in detail.

Case report

A 19-year-old man who had osteosarcoma of the right arm with metastases in the lungs and bones was admitted for chemotherapy (doxorubicin, cisplatin, ifosfamide plus mesna and methylprednisolone). On day 30, while in remission with a normal white blood cell count, the patient presented fever (39 °C) with rigors. No source of infection was clinically apparent. Laboratory investigations revealed the following: white blood cell count, $18.2 \times 10^9 \text{ l}^{-1}$ (92 % granulocytes); haemoglobin, 9.2 g dl⁻¹; and blood glucose, 11.3 mmol l⁻¹ (normal range, 3.85–6.05 mmol l⁻¹). All other biochemical laboratory tests were unremarkable. A chest X-ray as well as a CT scan of the brain, chest and abdomen showed no evidence of infection. Urine, stool and sputum cultures were negative. Three sets of blood cultures, which had been taken from a peripheral vein on day 30 while no central venous catheter or other prosthetic material was in place, yielded a Gram-negative rod, later identified as *R. radiobacter*. The patient was treated with amikacin (1 g per day) and piperacillin/tazobactam (13.5 g per day) for 10 days. The fever resolved 3 days later. There was no evidence of relapse during the following month, when he was discharged.

Environmental samples, e.g. sinks, showerhead, taps, detergents and disinfectants, from the patient's medical ward were collected and cultured as described by Perola *et al.* (2002). All environmental samples failed to yield this organism. Finally, investigation revealed strict adherence to the well-established guidelines for blood collection and blood culture processing (Dunne *et al.*, 1997) and no further

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence reported in this paper is AM157353.

instances of colonization or infection with *R. radiobacter* occurred in the months shortly before or after admission of the patient in question.

Microbiological investigation

Three sets of standard-medium blood cultures in a total of six bottles were drawn within 24 h. The patient was not receiving any antimicrobial agent at the time of collection. Cultures were processed with the BacT/ALERT System (bioMérieux). Bacterial growth was detected within 24–48 h after incubation at room temperature or at 35 °C, but not at 42 °C. The three aerobic bottles grew on MacConkey agar as pink colonies that became extremely mucoid after prolonged incubation. Conventional biochemical tests using standard methods were positive for reaction with catalase and cytochrome oxidase, reduction of NO₃ and hydrolysis of urea, aesculin and ONPG. 3-Ketolactose production was negative. The organism was identified as *R. radiobacter* by the API 20NE (biotype 1667744, probability 99.9%) and Vitek GNI+ (bionumber 66147600140, probability 99%) identification systems (bioMérieux).

In order to identify the primary structure of the 16S rRNA gene of the clinical strain, chromosomal bacterial DNA was isolated. Chromosomal DNA extraction, PCR amplification and sequencing of the 16S rRNA gene were performed as described elsewhere (Woo *et al.*, 2000). The PCR primers used were RADF1 (5'-TGGCAGACGGGTGAGTAACG-3') and RADR1 (5'-CCCTACGGCTACCTTGTTACG-3'). The PCR product (1373 bp) was cloned in a pGEM-T plasmid vector (Promega) and sequenced by universal primers at least twice for both strands. The sequence of the PCR product was compared with known 16S rRNA gene sequences in GenBank by BLAST searches. Multiple sequence alignment was performed using CLUSTAL W (Thomson *et al.*, 1994), and a phylogenetic tree was constructed with the neighbour-joining method using the TREE TOP program (data not shown). The nucleotide sequence of the PCR product showed the highest identity score (seven base differences, 99.5% identity) with six strains of *A. tumefaciens* and one strain of *A. radiobacter* deposited in GenBank, indicating that our isolate was closely related to the reference strains referred to above; the strain was therefore identified as *R. radiobacter* strain GCIZ.

The tumorigenicity of the isolate on kalanchoe, tomato, tobacco, sunflower and African marigold plants was tested by stem prick inoculation of young seedlings with a suspension of 10⁷ c.f.u. ml⁻¹ (from slope cultures on nutrient agar medium). Plants were incubated at 28 °C and checked for up to 2 months. The isolate was not tumorigenic on any of the plants tested.

A PCR test was used to detect the presence of Ti plasmid DNA in the clinical isolate (Haas *et al.*, 1995). Two oligonucleotides, sense-strand primer A (5'-ATGCCCGATCGAGCTCAAGT-3') and antisense-strand primer C' (5'-TCGTCTGGCTGACTTTCGTCATAA-3'), based on the

sequence of the *virD2* gene (corresponding to nt 1–20 and 201–224, respectively; Haas *et al.*, 1995) were synthesized by the Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-HELLAS, Heraklion, Crete, Greece. Briefly, DNA was extracted from the clinical isolate, two phytopathogenic strains (*A. tumefaciens* and *A. vitis*) and a negative control (*A. radiobacter* strain K-84). PCR was performed on a Perkin Elmer Cetus 480 DNA thermal cycler as described elsewhere (Haas *et al.*, 1995). PCR products (224 bp) were analysed by electrophoresis on a 2.5% agarose gel (Fig. 1) and visualized by staining with ethidium bromide (0.5 µg ml⁻¹) for 30 min in the dark followed by exposure of the gel to UV light (302 nm). Bands were photographed using Polaroid 667 fast film. No 224 bp fragment was amplified from the clinical isolate.

Antibiotic-sensitivity testing of the isolate was performed using the Neg MIC Type 30 panel (MicroScan; Dade Behring) according to the manufacturer's guidelines. MICs were read 24 h after inoculation using Clinical Laboratory Standard Institute (CLSI) interpretive criteria for non-fermentative Gram-negative bacteria (CLSI, 2005). The strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls. The isolate was sensitive to ciprofloxacin (≤0.5 µg ml⁻¹), imipenem and meropenem (≤1 µg ml⁻¹), levofloxacin, gatifloxacin and cefepime (≤2 µg ml⁻¹), tetracycline (≤4 µg ml⁻¹) and piperacillin/tazobactam (≤8/4 µg ml⁻¹), but resistant to gentamicin and tobramycin (≥16 µg ml⁻¹), ceftazidime, aztreonam and chloramphenicol (≥32 µg ml⁻¹), amikacin (≥64 µg ml⁻¹) and trimethoprim/sulfamethoxazole (≥4/76 µg ml⁻¹).

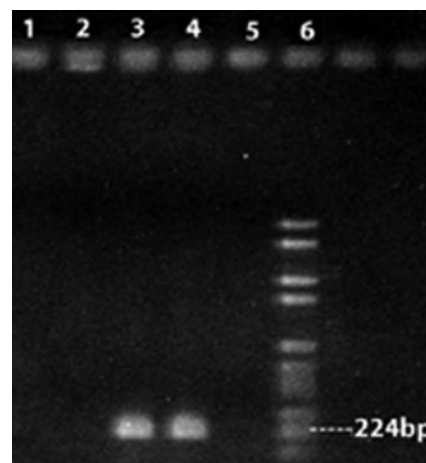


Fig. 1. Analysis of amplified DNA products by PCR using specific *virD* primers. Lanes: 1, clinical isolate; 2, *A. radiobacter* strain K-84 (negative control); 3 and 4, phytopathogenic strains of *A. tumefaciens* and *A. vitis*, respectively; 5, negative control (H₂O); 6, molecular mass markers VI (Roche).

Discussion

Tumorigenic strains of *Agrobacterium* species have different host plants, but all such strains produce crown galls on at least one of tomato, sunflower, African marigold and kalanchoe plants (Hammerberg *et al.*, 1991). The oncogenic and phytopathogenic potential of these bacteria is conferred by a Ti plasmid (Sawada *et al.*, 1993). To date, none of the six clinical isolates that have been tested by phyto-genicity tests induced tumours upon inoculation of plants (Harrison *et al.*, 1990; Alnor *et al.*, 1994).

Infection occurs at plant wound sites and involves the transfer of oncogenic DNA (T-DNA) from plasmid-harboured phytopathogenic strains to the plant cell nucleus. Virulence (*vir*) genes are responsible for the T-DNA transfer (Haas *et al.*, 1995). Haas *et al.* (1995) designed two primer pairs based on the *virD2* and *ipt* genes and detected a wide variety of pathogenic *Agrobacterium* strains. The *virD2* primers detected all pathogenic strains as well as one non-pathogen; the *ipt* primers detected only tumorigenic strains and not rhizogenic strains or non-pathogens. In this study, we used a PCR based on the *virD2* primers. The positive controls showed amplification bands of the expected size of 224 bp, suggesting the potential phytopathogenicity of these bacteria. In contrast, the clinical isolate and negative control showed no amplification of the 224 bp band (Fig. 1). As in the case of our isolate, none of the five isolates that have been tested previously by molecular methods harboured a Ti plasmid (Freney *et al.*, 1985; Harrison *et al.*, 1990; Hammerberg *et al.*, 1991; Dunne *et al.*, 1993).

The production of 3-ketolactose is a feature of *Rhizobium* (*Agrobacterium*) species that serves to differentiate the two biotypes (Sawada *et al.*, 1993). Our isolate did not produce 3-ketolactose, a characteristic indicative of biotype 2. 3-Ketolactose-negative clinical isolates have been reported previously, but have not been characterized further (Freney *et al.*, 1985; Harrison *et al.*, 1990; Alnor *et al.*, 1994). However, identification of *Rhizobium* (*Agrobacterium*) species relying solely on phenotypic criteria is not very reliable, and sequence analysis of 16S rRNA genes should be added (Giammanco *et al.*, 2004).

R. radiobacter has been recognized as an opportunistic human pathogen. Human infections caused by *R. radiobacter* are most commonly community-acquired (Mastroianni *et al.*, 1996). Catheter-related bacteraemia, continuous ambulatory peritoneal dialysis peritonitis, urinary tract infections and pneumonia are the most common clinical conditions caused by this micro-organism (Mastroianni *et al.*, 1996; Lai *et al.*, 2004). Infections due to *R. radiobacter* are strongly related to the presence of foreign plastic materials, and effective treatment often requires removal of the device (Alnor *et al.*, 1994; Mastroianni *et al.*, 1996). In addition, antibiotic-inactivating enzymes have been described in a clinical isolate of *R. radiobacter* (Martínez *et al.*, 1989). *R. radiobacter* is uniformly

susceptible to cefepime, carbapenems, tetracyclines, piperacillin/tazobactam and ciprofloxacin, whereas resistance to other antibiotics is common but variable (Alnor *et al.*, 1994; Lai *et al.*, 2004). The outcome of infection due to *R. radiobacter* is favourable (Mastroianni *et al.*, 1996; Lai *et al.*, 2004).

To the best of our knowledge, this is the first case in which both molecular identification and phytopathogenicity of the isolate were checked in detail. In addition, only one other case of primary bacteraemia in a non-neutropenic patient with solid tumours has been described (Lai *et al.*, 2004). In our patient, the portal of entry of the organism, as well as the source of infection, was unknown. Corticosteroid therapy and diabetes mellitus were the predisposing factors. It is worth noting that the patient was successfully treated with amikacin and piperacillin/tazobactam in spite of *in vitro* resistance of the clinical isolate to amikacin.

In conclusion, *R. radiobacter* is an opportunistic organism that has demonstrated a propensity to cause infections in humans, particularly in those with indwelling plastic devices. Appropriate antimicrobial therapy plus removal of the medical device is usually necessary in order to control infection. We suggest that environmental and clinical isolates of *Rhizobium* (*Agrobacterium*) species represent distinct groups, that clinical strains lack the ability to act as phytopathogens and that environmental isolates are unlikely to be responsible for human infections. However, more extensive studies with a wide collection of human and environmental isolates are needed to evaluate the role of tumorigenic strains in human diseases.

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