ORIGINAL ARTICLE

Pseudobacteraemia in a patient with neutropenic fever caused by a novel paenibacillus species: *Paenibacillus hongkongensis* sp. nov.

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Aims: To characterise a strain of Gram negative aerobic straight or slightly curved rods (HKU3) isolated from the blood culture of a 9 year old Chinese boy with neutropenic fever and pseudobacteraemia.

Methods: The isolate was phenotypically investigated by standard biochemical methods using conventional biochemical tests, scanning electron microscopy, and transmission electron microscopy. Genotypically, the 16S rRNA gene of the bacterium was amplified by the polymerase chain reaction (PCR) and sequenced. The sequence of the PCR product was compared with known 16S rRNA gene sequences in the Genbank by multiple sequence alignment. The G + C content was determined by thermal denaturation. A phylogenetic tree was constructed by the PileUp method.

Results: The cells of the bacterial strain were aerobic, sporulating, Gram negative straight or slight curved rods. The bacterium grew on horse blood agar as non-haemolytic, grey colonies of 1 mm in diameter after 24 hours of incubation at 37°C in ambient air. No enhancement of growth was seen in 5% CO_2. It grew at 50°C as pinpoint colonies after 72 hours of incubation, but did not grow at 65°C or on MacConkey agar. It was non-motile. It produced catalase (weakly positive) and cytochrome oxidase. It reduced nitrate, produced β galactosidase, hydrolysed esculin, and utilised sodium acetate. A scanning electron micrograph of the bacterium showed straight or slightly curved rods. A transmission electron micrograph of the cell wall of the bacterium revealed multiple electron dense layers, including the outer membrane, middle murein layer, and inner cytoplasmic membrane, compatible with its Gram smear appearance. 16S rRNA gene sequencing showed that there were 7.7%, 8.0%, 8.2%, and 8.6% differences between the 16S rRNA gene sequence of the bacterium and those of *Paenibacillus macerans*, *Paenibacillus borealis*, *Bacillus ehimensis*, and *Paenibacillus amylolyticus*, respectively. The mean (SD) G + C content of the bacterium was 47.6 (2.1) mol%. Phylogenetically, it belongs to the genus paenibacillus (previously called group 3 bacillus).

Conclusions: A bacterium that exhibited phenotypic and genotypic characteristics that are very different from closely related members of paenibacillus was the cause of pseudobacteraemia in a patient with neutropenic fever. A new species, *Paenibacillus hongkongensis* sp. nov. is proposed, for which HKU3 is the type strain.

Since the discovery of the polymerase chain reaction (PCR) and DNA sequencing, comparison of the gene sequences of bacterial species has shown that the 16S rRNA gene is highly conserved within a species and among species of the same genus, and hence can be used as the new gold standard for speciation of bacteria. Using this new standard, phylogenetic trees, based on base differences between species, can be constructed and bacteria classified and re-classified into new genera.[1] Furthermore, non-cultivable organisms and organisms with ambiguous biochemical profiles can be classified and identified.[2 3] Recently, we have reported the use of this technique for the identification of bacterial strains with ambiguous biochemical profiles,[4–8] species that are rarely encountered clinically,[9–13] and a bacterium that is non-cultivable[14]; the discovery of a novel clinical syndrome[15 16] and two novel species[17 18]; and the characterisation of β haemolytic Lancefield group G streptococcal bacteraemia[19] and thermotolerant *Campylobacter fetus* bacteraemia.[20]

> "The 16S rRNA gene is highly conserved within a species and among species of the same genus, and hence can be used as the new gold standard for speciation of bacteria"

In our present study, we report the isolation of a bacterial strain from the blood culture in a patient with neutropenic fever. The strain, named HKU3, exhibited phenotypic characteristics that do not fit into the patterns of a known species. 16S rRNA gene sequencing showed that there was 92.3% base identity between the 16S rRNA gene of HKU3 and that of *Paenibacillus macerans*. On the basis of these studies, we propose a new species, *Paenibacillus hongkongensis* sp. nov., to describe this bacterium.

MATERIALS AND METHODS

Patient and microbiological methods

All clinical data were collected prospectively as described in our previous publication.[21] The BACTEC 9240 blood culture system (Becton Dickinson, Maryland, USA) was used. The bacterium was identified by standard conventional biochemical methods.[22] All tests were performed in triplicate with freshly prepared media on separate occasions. In addition, the Vitek System (BACIL; BioMerieux Vitek, Hazelwood, Missouri, USA) and the API system (50CHB/20E; BioMerieux

Abbreviations: MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; SSC, saline sodium citrate

Table 1 Biochemical profile of strain HKU3 by conventional biochemical tests and the Vitek system (BACIL) and API system (50CHB/20E)

Biochemical reactions/enzymes	Conventional	Vitek BACIL	API 50CHB/20E
Catalase	Weak +		
Cytochrome oxidase	+		
Nitrate reduction	+		+
β Galactosidase	+		+
Arginine dihydrolase	–		–
Lysine decarboxylase	–		–
Ornithine decarboxylase	–		–
Citrate utilisation	–		–
Malonate utilisatiion	–		
MBM acetate utilisation	–		
H_2S			–
Urease	–		–
Tryptophan deaminase			–
Indole	–		–
Acetoin	–		–
Gelatinase	–		–
Esculin hydrolysis	+	+	+
Casein hydrolysis	–		
Fermentation/oxidation/assimilation of			
Glycerol			–
Erythritol			–
D-Arabinose		–	–
L-Arabinose			–
Ribose		–	–
D-Xylose		–	–
L-Xylose			–
Adonitol			–
β Methylxyloside			–
Galactose	–	–	–
D-Glucose	–	–	–
D-Fructose			–
D-Mannose	–		–
L-Sorbose			–
Rhamnose			–
Dulcitol			–
Inositol		–	–
Mannitol		–	–
Sorbitol		–	–
α Methyl-D-mannoside			–
α Methyl-D-glucoside			–
N-Acetyl glucosamine		–	–
Amygdalin		–	–
Arbutin			–
Salicin		–	–
Cellobiose			–
Maltose		–	–
Lactose	–		–
Melibiose			–
Saccharose			–
Trehalose		–	–
Inulin		–	–
Melezitose			–
D-Raffinose		–	–
Amidon			–
Glycogene			–
Xylitol			–
β Gentiobiose			–
D-Turanose			–
D-Lyxose			–
D-Tagatose		–	–
D-Fucose			–
L-Fucose			–
D-Arabitol		–	–
L-Arabitol			–
Gluconate			–
2 cetogluconate			–
5 cetogluconate			–
Sucrose		–	
Palatinose		–	
Amylopectin		–	
Potassium thiocyanate		–	
7% NaCl		–	
Mandelic acid		–	
Oleandomycin		–	
Sodium acetate		+	
Polyamidohygrostreptin		–	
Nalidixic acid		–	
Reduction of tetrazolium red		–	
Identification		99% *Bacillus sphaericus*	*77.5% Brevibacillus brevis* *22.5% Bacillus sphaericus*

Figure 1 Scanning electron micrograph of *Paenibacillus hongkongensis*. The bacterium is straight or slightly curved and is aflagellated. Cells vary in length from 1.44 to 2.50 μm and in diameter from 0.28 to 0.39 μm (mean, 1.82 × 0.31 μm; n = 20). Bar, 1 μm.

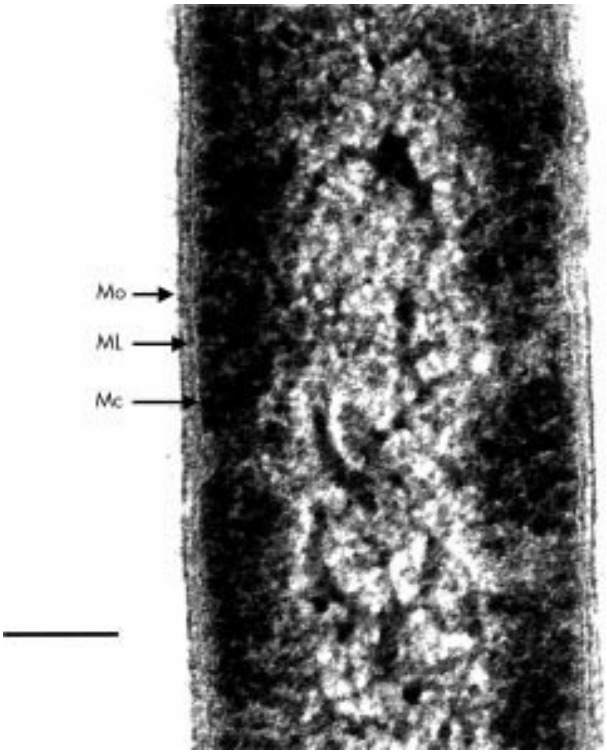

Figure 2 Transmission electron micrograph of *Paenibacillus hongkongensis*. The electron dense layers represent the outer membrane (Mo), murein layer (ML), and the inner cytoplasmic membrane (Mc). Bar, 100 nm.

Vitek) were used for the identification of the bacterial isolate in our study. The minimum inhibitory concentration (MIC) of penicillin, cefotaxime, and vancomycin on HKU3 was performed using the E-test method.

Scanning electron microscopy

Bacterial cells were washed twice using milli-Q water. A suspension of the bacterium was settled on to a polycarbonate membrane (Nucleopore) with pore size 5 μm for five minutes. The membrane was fixed in 2.5% glutaraldehyde (wt/vol) for one hour and washed once in 0.1M sodium cacodylate buffer. Fixed material was dehydrated through a graded ethanol series from 30% to 90% in 20% steps, followed by two changes of absolute ethanol. Each of the stepwise changes was for 15 minutes. Dehydrated material in absolute ethanol was critical point dried in a BAL-TEC CPD O30 critical point drier using carbon dioxide as the drying agent. Critical dried material was mounted on to an aluminum stub and coated with palladium in a BAL-TEC SCD 005 scanning electron microscopy coating system. Coated material was examined in a Leica Cambridge Stereoscan 440 scanning electron microscope operating at 12 kV and the specimen stage was tilted at zero degrees.

Transmission electron microscopy

Bacterial cells were fixed in 2.5% (wt/vol) glutaraldehyde at 4°C overnight followed by 1% (wt/vol) osmium tetroxide at room temperature for 30 minutes. Fixed cells were embedded in 2% (wt/vol) agar, which was then cut into 1 mm^3 blocks. Agar blocks with fixed cells were dehydrated through a graded ethanol series from 30% to 90% in 20% steps, followed by three changes of absolute ethanol. Each of the stepwise changes was for 15 minutes. Dehydrated agar blocks were infiltrated by 33% and 66% Möllenhauer's resins in propylene (1.5 hours each). The material was embedded in 100% resin and polymerised in an oven at 60°C for 24 hours. Ultrathin sections of 90 nm were prepared and stained with saturated uranyl acetate for 30 minutes and lead citrate for 20 minutes. The samples were examined using a JEOL 100SX (Philips) transmission electron microscope at an accelerating voltage of 80 kV.

Extraction of bacterial DNA for 16S rRNA gene sequencing

Bacterial DNA extraction was modified from our previously published protocol.[23] Briefly, 80 μl of NaOH (0.05M) was added to 20 μl of bacterial cells suspended in distilled water and the mixture was incubated at 60°C for 45 minutes, followed by the addition of 6 μl of Tris/HCl (pH 7.0), achieving a final pH of 8.0. The resultant mixture was diluted 100× and 5 μl of the diluted extract was used for PCR.

PCR, gel electrophoresis, and 16S rRNA gene sequencing

PCR amplification and DNA sequencing of the 16S rRNA gene was performed according to our previous publications.[17 18 24] Briefly, DNase I treated distilled water and PCR master mix (which contains deoxynucleoside triphosphates (dNTPs), PCR buffer, and Taq polymerase) were used in all PCR reactions by adding 1 U of DNase I (Pharmacia, Uppsala, Sweden) to 40 μl of distilled water or PCR master mix, incubating the mixture at 25°C for 15 minutes, and subsequently at 95°C for 10 minutes to inactivate the DNase I. The bacterial DNA extract and control were amplified with 0.5μM primers (LPW55: 5′-AGTTTGATCCTGGCTCAG-3′ and LPW324: 5′-TTGTTACGACTTCACCCCA-3′; Gibco BRL, Rockville, Maryland, USA). The PCR mixture (50 μl) contained bacterial DNA, PCR buffer (10mM Tris/HCl, pH 8.3, 50mM KCl, 2mM $MgCl_2$, and 0.01% gelatin), 200 μM of each dNTP, and 1.0 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany).

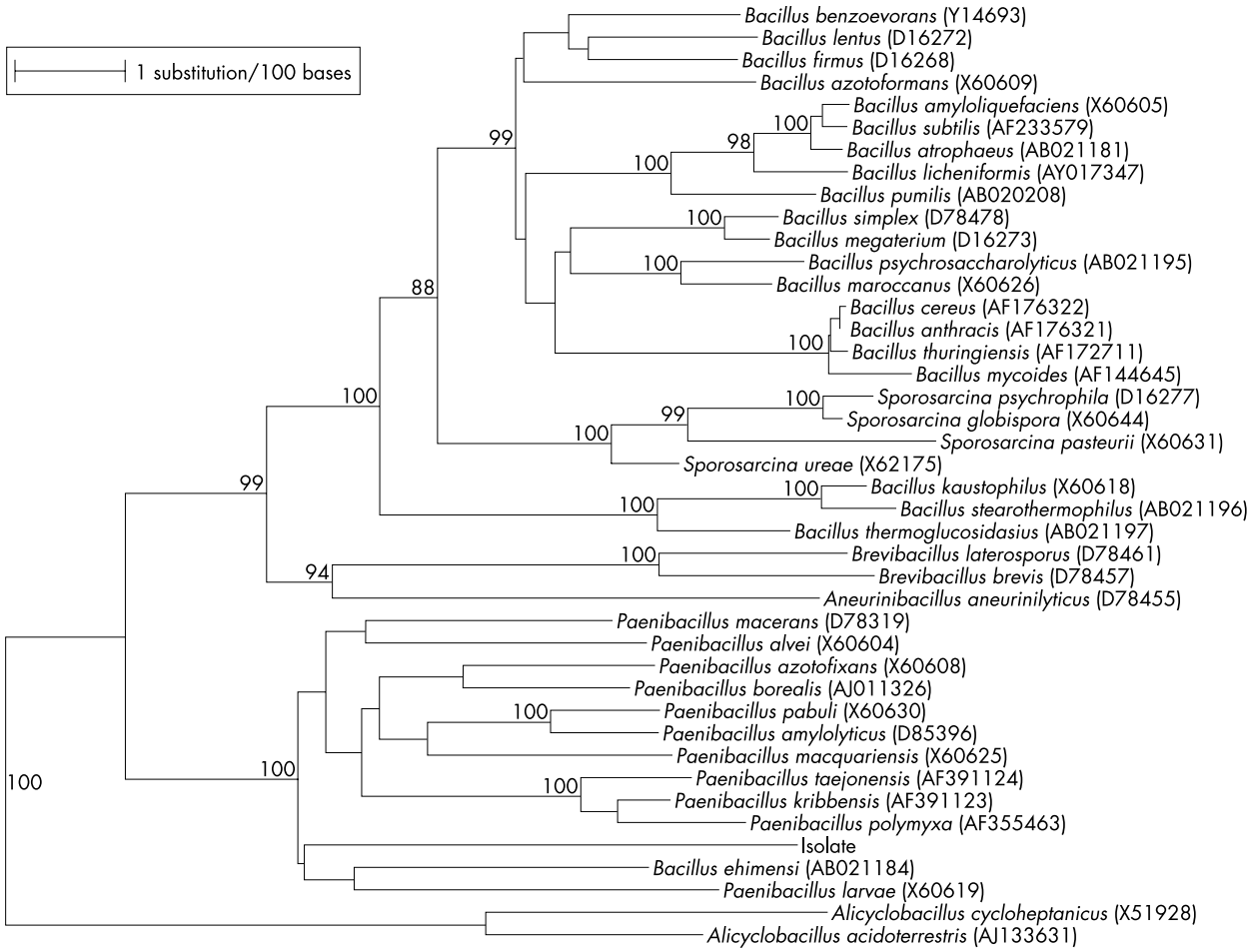

Figure 3 Phylogenetic tree showing the relationships between *Paenibacillus hongkongensis* sp. nov. and other paenibacillus species and representative species of related genera. The tree was inferred from 16S rRNA data by the neighbour joining method and bootstrap values calculated from 1000 trees. The scale bar indicates the estimated number of substitutions for each 100 bases using the Jukes-Cantor correction. Names and accession numbers are given as cited in the GenBank database.

The mixtures were amplified for 40 cycles of 94°C for one minute, 55°C for one minute, and 72°C for two minutes, with a final extension at 72°C for 10 minutes in an automated thermal cycler (Perkin-Elmer Cetus, Gouda, The Netherlands). DNase I treated distilled water was used as the negative control. A 10 µl aliquot of each amplified product was electrophoresed in 1.0% (wt/vol) agarose gel, with a molecular size marker (λ DNA AvaII digest; Boehringer Mannheim) in parallel. Electrophoresis in Tris/borate/EDTA buffer was performed at 100 V for 1.5 hours. The gel was stained with ethidium bromide (0.5 µg/ml) for 15 minutes, rinsed, and photographed under ultraviolet light illumination.

The PCR product was gel purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Both strands of the PCR product were sequenced twice with an ABI 377 automated sequencer according to the manufacturer's instructions (Perkin-Elmer, Foster City, California, USA), using the PCR primers (LPW55 and LPW324) and additional sequencing primers (LPW278: 5'-CCCTTATGACCTGGGCTAC-3' and LPW279: 5'-CTGGCAACAGAGCTTTACG-3'). The sequence of the PCR product was compared with known 16S rRNA gene sequences in the GenBank by multiple sequence alignment using the CLUSTAL W program.[25]

Determination of G + C content

Preparation of genomic DNA was performed according to a published protocol,[26] and the G + C content was determined by thermal denaturation.[27] Briefly, the temperature of the genomic DNA in SSC (saline sodium citrate; 0.15M NaCl with 0.015M sodium citrate) buffer (25 µg/ml) was increased slowly (0.5°C/minute) from 25°C and the absorbance of the solution at 260 nm was monitored continuously against a blank containing SSC buffer only. The T_m of the DNA is defined as the temperature at 50% hyperchromicity. The G + C content of the genomic DNA was calculated by the formula: $(G + C)\% = 2.44T_m - 169$.

Phylogenetic characterisation

The phylogenetic relationships between strain HKU3 and other paenibacillus species and representative species of related genera were determined using the PileUp method with GrowTree (Genetics Computer Group, Wisconsin, USA). In total, 1385 nucleotide positions were included in the analysis.

Nucleotide sequence accession number

The 16S rRNA gene sequence of HKU3 has been lodged within the GenBank sequence database under accession number AF433165.

RESULTS

Patient

A 9 year old Chinese boy was admitted in January 1996 because of neutropenic fever after chemotherapy. He had posterior fossa medulloblastoma diagnosed in 1994. Surgical excision followed by craniospinal radiotherapy was performed but the disease relapsed as multiple bone metastases one year

later. He was then started on monthly chemotherapy (Baby Brain protocol) and achieved remission in November 1995 after five cycles of treatment. The sixth cycle of chemotherapy was administered in December 1995 uneventfully and he was scheduled for the next cycle one month later. However, he was readmitted 23 days after chemotherapy because of fever for one day without other symptoms. On admission, his oral temperature was 38°C. Physical examination revealed no foci of infection. Blood cultures were taken from both the central catheter and percutaneous venepuncture. He was started on empirical intravenous ceftazidime and amikacin. On day three after incubation, blood culture taken from percutaneous venepuncture, but not the central catheter, turned positive with a Gram negative bacillus (strain HKU3). Blood cultures were repeated through the central catheter and percutaneous venepuncture and they were negative. Fever responded to ceftazidime and amikacin and the patient received the seventh cycle of chemotherapy after one week of antibiotic treatment.

Phenotypic characteristics

Strain HKU3 is a straight or slightly curved, sporulating, Gram negative rod. It grows on horse blood agar as non-haemolytic, grey colonies of 1 mm in diameter after 24 hours of incubation at 37°C in ambient air. No enhancement of growth is seen in 5% CO_2. It also grows in a microaerophilic, but not in an anaerobic, environment. It grows at 50°C as pinpoint colonies after 72 hours of incubation, but does not grow at 65°C or on MacConkey agar. It is non-motile. It produces catalase (weakly positive) and cytochrome oxidase. The Vitek system (BACIL) showed that it was 99% *Bacillus sphaericus* and the API system (50CHB/20E) showed that it was 77.5% *Brevibacillus brevis* and 22.5% *B sphaericus* (table 1). It reduces nitrate, produces β galactosidase, hydrolyses esculin, and utilises sodium acetate. The MIC of penicillin, vancomycin, and cefotaxime on HKU3 were 0.125 μg/ml, 4 μg/ml, and 0.006 μg/ml, respectively.

Scanning electron microscopy

Figure 1 shows a scanning electron micrograph of *P hongkongensis*. Bacterial cells were straight or slightly curved rods.

Transmission electron microscopy

Figure 2 shows a transmission electron micrograph of *P hongkongensis*. Bacterial cells contained multiple electron dense layers, including the outer membrane, middle murein layer, and inner cytoplasmic membrane. The possession of this multilayered wall structure, typical of that of a Gram negative bacterium, is compatible with its Gram smear appearance.

Molecular characteristion by 16S rRNA gene sequencing, determination of G + C content, and phylogenetic characteristion

PCR of the 16S rRNA gene of strain HKU3 showed a band at about 1470 bp. There was a 7% difference between the 16S rRNA gene sequence of strain HKU3 and that of *P macerans* (GenBank accession number, D78319), 8.0% difference between the 16S rRNA gene sequence of strain HKU3 and that of *P borealis* (GenBank accession number, AJ011326), 8.2% difference between the 16S rRNA gene sequence of strain HKU3 and that of *B ehimensis* (GenBank accession number, AB021184), and 8.6% difference between the 16S rRNA gene sequence of strain HKU3 and that of *P amylolyticus* (GenBank accession number, D85396) (fig 3). The mean (SD) G + C content of strain HKU3 was 47.6 (2.1) mol%.

DISCUSSION

All paenibacillus species were originally classified as part of the bacillus genus. In 1991, using 16S rRNA sequence data, Ash *et al* classified the bacillus species into five phylogenetically distinct groups, with 10 species in group 3.[28] Subsequently, the bacillus genus was split into multiple genera, and group 3 bacillus was reclassified as a novel genus, paenibacillus in 1993.[29] Currently, there are 33 species in the paenibacillus genus, under the family bacillaceae.

In our study, we report the isolation of HKU3 from a Chinese patient with neutropenic fever. Because the bacterium was only recovered from one of the four blood cultures, it was probably a contaminant of the blood culture, and did not cause genuine bacteraemia. The 16S rRNA gene of HKU3 exhibited less than 97% nucleotide identity with the 16S rRNA gene of all previously described bacterial strains of the paenibacillus genus. The most closely related species is *P macerans*, another paenibacillus that was also reported to cause an outbreak of pseudobacteraemia.[30] In that report, *P macerans* was recovered from blood cultures of eight neonates. Epidemiological investigations showed that the most likely source of the pseudobacteraemia outbreak was environmental contamination of the rubber stoppers in blood culture bottles, and this hypothesis was subsequently confirmed by environmental sampling and simulated inoculation studies. In fact, bacillus species are very common contaminants of blood cultures, and outbreaks of pseudobacteraemia as a result of bacillus species have been reported to be caused by contaminated intravenous cannulae,[31] contaminated commercial blood culture media,[32] contaminated alcohol swabs,[33] contaminated gloves used in the collection of blood,[34] and hospital construction.[35]

> "The 16S rRNA gene of HKU3 exhibited less than 97% nucleotide identity with the 16S rRNA gene of all previously described bacterial strains of the paenibacillus genus"

HKU3 exhibited microbiological and clinical characteristics that are very different from closely related members of the paenibacillus genus (table 2). Microscopically, HKU3 (confirmed by transmission electron microscopy) and *P borealis* appear as Gram negative rods, but *P macerans*, *P amylolyticus*, *Paenibacillus alvei*, and *Paenibacillus polymyxa* as Gram positive rods. HKU3 is non-motile, whereas *P macerans*, *P amylolyticus*, *P borealis*, *P alvei*, and *P polymyxa* are motile. *Paenibacillus macerans*, *P amylolyticus*, *P borealis*, *P alvei*, and *P polymyxa*, but not HKU3, grow in an anaerobic environment. HKU3 and *P macerans*, but not *P amylolyticus*, *P borealis*, *P alvei*, and *P polymyxa*, grow at 50°C. HKU3, *P macerans*, *P amylolyticus*, and *P polymyxa*, but not *P borealis* and *P alvei*, reduce nitrate. *Paenibacillus macerans*, *P amylolyticus*, *P borealis*, *P alvei*, and *P polymyxa*, but not HKU3, produce acid from glucose. *Paenibacillus amylolyticus*, *P borealis*, *P alvei*, and *P polymyxa*, but not HKU3 and *P macerans*, hydrolyse casein. *Paenibacillus alvei* and *P polymyxa*, but not HKU3, *P macerans*, *P amylolyticus*, and *P borealis*, are positive for the Voges-Proskauer reaction. *Paenibacillus macerans*, *P alvei*, and *P polymyxa*, but not HKU3, *P amylolyticus*, and P *borealis*, have been isolated from clinical specimens. *Paenibacillus macerans* has been reported to be associated with brain abscesses after penetrating periorbital injury, catheter associated infection, and wound infection[36 37]; *P alvei* with endoophthalmitis, neonatal meningitis, and prosthetic hip infection[38–40]; and *P polymyxa* with ovine abortion.[41]

Description of *Paenibacillus hongkongensis* sp. nov

Paene means almost and *hongkongensis*, in honour of Hong Kong, means the place where the bacterium was discovered.

Cells are aerobic, sporulating, Gram negative, straight or slightly curved rods. The organism grows on horse blood agar as non-haemolytic, grey colonies of 1 mm in diameter after 24 hours of incubation at 37°C in ambient air. No enhancement of growth is seen in 5% CO_2. It grows at 50°C as pinpoint colonies after 72 hours of incubation, but does not grow at 65°C or on MacConkey agar. It is non-motile. It produces catalase (weakly positive) and cytochrome oxidase. It reduces nitrate, produces β galactosidase, hydrolyses esculin, and utilises

Table 2 Comparison of microbiological and clinical characteristics of HKU3 and those of closely related members of paenibacillus

Characteristics	HKU3	*Paenibacillus macerans*	*Paenibacillus amylolyticus*	*Paenibacillus borealis*	*Paenibacillus alvei*	*Paenibacillus polymyxa*
Microbiological						
Gram smear	Gram negative rod	Gram positive rod	Gram positive rod	Gram negative rod	Gram positive rod	Gram positive rod
Cell width ⩾10 μm	–	–	–	–	V	–
Motility	Non–motile	Motile	Motile	Motile	Motile	Motile
Anaerobic growth	–	+	+	+	+	+
Growth at 50°C	+*	V	–	–	–	–
Growth at 65°C	–	–	–	–	–	–
Growth in 7% NaCl	–	–	–	–	–	–
Catalase production	+†	+	+	+	+	+
Nitrate reduction	+	+	+	–	–	+
Acid from glucose	–	+	+	+	+	+
Casein hydrolysis	–	–	+†	+	+	+
Voges-Proskauer reaction	–	–	–	–	+	+
Clinical						
Cause of pseudobacteraemia	Yes	Yes	No	No	No	No
Isolation from clinical specimens	No	Yes	No	No	Yes	Yes

*Pinpoint colonies at 72 hours; †weakly positive.
V, variable.

Take home messages

- We report the isolation of a novel bacterial strain from the blood culture in a patient with neutropenic fever
- The strain, named HKU3, exhibited phenotypic characteristics that do not fit into the patterns of a known species
- 16S rRNA gene sequencing showed that there was 92.3% base identity between the 16S rRNA gene of HKU3 and that of *Paenibacillus macerans*
- On the basis of these studies, we propose a new species, *Paenibacillus hongkongensis* sp. nov., to describe this bacterium

sodium acetate (table 1). The mean (SD) G + C content of the DNA of the strain is 47.6 (2.1) mol%. The organism was isolated from a patient with neutropenic fever. The type strain of *P hongkongensis* is strain HKU3. Its 16S rRNA gene sequence has been lodged within the GenBank sequence database under accession number AF433165.

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ECHO

$VEGF_{121}$ is downregulated most in idiopathic dilated cardiomyopathy

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Patients with idiopathic dilated cardiomyopathy (IDC) may benefit from future treatment to boost vascular endothelial growth factor $(VEGF)_{121}$ and thereby development of capillaries in the myocardium. A molecular study has shown for the first time that, of all three isomers of VEGF, $VEGF_{121}$ is downregulated the most in IDC.

$VEGF_{121}$ mRNA was expressed less than $VEGF_{189}$ and $VEGF_{165}$ isomers in ventricular endomyocardium from patients, whereas the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was expressed equally in patients and controls. The ratio of cDNA for VEGF to cDNA for GAPDH was significantly less in samples from patients than controls and least for $VEGF_{121}$ (40% *v* $VEGF_{165}$ 82% and $VEGF_{189}$ 83%). The mean protein ratio of VEGF to GAPDH for patients was significantly below that of the controls. The results were unaffected by the severity of disease.

The study was based on 28 patients with IDC and 10 brain dead controls without heart disease, all of whom had endomyocardial biopsy of the right ventricle. Total RNA was isolated from the tissue, then first strand cDNA subjected to reverse transcriptase (RT) PCR. cDNA for each VEGF isomer and the GADPH housekeeping gene was amplified by semiquanitative PCR. The method was validated by correlating the changes in ratios for each isomer with the amount of input template.

Recent evidence suggests that $VEGF_{165}$ and $VEGF_{189}$ isomers are downregulated in IDC, but previous work seems to have overlooked $VEGF_{121}$ despite its strong stimulation of capillary growth.

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