ORIGINAL ARTICLE

Identification by 16S ribosomal RNA gene sequencing of *Arcobacter butzleri* bacteraemia in a patient with acute gangrenous appendicitis

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Aims: To identify a strain of Gram negative facultative anaerobic curved bacillus, concomitantly isolated with *Escherichia coli* and *Streptococcus milleri*, from the blood culture of a 69 year old woman with acute gangrenous appendicitis. The literature on arcobacter bacteraemia and arcobacter infections associated with appendicitis was reviewed.

Methods: The isolate was phenotypically investigated by standard biochemical methods using conventional biochemical tests. Genotypically, the 16S ribosomal RNA (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. Literature review was performed by MEDLINE search (1966–2000).

Results: The bacterium grew on blood agar, chocolate agar, and MacConkey agar to sizes of 1 mm in diameter after 24 hours of incubation at 37°C in 5% CO_2. It grew at 15°C, 25°C, and 37°C; it also grew in a microaerophilic environment, and was cytochrome oxidase positive and motile, typically a member of the genus arcobacter. Furthermore, phenotypic testing showed that the biochemical profile of the isolate did not fit into the pattern of any of the known arcobacter species. 16S rRNA gene sequencing showed one to two base differences between the isolate and *A butzleri*, but 35 to 39 base differences between the isolate and *A cryaerophilus*, indicating that the isolate was a strain of *A butzleri*. Only three cases of arcobacter bacteraemia with detailed clinical characteristics were found in the English literature. The sources of the arcobacter species in the three cases were largely unknown, although the gastrointestinal tract is probably the portal of entry of the *A butzleri* isolated from the present case because the two concomitant isolates (*E coli* and *S milleri*) in the blood culture were common flora of the gastrointestinal tract. In addition, *A butzleri* has previously been isolated from the abdominal contents or peritoneal fluid of three patients with acute appendicitis.

Conclusions: 16S rRNA gene sequencing was useful in the identification of the strain of *A butzleri* isolated from the blood culture of a patient with acute gangrenous appendicitis. Arcobacter bacteraemia is rare. Further studies using selective medium for the delineation of the association between *A butzleri* and acute appendicitis are warranted.

The identification of bacteria in the clinical microbiology laboratory is traditionally performed by the isolation of the organism and the study of its phenotypic characteristics, including Gram staining, morphology, cultural requirements, and biochemical reactions, which have been the gold standard of bacterial identification. However, these methods of bacterial identification have two major drawbacks. First, they cannot be used for non-cultivable organisms and, second, organisms with biochemical characteristics that do not fit into the patterns of any known genus and species pose diagnostic problems.

> "The 16S ribosomal RNA 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 the speciation of bacteria"

Since the discovery of polymerase chain reaction (PCR) and DNA sequencing, the comparison of the gene sequences of bacterial species has shown that the 16S ribosomal RNA (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 the speciation of bacteria. Using this new standard, phylogenetic trees, based on base differences between species, are constructed, and bacteria are classified and reclassified into new genera.[1 2] Furthermore, non-cultivable organisms and organisms with ambiguous biochemical profiles can be classified and identified.[3 4] Recently, we have reported the application of 16S rRNA gene sequencing in the identification of clinical isolates with ambiguous biochemical profiles,[5–8] and a bacterium that was non-cultivable.[9] In our study, we report the application of such a technique in the identification of a strain of *Arcobacter butzleri* isolated from the blood culture of a patient with acute appendicitis.

METHODS

Patient and microbiological methods

All clinical data were collected prospectively. Clinical specimens were collected and handled according to standard protocols, and all suspect colonies were identified by standard conventional biochemical methods.[10]

Extraction of bacterial DNA for 16S rRNA gene sequencing

Bacterial DNA extraction was modified from our previously published protocol.[11] Briefly, 80 µl of NaOH (0.05M) was added

Abbreviations: PCR, polymerase chain reaction; rRNA, ribosomal RNA

Table 1 Phenotypic characteristics of the blood culture arcobacter isolate and other arcobacter species

Species	Growth on/in/at									Susceptibility to	
	MacConkey agar	1% glycine	1.5% NaCl	3.5% NaCl	42°C	Catalase	Nitrate reduction	Nitrite reduction	Indoxyl acetate hydrolysis	Nalidixic acid	Cephalothin
A cryaerophilus group 1A	–	–	–	–	–	+	V	–	+	V	R
A cryaerophilus group 1B	+	–	–	–	–	+	V	ND	+	S	V
A butzleri	+	+	V	V	V	W	+	–	+	S	R
A nitrofigilis	–	–	+	+	–	+	+	–	–	S	S
A skirrowii	–	V	+	V	V	+	+	ND	+	S	S
Blood culture isolate	+	–	+	–	–	W	+	–	+	S	R

V, variable; W, weakly positive; ND, not determined; R, resistant; S, sensitive.

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 times 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.[5–9 12 13] Briefly, DNase I treated distilled water and PCR master mix (which contains 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 (LPW57, 5′-AGTTTGATCCTGGCTCAG-3′; and LPW58, 5′-AGGCCCGGGAACGTATTCAC-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), 200µM of each dNTP, and 1.0 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany). 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 1.5% (wt/vol) agarose gel, with a molecular size marker (ϕX174 HaeIII 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 manufacturer's instructions (Perkin-Elmer, Foster City, California, USA), using the PCR primers (LPW57 and LPW58) and additional primers designed from the sequencing data of the first round of sequencing reaction (LPW69, 5′-AGCACCGGCTAACTCCGT-3′; and LPW232, 5′-AGTTTTAATCTTGCGA-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.[14]

RESULTS

Patient and identification of the bacterial strain by conventional methods

A 69 year old woman was admitted to hospital because of fever and right lower quadrant pain for one day. On admission, her oral temperature was 38°C. Examination of her abdomen revealed right lower quadrant tenderness, guarding, and rebound tenderness. Blood culture was performed. The total white blood cell count was 18.1×10^9/litre, with neutrophils at 16.5×10^9/litre, lymphocytes at 0.9×10^9/litre, and monocytes at 0.7×10^9/litre. The haemoglobin concentration was 137 g/litre and the platelet count was 342×10^9/litre. The serum urea, creatinine, and liver enzymes were within normal ranges. A clinical diagnosis of acute appendicitis was made. She was started on intravenous cefuroxime and metronidazole empirically and emergency appendicectomy was performed. At surgery, a gangrenous retrocaecal appendix was found, with local perforation near the tip and surrounding turbid peritoneal fluid. Her postoperative course was uneventful and she was discharged three days after the operation with oral antibiotics.

On day 1 post-incubation, the aerobic blood culture bottle turned positive with a Gram negative facultative anaerobic curved bacillus, a Gram negative facultative anaerobic bacillus, and a Gram positive facultative aerobic coccus. The last two organisms were subsequently identified as *Escherichia coli* and *Streptococcus milleri*, respectively. The first organism grew on blood agar and chocolate agar to sizes of 1 mm in diameter after 24 hours of incubation at 37°C in 5% CO_2. It also grew at 15°C, 25°C, and 37°C, it grew in a microaerophilic environment, and it was cytochrome oxidase positive and motile, typically a member of the genus arcobacter. Table 1 summarises further phenotypic tests used to distinguish between the blood culture arcobacter isolate and the known arcobacter species. The blood culture arcobacter isolate was weakly catalase positive but urease negative, grew on MacConkey agar, in 1.5% NaCl, but not in 1% glycine or 3.5% NaCl, reduced nitrate but not nitrite, hydrolysed indoxyl acetate but not hippurate, did not produce H_2S, and was resistant to cephalothin but sensitive to nalidixic acid. The profile did not fit into the patterns of any of the known arcobacter species.

16S rRNA gene sequencing

PCR of the 16S rRNA gene of the bacteria showed a band at 1414 bp. There were two base differences between the isolate and *A butzleri* (GenBank accession number L14626), one base difference between the isolate and *A butzleri* (GenBank accession number AF314538), 35 base differences between the isolate and *A cryaerophilus* (GenBank accession number L14624), and 39 base differences between the isolate and *A cryaerophilus* (GenBank accession number U25805), indicating that the isolate was a strain of *A butzleri*.

DISCUSSION

Small rRNA gene sequencing, particularly 16S rRNA sequencing in bacteria, has led to advances on multiple fronts in microbiology. First, the construction of a universal phylogenetic tree classifies organisms into three domains of life: bacteria, Archaea, and Eucarya.[1 2] Second, it revolutionises the classification of microorganisms, and makes the classification of non-cultivable microorganisms possible.[3 4] Third, it helps to

Table 2 Characteristics of patients with bacteraemia caused by *Arcobacter* spp

Characteristics	Patients			
	1	2	3	4
Refs	On *et al* (1995)[21]	Hseuh *et al* (1997)[20]	Yan *et al* (2000)[22]	Present case
Sex/age	M/1 day	F/72 years	M/60 years	F/69 years
Predisposing factors	Preterm labour	Chronic renal failure, haemodialysis with arteriovenous fistula	Chronic hepatitis B carrier, liver cirrhosis	None
Presenting features	Neonatal sepsis	Fever, cough, sputum, pleuritic chest pain for 2 months; anorexia, frequent loose stool for 1 month	Fever for 1 day	Fever and right lower quadrant pain for 1 day
Source of infection/route of transmission	Vertical?	Infected arteriovenous fistula	Unknown	Oral
Arcobacter species	*A butzleri*	*A cryaerophilus*	*A butzleri*	*A butzleri*
Method of identification	Numerical analysis using 66 phenotypic characteristics	Conventional phenotypic tests, API CAMPY system	16S rRNA gene sequencing	16S rRNA gene sequencing
Treatment	Penicillin, cefotaxime	Ceftizoxime, tobramycin	Cefuroxime	Cefuroxime, metronidazole
Outcome	Cured	Cured	Cured	Cured

elucidate the relationship between unknown bacterial species and known ones. New species of bacteria such as *Gemella sanguinis*, *Mycobacterium heidelbergense*, and *Massilis timonae* have been discovered using 16S rRNA.[15–17] Bacteria such as *Pseudomonas veronii*, *Mycobacterium celatum*, and *Methylobacterium zatmanii*, which were not previously known to cause infections in humans, have been identified in clinical specimens using this technique.[9 18 19] Furthermore, bacteria that were difficult to identify were successfully speciated using this technique. Recently, we have reported the use of this technique for the identification of a strain of *Mycobacterium neoaurum* with ambiguous biochemical and whole cell fatty acid profiles isolated from a patient with acute lymphoblastic leukaemia,[6] a strain of *E coli* with an ambiguous biochemical profile isolated from a bone marrow transplant recipient,[5] a strain of *Enterobacter cloacae* with an ambiguous biochemical profile isolated from a renal transplant recipient,[7] and a strain of tube coagulase negative *Staphylococcus aureus* isolated from a patient with refractory anaemia with excessive blasts in transformation.[8]

The identification of arcobacter species has been difficult, especially when it is primarily isolated under aerobic conditions in clinical microbiology laboratories that are equipped only with routine phenotypic tests. Similar to our present case, the isolation of arcobacter species from aerobic cultures has been reported previously.[20–22] Although its curved morphology when examined under the microscope may indicate that it is an arcobacter species, inconclusive biochemical reactions (as in our case) may result in failure to speciate the bacterium. In the two cases of *A butzleri* bacteraemia reported in the literature, both were "unidentifiable" by routine phenotypic tests. One was finally identified in the Central Public Health Laboratory in London by numerical analysis,[21] whereas the other was identified by 16S rRNA gene sequencing.[22] Interestingly, in one report, approximately 10% of isolates identified phenotypically as *A butzleri* were identified by DNA hybridisation as *A cryaerophilia*.[23]

Invasive arcobacter infections in humans have been described only rarely. Among the four species of this genus, only *A butzleri* and *A cryaerophilus* have been associated with human infection, with most being identified in diarrhoeal diseases.[23 24] Only three cases of arcobacter bacteraemia with detailed clinical characteristics were found in the English literature (MEDLINE search 1966–2000) (table 2),[20–22] although additional blood culture isolates were described.[23] The sources of the arcobacter in the three cases were largely unknown, although it was speculated that an infected arteriovenous fistula was the cause in one case. All four patients with arcobacter bacteraemia (including our present patient) recovered after antibiotic treatment.

> "Whether certain microorganisms can directly cause acute appendicitis or whether they are just innocent bystanders is still controversial"

The gastrointestinal tract is probably the portal of entry of the *A butzleri* isolated from our patient. Both *A butzleri* and *A cryaerophilus* species have been isolated in fresh and salt water environments, dairy cows, ground pork, bovine and porcine gut, and abortes, suggesting a possible oral route of transmission.[25] Because our patient had not been exposed to farm animals and the two concomitant isolates (*E coli* and *S milleri*) in the blood culture are common flora of the gastrointestinal tract, it is most likely that the bacteria penetrated through the inflamed intestinal mucosa after the ingestion of contaminated food or water.

The role of *A butzleri* in acute appendicitis remains to be determined. The association of pseudo-appendicitis with bacterial agents such as campylobacter, yersinia, and salmonella has been well described.[26 27] However, whether certain microorganisms can directly cause acute appendicitis or whether they are just innocent bystanders is still controversial. In one study, *Bacteroides fragilis* and *E coli* were isolated from almost all specimens of peritoneal fluid, appendiceal tissue, and abscess contents of patients with perforated or gangrenous appendicitis, whereas other frequent isolates included peptostreptococcus, pseudomonas, lactobacillus, and other bacteroides species.[28] In another study, *Haemophilus segnis*, which was rarely reported to be associated with infections, was recovered from the peritoneal fluid in five patients with acute appendicitis.[29] Interestingly, *A butzleri* has previously been isolated from the abdominal contents or peritoneal fluid of three patients with acute appendicitis,[23] and it was also present in the blood culture in our present case. Further studies using selective medium for the delineation of the association between *A butzleri* and acute appendicitis are warranted.

16S rRNA gene sequencing will continue to be the gold standard for the identification of bacteria, and the automation of such a technique may put it into routine use in clinical microbiology laboratories, replacing the traditional phenotypic tests. Modern technologies have made it possible to construct a high density of oligonucleotide arrays on a chip with oligonucleotides representing the 16S rRNA gene sequence of various bacteria.[30] Such a design will facilitate the automation of the annealing and detection of the PCR products of 16S

Take home messages

- 16S ribosomal RNA (rRNA) gene sequencing was useful in the identification of the strain of *Archobacter butzleri*, which is rarely encountered clinically, that was isolated from the blood culture of a patient with acute gangrenous appendicitis
- Further studies using selective medium for the delineation of the association between *A butzleri* and acute appendicitis are warranted
- 16S rRNA gene sequencing will continue to be the gold standard for the identification of bacteria, and the automation of such a technique may put it into routine use in clinical microbiology laboratories, replacing the traditional phenotypic tests

rRNA gene amplification, and hence the routine identification of most clinical isolates would be possible. The use of 16S rRNA gene sequencing has several advantages. First, the turnaround time is short. Because amplification of the 16S rRNA gene takes only four to six hours, and the annealing and detection of the PCR product takes only another few hours, theoretically, the identification can be completed within one day. Second, the problem of "unidentifiable strains" would be overcome and there would be minimal misidentification because the identification of a clinical strain is clearly defined by the number of base differences between it and the existing species. Third, oligonucleotides representing all bacterial species, including those rarely encountered clinically (as in the present case), can be included in the array, making it easy to identify the rare species. Fourth, such a technique would be applicable not only to pyogenic bacteria, but also to other organisms, such as mycobacteria, the identification of which is not performed in most clinical microbiology laboratories because special expertise and equipment such as gas liquid chromatography are required. Therefore, it will reduce not only manpower, but also capital costs and the cost of consumables in the long run. Although the cost effectiveness of using 16S rRNA gene sequencing in routine clinical microbiology laboratories remains to be evaluated, our present report represents another example of the usefulness of 16S rRNA gene sequencing for the identification of a rarely encountered clinical isolate with ambiguous biochemical profiles.

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