Identification by 16S rRNA Gene Sequencing of *Lactobacillus salivarius* Bacteremic Cholecystitis

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An anaerobic, nonsporulating, gram-positive bacterium was isolated from blood and bile pus cultures of a 70-year-old man with bacteremic acute cholecystitis. The API 20A system showed that it was 70% *Actinomyces naeslundii* and 30% *Bifidobacterium* species, whereas the Vitek ANI system and the ATB ID32A Expression system showed that it was “unidentified.” The 16S rRNA gene of the strain was amplified and sequenced. There were 3 base differences between the nucleotide sequence of the isolate and that of *Lactobacillus salivarius* subsp. *salivarius* or *L. salivarius* subsp. *salicinius*, indicating that the isolate was a strain of *L. salivarius*. The patient responded to cholecystectomy and a 2-week course of antibiotic treatment. Identification of the organism in the present study was important because the duration of antibiotic therapy would have been entirely different depending on the organism. If the bacterium had been identified as *Actinomyces*, penicillin for 6 months would have been the regimen of choice. However, it was *Lactobacillus*, and a 2-week course of antibiotic was sufficient.

Identification of anaerobic, nonsporulating, gram-positive bacteria in the clinical microbiology laboratory is traditionally performed by isolation of the organism and phenotypic study of the organism by elucidation of its morphological and biochemical characteristics and metabolic end products. However, these methods have two major drawbacks. First, for organic acid analysis by gas chromatography, special equipment and expertise are required but are generally not available in most routine clinical microbiology laboratories. Second, we are occasionally faced with organisms with biochemical characteristics that do not match the patterns for any known genus and species.

Since the discovery of PCR and DNA sequencing, comparison of the gene sequences of bacterial species showed 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 determination of the species of bacteria. By use of this new standard, phylogenetic trees, based on base differences between species, are constructed; and bacteria are classified and reclassified into new genera (12, 13). Furthermore, noncultivable organisms and organisms with ambiguous biochemical profiles can be classified and identified (15, 16). Recently, we have reported on the application of 16S rRNA gene sequencing to the identification of clinical isolates with ambiguous biochemical profiles (23, 24, 26, 27) and a bacterium that was noncultivable (6). Here, we report on the application of such a technique to the identification of a strain of *Lactobacillus salivarius* isolated from the blood and gallbladder pus of a patient with acute cholecystitis and bacteremia and discuss the significance of identification of the organism.

All clinical data were collected prospectively, as described in our previous publication (9). Clinical specimens were collected and handled by standard protocols. The Vitek ANI system

(bioMérieux Vitek, Hazelwood, Mo.), the API 20A system (bioMérieux Vitek), and the ATB ID32A Expression system (bioMérieux Vitek) were used for the identification of the bacterial isolate obtained in the present study.

Bacterial DNA extraction, PCR amplification, and DNA sequencing of the 16S rRNA gene were performed as described in our previous publications (6, 22, 23, 24, 25, 26, 27). Primers LPW57 (5'-AGTTTGATCCTGGCTCAG-3') and LPW205 (5'-CTTGTTACGACTTCACCC-3') were used as the PCR primers; and primers LPW57, LPW205, LPW290 (5'-ACGGCTAACTACGTGCCAG-3'), and LPW291 (5'-TTAAACCACATGCTCCACCG-3') were used as the sequencing primers. The sequence of the PCR product was compared with known 16S rRNA gene sequences in GenBank by multiple-sequence alignment with the CLUSTAL W program (20).

A 70-year-old Chinese man was admitted to a hospital because of right-upper-quadrant abdominal pain and repeated vomiting for 1 day. He had Parkinson's disease, stable chronic obstructive pulmonary disease, and a history of appendicitis with appendectomy that had been performed 30 years earlier. On admission, his oral temperature was 38°C. Examination of the abdomen revealed right-upper-quadrant tenderness and guarding but no rebound tenderness. The total white cell count was 21.8×10^9 /liter, with a neutrophil count of 19.4×10^9 /liter, a lymphocyte count of 0.8×10^9 /liter, and a monocyte count of 1.5×10^9 /liter. The patient's hemoglobin level was 17.8 g/dl, and his platelet count was 281×10^9 /liter. The serum urea level was 3.9 mmol/liter, the creatinine level was 91 μ mol/liter, the albumin concentration was 41 g/liter, and the globulin concentration was 37 g/liter. Liver enzyme levels were normal, and the total bilirubin level was 20 μ mol/liter. A blood culture was performed, and the patient was commenced on empirical intravenous cefuroxime and metronidazole therapy. Contrast computed tomography of the abdomen showed pericholecystic fluid with a thickened gallbladder wall and a mildly dilated common bile duct. Intrahepatic ducts were not dilated.

In view of the persistent leukocytosis and right-upper-quadrant

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rant tenderness, an open cholecystectomy and operative cholangiography were performed 2 days after admission. During the operation, the acutely inflamed gallbladder was found to be grossly distended and filled with pus. There were multiple gallstones, but the cystic duct was not dilated. No filling defect was found in the biliary tree, and the free flow of the contrast material into the duodenum was demonstrated. Pus from the gallbladder was sent for bacterial culture. Histology of the sectioned gallbladder confirmed acute cholecystitis on a background of chronic cholecystitis and cholesterosis.

On day 2 postincubation, the aerobic blood culture bottle turned positive with a gram-positive, non-spore-forming bacillus. The bacterium grew on blood agar, chocolate agar, and MacConkey agar to sizes of 1 mm in diameter after 24 h of incubation at 37°C in an anaerobic environment but to only pinpoint sizes after 24 h of incubation at 37°C in 5% CO₂. The API 20A system indicated that it was 70% *Actinomyces naeslundii* and 30% *Bifidobacterium* species, whereas the Vitek ANI system and the ATB ID32A Expression system indicated that it was "unidentified" (Table 1). A strain with the same biochemical profile was also recovered from the bile pus culture. The patient was continued on antibiotics postoperatively, and the infection resolved.

PCR of the 16S rRNA genes of both isolates from the blood culture and gallbladder pus showed bands at 1,515 bp. Sequencing of the 16S rRNA genes from both specimens showed that they possessed the same nucleotide sequence. There were 3 base differences between the sequence of the isolate and that of *L. salivarius* subsp. *salivarius* (GenBank accession no. AF089108), 3 base differences between the sequence of the isolate and that of *L. salivarius* subsp. *salicinius* (GenBank accession no. M59054), 73 base differences between the sequence of the isolate and that of *Lactobacillus aviarius* (GenBank accession no. AB001836), 325 base differences between the sequence of the isolate and that of *A. naeslundii* (GenBank Accession no. X53226), and 350 base differences between the sequence of the isolate and that of *Bifidobacterium catenulatum* (GenBank accession no. M58732), indicating that the isolate was a strain of *L. salivarius*.

We describe the identification of a strain of *L. salivarius* isolated from the blood and gallbladder pus of a patient with acute cholecystitis by 16S rRNA gene sequencing, and this report is the first to describe a case of bacteremic *Lactobacillus* cholecystitis. Lactobacilli are generally considered nonpathogenic and relatively avirulent. However, they can infrequently cause serious infections, including endocarditis, pleuropulmonary infection, intra-abdominal abscesses, meningitis, conjunctivitis, dental caries, and endometritis. As for biliary tract infections, in the English-language literature there is only a single report of *Lactobacillus casei* as a cause of empyema of the gallbladder, which occurred in a 73-year-old woman; she was successfully treated with drainage and antibiotic therapy, followed by elective cholecystectomy (3).

Identification of the organism in the present study was important because the duration of antibiotic therapy would have been entirely different if the organism had been different from the one identified. If the bacterium had been identified as *Actinomyces*, another anaerobic, nonsporulating, gram-positive bacillus, penicillin for 6 months would have been the regimen of choice. However, the organism was *Lactobacillus*, and a

TABLE 1. Biochemical profile and identification of the blood culture isolate with the Vitek ANI system, ATB ID32A Expression system, and API 20A system

Biochemical reaction, enzyme, or isolate identification	Test result		
	Vitek ANI	ATB ID32A	API 20A
Biochemical reactions			
Hydrolysis of:			
Esculin			—
Gelatin			—
Production of indole		—	—
Reduction of:			
Nitrate		—	
Triphenyl tetrazolium	—		
Oxidation or fermentation of:			
Arabinose	—		—
Cellobiose			—
Glucose	+		+
Glycerol			—
Lactose			+
Maltose			+
Mannitol			+
Mannose		+	+
Melezitose			—
Raffinose	+	+	+
Rhamnose			—
Salicin			—
Sorbitol			+
Sucrose			+
Trehalose	+		+
Xylose	—		—
Enzymes			
Arginine dehydrogenase	+	+	
Catalase			—
Glutamic acid decarboxylase		—	
Alkaline phosphatase	+	+	
Phosphate choline	—		
Urease	—	—	—
α-Arabinosidase	—	—	
α-Fucosidase	—	—	
α-Fucosidase	—		
α-Galactosidase	+	+	
β-Galactosidase	+	+	
β-Galactosidase-6-phosphate		—	
α-Glucosidase	+	+	
β-Glucosidase	—	—	
β-Glucuronidase	—	—	
α-Mannosidase	—		
β-Lactosidase	—		
β-Xylosidase	—		
N-Acetyl-glucosaminidase	—	—	
Alanine arylamidase	+	+	
Arginine arylamidase		+	
Benzoyl arginine arylamidase	—		
Glutamyl glutamic acid arylamidase	—	—	
Glycine arylamidase		+	
Histidine arylamidase		+	
Leucine arylamidase	+	+	
Leucyl glycine arylamidase		—	
Lysine arylamidase	+		
Phenylalanine arylamidase		+	
Proline arylamidase	+	+	
Pyroglutamic acid arylamidase		—	
Serine arylamidase		+	
Tyrosine arylamidase		+	
Isolate identification	Unidentified	Unidentified	<i>A. naeslundii</i> (70%) and <i>Bifidobacterium</i> species (30%)

2-week course of antibiotic treatment was sufficient. In fact, *Actinomyces* species have been well reported as the causative agents of cholecystitis, and the incidence of biliary tract infections caused by *Actinomyces* reported in the English-language literature (seven cases) (8, 10, 11, 14, 18, 21) is far greater than the incidence of infections caused by *Lactobacillus* (one case) (3).

Problems with identification have often been encountered with anaerobic gram-positive rods. In one report, only 30.2% of *Lactobacillus* isolates from human stool and vaginal specimens were correctly identified to the species level with the API 50CHL kit (19). For our isolate, although resistance to vancomycin (no zone of inhibition by the disk diffusion test) suggested that the organism could be *Lactobacillus*, commercially available kits failed to identify it to the genus level. Interestingly, the API 20A system identified our isolate as *A. naeslundii* with 70% confidence.

Resistance to gastric acid as well as bacteriocin and lactic acid production may explain why *L. salivarius* was recovered in pure culture from both blood and bile, while common pathogens of biliary tract infections were not found. *L. salivarius* is part of the normal flora in the human oral cavity, gastrointestinal tract, and genitourinary tract. It has been reported to cause only dental caries and a case of endocarditis in humans (1, 5). Several reasons may explain why the organism detected in the present study was found in pure culture. First, the acid tolerance of the organism may have contributed to its survival in the gastric acid environment and its ability to enter the duodenum and biliary tract. In fact, bacteria, including lactobacilli, have been found to exist within bile ducts but have caused little symptomatology, and cultures of gallbladder wall and gallbladder bile are positive for about 50% of patients with chronic cholecystitis (7). Second, bacteriocin or bacteriocin-like substances have been synthesized from different *L. salivarius* strains and have been found to have inhibitory or bactericidal activities against various bacteria, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* species, *Micrococcus luteus*, *Listeria monocytogenes*, *Lactobacillus* species, *Actinomyces viscosus*, *Propionibacterium acnes*, *Pediococcus* species, *Yersinia enterocolitica*, and *Neisseria gonorrhoeae* (4, 17). Third, *L. salivarius* has also been proved to be capable of producing a large amount of lactic acid, which completely inhibited the growth of *Helicobacter pylori* in a mixed culture (2). We speculate that the *L. salivarius* strain in our patient may have colonized the gallbladder after ascending from the gastrointestinal tract, and it may also possess the ability to produce bacteriocins and lactic acid, thus inhibiting the growth of other bacteria in the biliary tract. In the presence of a transient obstruction, the gallbladder became acutely inflamed, resulting in cholecystitis and, subsequently, bacteremia.

Nucleotide sequence accession number. The 16S rRNA gene sequence of the *L. salivarius* strain from the present study was submitted to GenBank and given accession no. AF420311.

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