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# *Haemophilus parainfluenzae* Endocarditis: Application of a Molecular Approach for Identification of Pathogenic Bacterial Species

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*Haemophilus parainfluenzae* is both a human oropharyngeal commensal bacterium and a cause of serious invasive disease. The fastidious growth characteristics of this organism and the poor specificity of traditional methods for species identification are likely to have led to inaccuracies in the diagnosis of infections caused by *H. parainfluenzae* and related organisms. We report a case of *H. parainfluenzae* endocarditis in which confusion related to microbial identification was resolved by the analysis of 16S ribosomal RNA sequences. Rapid identification was facilitated by amplification of 16S ribosomal DNA directly from cultured cells with use of the polymerase chain reaction and by direct DNA sequence determination of the amplified product. This procedure is potentially useful for the identification of fastidious bacterial pathogens by reference laboratories.

*Haemophilus parainfluenzae* is a common inhabitant of the upper respiratory tract as well as an occasional pathogen. In one study, it was isolated from the throats of all of 10 healthy children and all of 10 healthy adults and constituted 74% of cultivated pharyngeal *Haemophilus* organisms [1]. Among *Haemophilus* species, this organism is the second most frequently isolated pathogen after *Haemophilus influenzae*. Reported disease syndromes caused by *H. parainfluenzae* include endocarditis, meningitis, pharyngitis, epiglottitis, otitis media, dental abscess, conjunctivitis, brain abscess, pneumonia, empyema, septicemia, septic arthritis, osteomyelitis, soft-tissue abscess, peritonitis, hepatic abscess, and urinary tract infection [2, 3].

Endocarditis caused by *Haemophilus* species accounts for more than half of all cases caused by a group of fastidious coccobacillary organisms known as the HACEK group. The other organisms in this group are *Actinobacillus actinomycescomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*. HACEK organisms, in turn, cause more than half of all cases of gram-negative bacterial endocarditis [4]. It is likely that many cases of endocarditis caused by these organisms have been classified as culture-negative or attributed to the wrong species because of difficulties in iso-

lation or identification, especially of *Haemophilus paraphrophilus* and *H. parainfluenzae*. These organisms remain difficult to cultivate in the laboratory and usually require at least 1 week for identification to the species level. Their identification has been based on metabolic or biochemical features that may be variable or nonspecific [5].

Species and, in some instances, strains can be reliably identified by the analysis of certain specific gene sequences—in particular, those of the ribosomal RNA (rRNA) genes. This type of approach has been applied clinically to the identification of “unculturable” microbial pathogens and fastidious microorganisms such as the mycobacteria [6–9]. In this paper, we use a case of endocarditis to illustrate how a 16S rRNA-based approach can be used to expeditiously resolve confusion regarding the identification of *H. parainfluenzae*.

## Materials and Methods

**Determination of 16S rRNA sequence.** A partial sequence of the 16S rRNA gene of the bacterial isolate—referred to as UNK—from the case described below was obtained as follows. The bacterial cell mass from several minute colonies of a blood-bottle subculture was digested overnight at 55°C in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Laureth-12 (Mazer Chemicals, Gurnee, IL), and 0.2 mg of proteinase K/mL (Boehringer Mannheim, Indianapolis), as previously described [6]. After inactivation of the proteinase K at 94°C for 10 minutes, DNA from 1- $\mu$ L and 10- $\mu$ L aliquots of the lysate was amplified by the polymerase chain reaction (PCR) with use of the bacterial broad-range 16S rRNA primers 8FPL and 806R [10, 11]. The PCR products were separated by agarose gel electrophoresis and purified with the Magic PCR Preps System (Promega Corporation, Madison, WI). The purified DNA fragments were directly sequenced with the *Taq* polymerase-based *fmol* sequencing system (Pro-

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mega). The sequencing primers 50F (5' AACACATGCAAGTCGAACG3') and 512R (5' CGAATTCCCGCGGCTGCTGGCACGGA3') were based on conserved bacterial 16S rRNA sequences [11, 12]. The sequencing reactions were chased with terminal deoxynucleotidyltransferase (Gibco BRL, Gaithersburg, MD), and <sup>35</sup>S-labeled sequencing products were analyzed by separation on buffer gradient polyacrylamide-urea gels and autoradiography [13].

Multiple-sequence alignment was performed with Pileup, a program in the Genetics Computer Group Sequence Analysis Software Package, version 7.1.1 [14]. Pileup uses a simplification of the progressive alignment method to create alignments and a dendrogram [15]. The sequences of 16S rRNA genes from members of the family Pasteurellaceae, including a representative of each subcluster defined by Dewhirst et al. [16], and from *Escherichia coli* were obtained from GenBank (Bethesda, MD) and used as a data set for the alignment. The species of origin for these sequences (as listed by Dewhirst et al. [16]), the strain designations, and the GenBank accession numbers are as follows: *A. actinomycetemcomitans*, ATCC 29522, M75036; *H. aphrophilus*, ATCC 33389<sup>T</sup>, M75041; *H. influenzae*, ATCC 33391, M35019 and M59433; *Actinobacillus seminis*, ATCC 15768<sup>T</sup>, M75047; *Pasteurella* species, CCUG 18782, M75055; *Pasteurella multocida*, NCTC 10322, M35018 and M59769; *Haemophilus parasuis*, NCTC 4557<sup>T</sup>, M75065; *Actinobacillus suis*, ATCC 15557, M75071; *Actinobacillus ureae* (listed by GenBank as *Pasteurella ureae*), Henrikson 3520/59<sup>T</sup>, M75075; *Actinobacillus* species (listed by GenBank as *Actinobacillus capsulatus*), CCUG 19799, M75067; *Actinobacillus pleuropneumoniae*, ATCC 27088<sup>T</sup>, M75074; *Haemophilus paraphrohaemolyticus*, NCTC 10670<sup>T</sup>, M75076; *Haemophilus ducreyi*, ATCC 33922, M75079; *Pasteurella haemolytica*, NCTC 9380<sup>T</sup>, M75080; *H. parainfluenzae*, ATCC 33392<sup>T</sup>, M75081; *H. parainfluenzae* (listed by GenBank as *H. paraphrophilus*), ATCC 29242, M75082; *Haemophilus* species, strain 202, M75077; *Pasteurella pneumotropica*, NCTC 8141<sup>T</sup>, M75083; and *E. coli*, K-12/MG1655, J01695. Base positions were identified by the numbering established for *E. coli* 16S rRNA [17]. In order to ensure unambiguous alignment, bases 94–489 of each sequence were used for analysis.

The entire procedure—from cell digestion to sequence analysis—consumed ~48 hours; however, this procedure can be significantly shortened, as will be described below.

## Case Report

A 24-year-old woman with nontropical sprue was admitted to Stanford University Medical Center with a 10-day history of nausea, emesis, and headache and a 4-day history of fever. She had no history of abdominal pain, diarrhea, photophobia, neck stiffness, or head trauma. The day before admission the patient had undergone a neurological examination that yielded normal results. A lumbar puncture at that

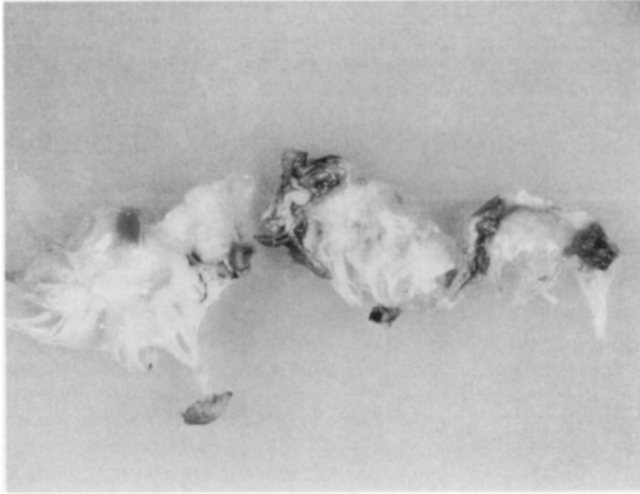
time yielded CSF containing 6 red blood cells/mm<sup>3</sup>, 9 leukocytes/mm<sup>3</sup> (100% monocytes), 27 mg of protein/dL, and 58 mg of glucose/dL; the blood glucose level was 85 mg/dL. Gram staining of the CSF revealed no organisms.

On admission the patient was lethargic and somewhat confused; her temperature was 39°C, her blood pressure was 100/68 mm Hg, and her pulse was 108/min, with orthostatic changes. Her fundi were unremarkable and her neck was supple. Examination of her mouth revealed good dental hygiene with multiple erupting wisdom teeth. A grade 1/6 systolic murmur was heard by one examiner. The lungs were clear to auscultation and percussion. Hepatosplenomegaly was detected. The patient was oriented to time, place, and person, and she had no focal neurological findings. The rest of the results of her physical examination were normal.

Initial laboratory findings included a peripheral-blood leukocyte count of 17,000/mm<sup>3</sup>, with 65% polymorphonuclear cells and 22% band forms; a hematocrit of 32.0%; a platelet count of 84,000/mm<sup>3</sup>; and the following serum levels: sodium, 135 mmol/L; potassium, 2.7 mmol/L; chloride, 95 mEq/L; bicarbonate, 30 mEq/L; albumin, 2.7 g/dL; and lactate dehydrogenase, 952 IU/L. Serum levels of creatinine, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase were within normal limits. Urinalysis revealed microscopic hematuria. A chest roentgenogram was normal. Blood was drawn for cultures, and therapy was begun with parenteral broad-spectrum antibiotics.

On the second day of hospitalization, the patient developed photophobia, increased lethargy, and disorientation to time. She also had meningismus but exhibited no other neurological abnormalities. A second lumbar puncture documented a total CSF leukocyte count of 9/mm<sup>3</sup>, with 61% polymorphonuclear cells, 14% lymphocytes, and 25% monocytes. No red blood cells were detected in CSF; the protein level was 24 mg/dL and the glucose level was 50 mg/dL. The glucose concentration in blood was 136 mg/dL. The initial diagnosis was meningoencephalitis of probable viral origin, and intravenous acyclovir was added to the regimen. However, magnetic resonance imaging of the head revealed hyperintense areas that did not display enhancement with gadolinium in the left basal ganglia and the left middle cerebellar peduncle on a T2-weighted image. Transesophageal echocardiography performed on the same day showed mitral valve prolapse, a large vegetation on the anterior mitral leaflet, and possible posterior mitral annular abscess. By this time the systolic murmur heard on admission had increased to grade 3/6, with radiation to the axilla.

On the fifth day of hospitalization, one of the cultures of blood drawn at admission became positive for small gram-negative rods with positive catalase but negative oxidase reactions. These organisms were preliminarily identified as *A. (Haemophilus) actinomycetemcomitans*. At this time two transient Janeway lesions were described on the palmar aspect of the fingers. The patient continued to be lethargic



**Figure 1.** Mitral-valve leaflet vegetations from a woman subsequently shown to have *Haemophilus parainfluenzae* endocarditis. Small gram-negative bacilli were found within these vegetations (courtesy of Dr. Donald Regula, Department of Pathology, Stanford University School of Medicine).

and to have high-grade fever, but she had neither clinical evidence of congestive heart failure nor electrocardiographic evidence of conduction defects. On the sixth day of hospitalization, there was an abrupt onset of intense left-upper-quadrant abdominal pain. A computerized tomogram of the abdomen with contrast showed multiple low-density regions throughout the spleen—the largest measuring 4 cm × 5 cm—that were consistent with infarctions. The scan also revealed hepatosplenomegaly. The following day the patient underwent mitral valve replacement with a bileaflet mechanical prosthetic valve. Loosely attached vegetations 2–7 mm in diameter were found on both leaflets of the native valve (figure 1). Microscopic examination of the valvular material revealed numerous small gram-negative bacilli.

The patient's postoperative course was generally uneventful, with resolution of fever on the second day. No CSF cultures yielded growth. The patient received intravenous ampicillin (12 g daily) for 3 weeks; because of the development of an ampicillin-associated rash, this drug was subsequently replaced with intravenous ceftriaxone (2 g daily), which was administered for an additional 3 weeks. In addition, intravenous gentamicin (2 mg/kg daily) was given for a total of 4 weeks. The patient was doing well 4 months after the initial episode.

An identical organism was eventually isolated from four of eight paired sets of blood cultures. Growth was detected in the aerobic bottle of each set 48–96 hours after inoculation as well as in one anaerobic bottle that was subcultured after 7 days. A Bactec 9240 system (Becton-Dickinson, Sparks, MD) was used with enriched soybean-casein digest broth bottles (BACTEC PLUS Aerobic/F and BACTEC Standard

Anaerobic/F). After 24 hours, pinpoint colonies were observed on chocolate agar plates only; gram staining revealed thin, delicate gram-negative rods. After 48 hours, these colonies became gray; in addition, fine pinpoint colonies were observed on Brucella agar plates. An oxidase test based on commercial tetramethylphenylenediamine reagent (BBL, Cockeysville, MD) was negative. The star-like colonial morphology and puff-like growth on the side of a brain-heart infusion broth tube that are sometimes seen with *A. actinomycetemcomitans* were not observed. Repeat oxidase testing of subcultured colonies gave a positive result. The organism required V Factor but not X Factor and was catalase-positive. It was subsequently identified as *H. parainfluenzae* biotype II, both by the RapID NH System (Innovative Diagnostic Systems, Atlanta) and by biochemical testing recommended by the Centers for Disease Control and Prevention (CDC). (However, the isolate did not ferment glucose and sucrose, probably because of poor growth.) The organism did not produce  $\beta$ -lactamase. The MICs for the strain were as follows: ampicillin, 0.25  $\mu$ g/mL; ceftriaxone,  $\leq$ 0.015  $\mu$ g/mL; and chloramphenicol,  $\leq$ 0.5  $\mu$ g/mL. This isolate was studied in further detail, as described under Results.

## Results

**16S rRNA sequence determination.** The bacterial blood isolate (UNK) cultured from the case just described was initially identified by the clinical microbiology laboratory as an oxidase-negative strain of *A. actinomycetemcomitans* and was subsequently reclassified as an oxidase-positive strain of *H. parainfluenzae*. To resolve this discrepancy, we sought to identify the isolate by sequence analysis. A sequence comprising 411 base pairs of the bacterial 16S rRNA gene (*E. coli* 16S rRNA positions 79–489) was determined unambiguously for UNK (figure 2). This sequence was deposited in the Genome Sequence DataBase (Los Alamos National Laboratory, Los Alamos, NM) under accession number L26363. 16S rRNA gene sequences from positions 94–489 were available for all members of a sequence data set drawn from *E. coli* and from representative members of the Pasteurellaceae (listed in Materials and Methods) and could be aligned unambiguously with the UNK sequence. In a phylogenetic tree constructed with this alignment, the UNK sequence and the only two available *H. parainfluenzae* 16S rRNA sequences formed a coherent, monophyletic lineage that was distinct from all other taxon lineages (data not shown). The UNK sequence was 99.2% identical to the sequence from one strain of *H. parainfluenzae* (GenBank accession number, M75082) and was 98.7% identical to the sequence from the second strain (M75081). The two published *H. parainfluenzae* sequences were 99.5% identical to each other in this region. The 16S rRNA gene sequence from *H. aphrophilus* (M75041) was the next most closely related to the UNK

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ECO      80           90           100          110          120
AAC      . . . . . T C . . . . . N
HAP      A G T a . . . T A C T . A . C . . . . . N
HPI      . . . . . . . . . . . . . . . . . . . .
UNK      G A A G C T T G C T T C T T T G A T G A C G A G T G G C G G A C G G G T G A G T A A T G C T T G G G A

ECO      130          140          150          160          170
AAC      . A . . G C . . G . . . . . G . T . . . G . . . . . A
HAP      . . N . g . . . . . . . . . . G . . . . . C . . . . .
HPI      . . N . N . . . . . . . . . . . . . . . . . . . . .
UNK      A T C T A G C T T A T G G A G G G G G A T A A C T A C G G G A A A C T G T A G C T A A T A C C G C G

ECO      180          190          200          210          220
AAC      . . A C G . . C A . . C C . . A . G . . . . T . N T . . G . T . . . C .
HAP      . . . G G . . . . . C . . . . . G . C . . . . . G A . . . . .
HPI      . . . . . . . . . . . . . . . . . . . . . . . . . . .
UNK      T A G A A T C G G G A G A T G A A A G T G T G G G A C C T T C G G G C C A C A T G C C A T A G G A T

ECO      230          240          250          260          270
AAC      . . T . . . G A . . . C . . . A . . . G . . . G . . . C T . . T . G .
HAP      . . A . . . . . T . . . . . . . . . . G . . . . G . . . C T . . . .
HPI      . . . . . . . . . . . . . . . . . . . . . . . . . . .
UNK      G A G C C C A A G T G G G A T T A G G T A G T T G G T G A G G T A A A G G C T C A C C A A G C C G A

ECO      280          290          300          310          320
AAC      . . . . . C . . . . . . . . . . G . . . . . C . . . . .
HAP      . . . . . . . . . . . . . . . . . . . . . . . . . . .
HPI      . . . . . . . . . . . . . . . . . . . . . . . . . . .
UNK      C G A T C T C T A G C T G G T C T G A G A G G A T G A C C A G C C A C A C T G G G A C T G A G A C A

ECO      330          340          350          360          370
AAC      . . . T . . . N . . . . . . . . . . . . . . . . . . . . . C
HAP      . . . G . . . N N N . . . . . . . . . . . . . . . . . . . .
HPI      . . . . . . . . . . . . . . . . . . . . . . . . . . .
UNK      C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G C G C A A T G G G G

ECO      380          390          400          410          420
AAC      . . . G . . . T . . . . . . . . . . . T . . . . . . . . .
HAP      . . . . . . . . . . . . . . . . . . . . . . . . . . .
HPI      . . . . . . . . . . . . . . . . . . . . . . . . . . .
UNK      G C A A C C C T G A C G C A G C C A T G C C G C G T G A A T G A A G A A G G C C T T C G G G T T G T

ECO      430          440          450          460          470
AAC      . . . . . A . . . . . A C G . G . . . . . G . G A . A G . . . . . C C T . T C C .
HAP      . . . . . . . . . . . T T . . . . . T T G G . G T G . . . . . G C A . C C A A .
HPI      . . . . . . . . . . . G A . . . . . T T G . G T G . . . . . G C G C A A C A A .
HPI      . . . . . . . . . . . N . . . . . . . . . . . . . . . . . . . . . G . A . G .
UNK      A A A G T T C T T T C G G T A G C G A G G A A G G C A T T T A G T T A A T A A A C T G G A T G A T

ECO      480
AAC      . . . . . C .
HAP      . . . . . T
HPI      . . . . .
UNK      T G A C G T T A A C

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**Figure 2.** Sequences of 16S rRNA genes from UNK (the bacterial blood isolate from a woman subsequently shown to have *Haemophilus parainfluenzae* endocarditis), *Escherichia coli*, and selected members of the Pasteurellaceae. Positions 79–489 are shown, numbered according to the system for *E. coli*. The International Union of Biochemistry nucleotide code is employed, with lower-case letters denoting ambiguous nucleotides in published sequences, *Ns* denoting undetermined bases, and dots denoting identity with the UNK sequence. *ECO* denotes *E. coli* (GenBank accession number, J01695); *AAC*, *Actinobacillus actinomycetemcomitans* (M75036); *HAP*, *Haemophilus aphrophilus* (M75041); and *HPI*, *H. parainfluenzae* (M75081). The UNK partial 16S rRNA sequence has been deposited in the Genome Sequence DataBase (accession number, L26363).

sequence, with 92.9% identity. The sequence from *A. actinomycetemcomitans* (M75036) was 89.9% identical to the UNK sequence.

Three nucleotide differences between the UNK sequence and either or both of the *H. parainfluenzae* sequences occurred at non-base-paired positions (numbered 182, 183, and 469) of 16S rRNA. The two other nucleotide differences between the UNK sequence and both *H. parainfluenzae* sequences occurred at base-paired positions within established

16S rRNA secondary structures [18]. The latter substitutions preserve canonical and noncanonical base pairing within these structures (position 473, A-U → G-U; position 475, G-U → A-U). Selected comparisons with use of more complete 16S rRNA gene sequences (1,483 and 1,479 bases in length) from the *H. parainfluenzae* strains indicate that the region from position 94 to position 489 shows more variability than does the more complete sequence (data not shown). Since 16S rRNA sequences from different species generally

**Table 1.** Characteristics differentiating HACEK organisms.

Organism	Factor required for growth		Expression or biochemical activity of indicated enzyme						Production of acid from		
	X	V	Indole production	Urease	Oxidase	Catalase	Lysine decarboxylase	Nitrate reduction	Glucose	Lactose	Sucrose
<i>Haemophilus parainfluenzae</i>	-	+	V	V	+	V	V	+	+	+	-
<i>Haemophilus paraphrophilus</i>	-	+	-	-	+	-	-	+	+	+	+
<i>Haemophilus aphrophilus</i>	+*	-	-	-	V	-	-	+	+	+	+
<i>Actinobacillus actinomycetemcomitans</i>	-	-	-	-	V	+	-	+	+	-	-
<i>Cardiobacterium hominis</i>	-	-	+	-	+	-	-	-	+	+	-
<i>Eikenella corrodens</i>	-	-	-	-	+	-	+	+	-	-	-
<i>Kingella kingae</i>	-	-	-	-	+	-	-	-	+	+	-

NOTE. Key: + = more than 90% of strains positive; - = more than 90% of strains negative; V = 10%-90% of strains positive.

\* Requirement for Factor X is reported at initial isolation but is lost on subculture.

differ by >1.5% [19], our similarity scores as well as our dendrogram are consistent with the placement of UNK within the species boundaries for *H. parainfluenzae*.

## Discussion

Russell and Fildes [20] first reported human disease due to *H. parainfluenzae* in 1928, some 6 years after the description of the species by Rivers [21]. *H. parainfluenzae* is a fastidious organism whose isolation often requires a long incubation period, specialized media, or terminal subculture. Colonies of *H. parainfluenzae* may grow to 3 mm in diameter after incubation at 35°C–37°C for 24 hours and may appear either flat, grayish, translucent, and smooth or rough and wrinkled. Gram staining may reveal pleomorphic gram-negative bacilli. In the identification of *H. parainfluenzae*, a requirement for Factor V should be established by the documentation of satellite growth around nicotinamide adenine dinucleotide-containing disks on nutritionally deficient medium, and the lack of a requirement for Factor X should be established by the porphyrin test, with  $\delta$ -aminolevulinic acid as substrate. Many clinical laboratories report all strains that require only Factor V as *H. parainfluenzae*. Thus, it is possible that the true prevalence of infection with this organism has never been established and that many cases reported as due to *H. parainfluenzae* may in fact be caused by other species that require only Factor V, such as *H. paraphrophilus*. Confirmation of species identity is sought only in certain circumstances by most laboratories and is based on growth-

factor requirements, enzymatic activities, and fermentation patterns (table 1). These tests often require 10–14 days and may give misleading or variable results, depending on the degree of bacterial laboratory passage (e.g., the dependence of *H. aphrophilus* on Factor X [22]), the growth stage of the organism (a point particularly relevant to microorganisms that are difficult to cultivate in the laboratory), the manner in which the test is performed (e.g., the oxidase assay in the case we have reported herein), and naturally occurring differences among strains. Several commercial kits are available for relatively rapid identification of *Neisseria* species, *Haemophilus* species, and *Moraxella catarrhalis* (e.g., the HNID, API NH, and RapID NH systems) [23, 24]; however, additional tests are sometimes needed for the identification of *H. parainfluenzae*, other *Actinobacillus* species have not been adequately evaluated, and misidentifications do occur.

We reviewed the medical literature from 1966 to July 1993 and found 37 reports describing 73 cases of *H. parainfluenzae* endocarditis [25–30]. (A complete list of references is available from the authors upon request.) The mean duration of incubation of primary blood cultures until the detection of microbiological growth (reported in 54 cases) was 6.6 days, with a range of 1 day to 21 days. The interval from the detection of microbiological growth in liquid media to the identification of *H. parainfluenzae* was not stated in most instances but was as long as 12 days in one case report [31]. In 48 of the 73 cases, the method used for identification to the species level was given. In 11 cases, species identification was described only in terms of requirements for Factors X

and V; the results of hemolysis assessment were also included in two of these cases. Both growth-factor requirements and biochemical activities (as outlined in table 1) were reported in 37 cases. In 10 of these cases, the identity of *H. parainfluenzae* was confirmed by the CDC.

The identification of fastidious microbial pathogens in general is a problem of potentially wide magnitude. Even if a fastidious microorganism can be reliably propagated in the laboratory, microbial phenotypic characteristics are often variable and therefore unreliable for species identification; in addition, their determination may require lengthy periods. The identification of *H. parainfluenzae* by traditional methods illustrates these points. Many of the phenotypic characteristics used to distinguish among *Haemophilus* species and other HACEK organisms can vary among strains of the same purported species (table 1), in part because assay methods are poorly standardized. In addition, these phenotypes probably do not provide a means for unified and unambiguous classification of members of the family Pasteurellaceae [16].

Small-subunit (16S) rRNA sequence analysis may prove a rapid and specific approach to the identification of fastidious microorganisms. These sequences can be amplified directly from infected host tissue—with consequent elimination of the need for laboratory cultivation—and from minute quantities of culture material [6, 10]. Small-subunit rRNA sequences currently form the basis for a revised approach to microbial taxonomy [11] and have been used to establish phylogenetic relationships among many bacteria, including members of the genus *Mycobacterium* [9, 32, 33], members of the family Pasteurellaceae [16, 34], and several previously uncharacterized human pathogens [6–8]. Analyses of the Pasteurellaceae have demonstrated four major clusters of strains, three of which correspond approximately to the genera *Haemophilus*, *Actinobacillus*, and *Pasteurella*. However, some members of each genus appear to have been erroneously classified by traditional methods. For example, *A. actinomycetemcomitans* should probably be reclassified as a species of *Haemophilus* [16]. Conversely, members of the species *H. parainfluenzae*, which seems to include genetically heterogeneous strains, are more closely related to *Actinobacillus* than to *Haemophilus*. Some strains of *H. parainfluenzae* may have been originally misidentified—e.g., as *H. paraphrophilus*—and vice versa. More recent 16S rRNA-based analysis suggests that *H. parainfluenzae* may consist of a monophyletic cluster distinct from other known related organisms [34].

We analyzed a partial 16S rRNA sequence from a cultivated bacterial pathogen purported to be *H. parainfluenzae* in order to clarify ambiguities about its identity and to demonstrate the potential usefulness of this approach for the identification of fastidious microorganisms to the species level. The region of the 16S rRNA gene that we analyzed is one of the most variable for members of the Pasteurellaceae and is sufficient to reproduce the basic phylogenetic relation-

ships among this group of organisms (data not shown). On the other hand, this region would not be adequate for the phylogenetic analysis of all clinical bacterial isolates. The UNK sequence is more closely related to the two available 16S rRNA sequences for *H. parainfluenzae* than to any other sequences; it differs from each of the former two sequences at fewer than 1.5% of positions. A phylogenetic tree places these three strains of *H. parainfluenzae* in a monophyletic group that is distinct from other species. Thus, our data demonstrate convincingly that the clinical isolate UNK belongs within the species *H. parainfluenzae*. The minor sequence heterogeneity that we observed may indicate strain-related differences [9] or differences among the multiple copies of the rRNA operon in some organisms [35].

Although our procedure—from cell digestion to sequence analysis—consumed ~48 hours, it could easily have been shortened. Specifically, the time required for cell digestion could have been reduced to 1 hour, and one of the automated DNA-sequencing systems, which permit much more rapid collection of data [36, 37], could have been used. With these modifications, the total time required for species identification might be reduced to ~12 hours.

The clinical impact of this information, while limited, may include more precise predictions of the course of disease and the outcome of therapy. For example, given the preceding discussion, the literature concerning *H. parainfluenzae* endocarditis must be viewed with some caution, since some isolates classified within this species may in actuality have been members of other species or genera (e.g., *Actinobacillus*). In addition, a delay in species identification may permit the growth of relatively large valvular vegetations associated with the HACEK organisms, which—given the particularly friable nature of these vegetations—may lead to a high frequency of embolization in cases of HACEK endocarditis. Direct amplification of microbial small-subunit rRNA sequences from blood may further reduce such delays in diagnosis. Although the technical expertise required for this kind of approach may not be available in most clinical laboratories, the use of automated DNA-sequencing procedures and simplified protocols may allow regional reference centers to offer small-subunit rRNA-based identification of diverse, fastidious microbial isolates in the near future.

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