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Characterization of *Haemophilus segnis*, an Important Cause of Bacteremia, by 16S rRNA Gene Sequencing

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We describe the application of 16S rRNA gene sequencing in defining eight cases of bacteremia due to *Haemophilus* species other than *Haemophilus influenzae* (non-*H. influenzae* bacteremia) during a 7-year period. The first case of acute pyelonephritis due to *Haemophilus segnis* is also reported. In contrast to the extremely rare incidence of *H. segnis* infections reported previously, our results suggested that *H. segnis* is an important cause of non-*H. influenzae* bacteremia.

Apart from *Haemophilus influenzae*, other *Haemophilus* species, in particular *H. segnis*, *H. haemolyticus*, and *H. parahaemolyticus*, are considered uncommon causes of infections (14). These organisms are often fastidious and may exhibit ambiguous phenotypic profiles. Therefore, the epidemiology of *Haemophilus* infections not due to *H. influenzae* (non-*H. influenzae* infections) may have not been accurately defined. 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 (18, 19). 16S rRNA gene sequences can be used to more accurately identify and classify fastidious organisms and organisms with ambiguous biochemical profiles (9, 11, 22, 24, 25, 27, 28). Recently, we reported the application of 16S rRNA gene sequencing in defining two cases of *H. segnis* bacteremia (10). In this study, we used 16S rRNA gene sequencing for the species level identification of non-*H. influenzae* blood culture isolates in a 7-year period. The clinical spectrum of diseases and outcomes for patients with non-*H. influenzae* bacteremia were also analyzed and are discussed.

The bacterial strains used in this study were isolates from blood cultures of patients hospitalized at the Queen Mary Hospital in Hong Kong during a 7-year period (January 1996 to December 2002). All clinical data were collected prospectively as described previously by Luk et al. (13). The BACTEC 9240 blood culture system (Becton Dickinson) was used, and all suspected colonies were identified by standard conventional biochemical methods (15). All isolates of gram-negative bacilli or coccobacilli that grew on chocolate agar but not on MacConkey agar were tested for factor X and factor V requirements (15), hemolysis on horse blood agar, CO_2 enhancement of growth, catalase, and indole production and were identified by the Vitek system (NHI; bioMerieux Vitek). All isolates identified as *Haemophilus* species other than *H. influenzae* by either conventional phenotypic tests or the Vitek System (*Neisseria*/*Haemophilus* identification [NHI]) were subjected to 16S rRNA gene sequencing. Antimicrobial susceptibility was tested by the Kirby-Bauer disk diffusion method, and results were interpreted according to the NCCLS criteria (16).

Bacterial DNA extraction, PCR amplification, and DNA sequencing of the 16S rRNA genes were performed as described previously (10, 12, 26, 29). LPW55 (5′-AGTTTGATCCTGGCTCAG-3′) and LPW56 (5′-AGGCCCGGGAACGTATTCAC-3′) were used as the PCR primers, and LPW55, LPW56, LPW69 (5′-AGCACCGGCTAACTCCGT-3′), and LPW106 (5′-TAATCCTGTTTGCTCCCCAC-3′) were used as the sequencing primers. The sequences of the PCR products were compared with known 16S rRNA gene sequences in the GenBank (http://www.ncbi.nlm.nih.gov) by multiple sequence alignment using the CLUSTAL W program (23); phylogenetic tree construction was performed with PileUp, and the neighbor-joining method was performed with GrowTree (Genetics Computer Group, Inc., San Diego, Calif.).

A total of 25 strains of *Haemophilus* spp. were isolated from the blood cultures of 25 patients during the 7-year study period. Of the 25 isolates, 17 were identified as *H. influenzae* and 8 were identified as other *Haemophilus* species by either standard phenotypic tests or the Vitek system (NHI). All of the eight non-*H. influenzae* isolates, including the two reported previously (10), were gram-negative coccobacilli and grew on chocolate agar to colonies of 0.5 to 1 mm in diameter after incubation for 24 h at 37°C in air with 5% CO_2. Their phenotypic characteristics, identification by the Vitek System (NHI), and antibiotic susceptibilities are shown in Table 1. PCR of the 16S rRNA genes of these eight isolates showed bands at about 1,400 bp. Three isolates (isolates 1, 2, and 8) were identified by 16S rRNA gene sequencing as *H. segnis*, two (isolates 4 and 6) were identified as *H. parainfluenzae*, two (isolates 3 and 7) were identified as *H. aphrophilus*, and one (isolate 5) was identified as *H. paraphrophilus* (Fig. 1). Only one of the eight isolates (isolate 4) was identified correctly by a combination of conventional biochemical tests and the Vitek System (NHI; bioMerieux Vitek).

The clinical characteristics of the eight patients with non-*H. influenzae* bacteremia are summarized in Table 1. All patients had community-acquired bacteremia. With the exception of those associated with *H. segnis*, the clinical mani-

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TABLE 1. Patient characteristics, phenotypic characteristics, identification and antibiotic susceptibilities of the eight non-*H. influenzae* blood culture isolates

Parameter	Result for patient:							
	1	2	3	4	5	6	7	8
Yr of isolation	1996	1996	1998	1999	1999	2000	2000	2000
Sex/age (yr)	F/83	M/32	M/62	F/70	M/48	M/49	F/65	F/78
Underlying condition(s)	*Clostridium difficile* colitis during the episode of bacteremia	Intravenous drug abuser	Fractured left medial malleolus due to accidental injury 1 week earlier	Diabetes mellitus, hypertension, intracerebral hemorrhage	Intravenous drug abuser, prosthetic valvular replacement, emphysema	Peptic ulcer treated several years earlier	Hypertension, diabetes mellitus, hyperlipidemia, hypertrophic obstructive cardiomyopathy	Carcinoma of cervix, vesicovaginal fistula, left ureteric obstruction
Diagnosis	Primary bacteremia	Bacteremic empyema	Bacteremic osteomyelitis of left distal tibia	Primary bacteremia	Infective endocarditis	Bacteremic spondylitis and discitis	Primary bacteremia	Bacteremic left pyelonephritis
Complication	Septic shock	None	None	None	None	None	None	Acute renal failure
No. of positive blood cultures	1	1	1	2	3	2	1	1
Type of bacteremia (concomitant isolates)	Monomicrobial	Polymicrobial (*Streptococcus intermedius*, *Streptococcus sanguinis*)	Monomicrobial	Monomicrobial	Monomicrobial	Monomicrobial	Polymicrobial (*Streptococcus anginosus*, *Bacteroides fragilis*, *Eikenella corrodens*)	Monomicrobial
X factor requirement	−	−	−	−	−	−	−	−
V factor requirement	+	+	−	+	+	+	−	+
Hemolysis of horse blood	−	−	−	−	−	+	−	−
CO_2 enhancement of growth	−	−	−	−	−	−	+	−
Catalase	−	−	−	+	−	+	−	−
Indole production	−	−	−	−	−	−	−	−
Identification by Vitek system (NHI)	95% *H. influenzae* VIII	56% *Actinobacillus actinomycetemcomitans*, 40% *Neisseria subflava*	62% *H. paraphrophilus*, 37% *H. aphrophilus*	98% *H. parainfluenzae* I	62% *H. paraphrophilus*, 37% *H. aphrophilus*	95% *H. parainfluenzae* III	67% *H. ducreyi*, 29% *Kingella kingae*	62% *H. paraphrophilus*, 37% *H. aphrophilus*
Identification by 16S rRNA sequencing	*H. segnis*	*H. segnis*	*H. aphrophilus*	*H. parainfluenzae*	*H. paraphrophilus*	*H. parainfluenzae*	*H. aphrophilus*	*H. segnis*
Antibiotic susceptibility[a]								
Ampicillin	S	S	S	S	S	S	S	S
Cefotaxime	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S
Cotrimoxazole	S	S	S	S	S	M	S	S
Chloramphenicol	S	S	S	S	S	S	S	S

[a] S, sensitive; M, intermediate resistant.

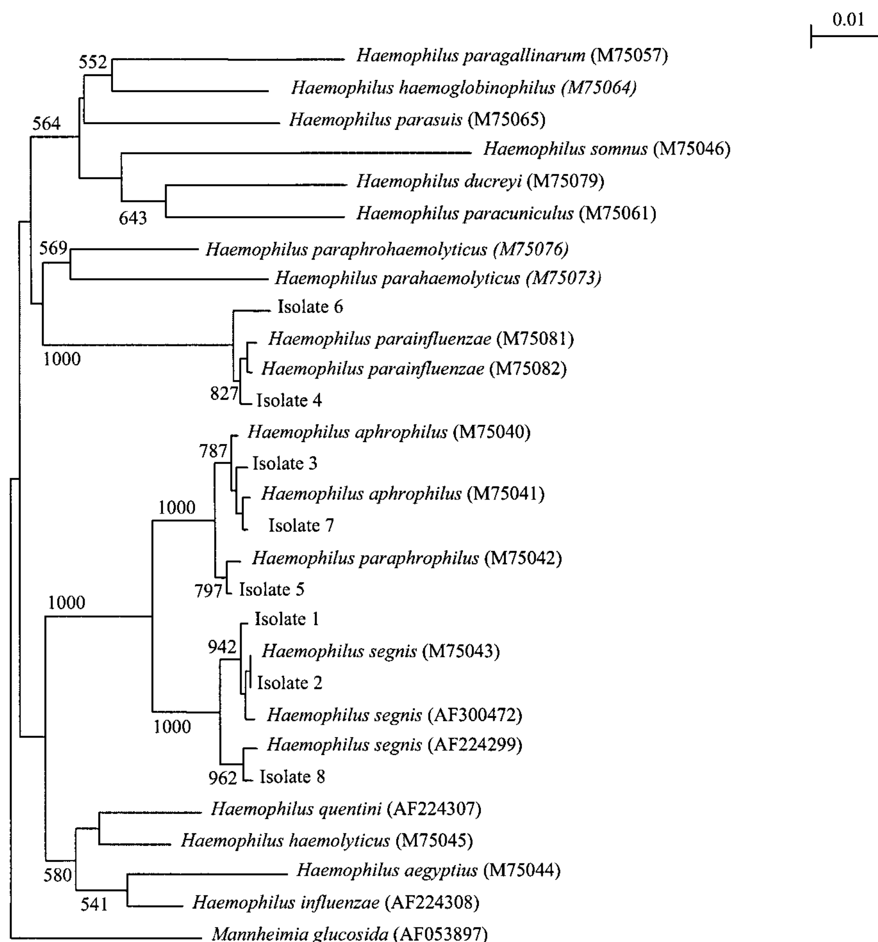

FIG. 1. Phylogenetic tree showing the relationship of the eight blood culture isolates from our patients to related species. The tree was inferred from 16S rRNA sequence data by the neighbor-joining method. Bootstrap values were calculated from 1,000 iterations. Scale bar, estimated number of substitutions per 100 bases by using the Jukes-Cantor correction. Names and accession numbers are given as cited in the GenBank database.

festations of *Haemophilus* bacteremia in our patients were compatible to those reported previously (2, 6, 17, 21). In contrast to *H. influenzae* bacteremia, which often affects young children and healthy adults, non-*H. influenzae* bacteremia was found to affect only adult patients (median age, 64 years; range, 32 to 83 years) who often had underlying diseases. Despite this, complications from non-*H. influenzae* bacteremia were not common. Apart from patient 1, who deteriorated rapidly after the bacteremia and died, all patients were cured with antibiotic treatment.

The present study suggests that the prevalence of *H. segnis* bacteremia may have been underestimated previously. Identification of *H. segnis* by conventional biochemical tests has been difficult (10). All three isolates in the present study could not be identified by phenotypic methods. *H. segnis* is phenotypically very similar to *H. parainfluenzae* and therefore may have been reported as *H. parainfluenzae* by many clinical laboratories (7). Although the numbers were small, *H. segnis* accounted for 37.5% (three of eight) of the cases of non-*H. influenzae* bacteremia in our locality, compared to 25% (two of eight) for both *H. parainfluenzae* and *H. aphrophilus* and 12.5% (one of eight) for *H. paraphrophilus*. This is in contrast to the rare isolation of *H. segnis* from blood cultures reported in the literature. Apart from the present three cases, there were only two reports of *H. segnis* bacteremia, one in a case of infective endocarditis and the other in a case of pancreatic abscess (1, 3). The first two cases in the present report, a case of primary bacteremia and a case of empyema thoracis, have been described previously, and the sources of the bacteremia were thought to be the gastrointestinal tract in one case and the oral cavity in the other (10). On the other hand, the last case represents the first report of pyelonephritis due to *H. segnis*. Since the patient had vesicovaginal fistula as a result of local radiotherapy for her carcinoma of the cervix, it is likely that the source of infection was her genital tract, which may have been transiently colonized by the bacterium. 16S rRNA gene sequencing should be used to identify more cases of *H. segnis* infections and better define its epidemiology, clinical spectrum, treatment, and outcome.

16S rRNA gene sequencing is the method of choice for the identification of *Haemophilus* species. As early as 1992, Dewhirst et al. reported the use of 16S rRNA gene sequences for phylogenetic study of *Pasteurellaceae* (5). Subsequently, its application for identification of isolated cases of *H. parainfluen-*

zae and *H. segnis* infections has been described (4, 7, 10). The present study also showed that, apart from its usefulness in identifying *H. segnis*, the technique is helpful for strains of other species, especially those with ambiguous biochemical profiles. One of our isolates (isolate 6) showed β-hemolysis on horse blood agar and was factor V, but not factor X, dependent. It would therefore be identified as *H. parahaemolyticus* by most clinical laboratories. 16S rRNA gene sequencing unambiguously identified it as *H. parainfluenzae*, an identification which is compatible with its other phenotypic characteristics. We speculate the isolate was a hemolytic variant of *H. parainfluenzae* that had acquired a hemolysin gene. Another isolate (isolate 7) also exhibited ambiguous biochemical profiles and was identified as 67% *H. ducreyi* by the Vitek system (NHI). It was confirmed to be *H. aphrophilus* only later by 16S rRNA gene sequencing. Although restriction enzyme analysis of PCR-amplified 16S rRNA genes has been used to distinguish among *Actinobacillus actinomycetemcomitans*, *H. aphrophilus*, and *H. paraphrophilus* (20), such a technique has not been demonstrated to be applicable to other species, and results may be compromised by single nucleotide substitutions at restriction sites. The translation initiation factor 2 has also been shown useful for phylogenetic analysis of *Haemophilus* (8) and may be an alternative molecular target useful for species identification.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of blood culture isolates 1, 2, 3, 4, 5, 6, 7, and 8 have been lodged within the GenBank sequence database under accession numbers AY365447, AY365448, AY365449, AY365450, AY365451, AY365452, AY365453, and AY365454, respectively.

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