J. Med. Microbiol. — Vol. 51 (2002), 635–640

ISSN 0022-2615

HOST RESPONSE TO INFECTION

Haemophilus segnis polymicrobial and monomicrobial bacteraemia identified by 16S ribosomal RNA gene sequencing

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This paper reports a case of *Haemophilus segnis* polymicrobial bacteraemia and a case of *H. segnis* monomicrobial bacteraemia identified by 16S ribosomal RNA gene sequencing. In the first case, a gram-negative aerobic coccobacillus was isolated with *Streptococcus intermedius* and *S. sanguis* from the blood culture of a 32-year-old intravenous drug addict with left thoracic empyema. In the second case, a gram-negative aerobic coccobacillus was isolated from the blood culture of an 82-year-old woman with *Clostridium difficile* colitis and septicaemic shock. Both gram-negative coccobacilli grew on chocolate agar as colonies of 1 mm in diameter after incubation for 24 h at 37°C in air with CO_2 5%, but only to pinpoint sizes on blood agar under the same incubation conditions. Both strains were factor V-dependent, but not factor X-dependent. For the first isolate, the Vitek system (NHI) showed that it was 56% likely to be *Actinobacillus actinomycetemcomitans* and 40% *Neisseria subflava*; whereas the API system (NH) showed that it was 58% likely to be *H. aphrophilus/paraphrophilus* and 42% *H. parainfluenzae*. For the second isolate, the Vitek system (NHI) showed that it was 95% likely to be *H. influenzae* VIII; whereas the API system (NH) showed that it was 58% likely to be *H. aphrophilus/paraphrophilus* and 42% *H. parainfluenzae*. 16S rRNA gene sequencing showed that there were four base differences between isolate 1 and *H. segnis* and two base differences between isolate 2 and *H. segnis*, indicating that both isolates most closely resembled a strain of *H. segnis*. Only two cases of *H. segnis* bacteraemia were found in the English scientific literature, one in a case of infective endocarditis and the other in a case of pancreatic abscess. Including the present two cases, the overall mortality of *H. segnis* bacteraemia was 50%.

Introduction

Haemophilus species, other than *H. influenzae*, have been considered uncommon causes of human disease. *H. segnis*, in particular, is rarely reported as being a pathogen. Infections reported to be associated with *H. segnis* include periodontal disease, infective endocarditis, acute cholecystitis, acute appendicitis and pancreatic abscess, amongst which *H. segnis* bacteraemia was reported only in a case of infective endocarditis and a case of pancreatic abscess [1–5]. *Haemophilus* spp. are traditionally identified on the basis of growth factor requirement, CO_2 dependence, haemolysis, enzymic activities and sugar fermentation. However, these biochemical tests may result in ambiguous biochemical profiles and give inconclusive results [5–7].

Since the introduction of PCR and DNA sequencing, 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. Phylogenetic trees, based on base differences between species, are constructed from 16S rRNA gene sequences; and bacteria are classified and re-classified into new genera [8, 9]. Furthermore, non-cultivable organisms and organisms with ambiguous biochemical profiles can be classified and identified [10, 11]. Recent reports described the application of

Received 29 June 2001; revised version received 2 Jan. 2002; accepted 9 Jan. 2002.
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16S rRNA gene sequencing in the identification of clinical isolates with ambiguous biochemical profiles [12–17] and a bacterium that was non-cultivable [18]. This article reports a case of *H. segnis* polymicrobial bacteraemia and a case of *H. segnis* monomicrobial bacteraemia identified by 16S rRNA gene sequencing. Other infections associated with *H. segnis* are also reviewed.

Materials and methods

Patients and microbiological methods

All clinical data were collected prospectively as described previously [19]. Clinical specimens were collected and handled according to standard protocols, and all suspect colonies were identified by standard conventional biochemical methods [20]. The Vitek System (NHI) (bioMérieux Vitek, Hazelwood, MO, USA) and API system (NH) (bioMérieux Vitek) were also used for the biochemical identification of the bacterial isolates in this study.

DNA extraction, PCR amplification and sequencing of 16S rRNA genes

Bacterial DNA extraction and PCR amplification and DNA sequencing of the 16S rRNA genes were performed as described previously [12–15, 21]. 5′-AGTTTGATCCTGGCTCAG-3′ (LPW55) and 5′-AG GCCCGGGAACGTATTCAC-3′ (LPW56) were used as the PCR primers and LPW55, LPW56, 5′-AGCAC CGGCTAACTCCGT-3′ (LPW69) and 5′-TAATCCT GTTTGCTCCCCAC-3′ (LPW106) were used as the sequencing primers. The sequences of the PCR products were compared with known 16S rRNA gene sequences in the GenBank database by BLAST searches and multiple sequence alignment was performed with the CLUSTAL W program [22]. The phylogenetic tree was constructed by the neighbour-joining method with GrowTree (Genetics Computer Group). A total of 1393 nucleotide positions was included in the analysis.

Results

Patients and identification of the bacterial isolates by conventional methods and commercially available systems

Case 1. A 32-year-old Chinese intravenous drug addict was admitted to hospital because of progressive shortness of breath and left pleuritic chest pain for 1 week. His past medical history was unremarkable. His oral temperature was 38°C. Chest X-ray showed left hydro-pneumothorax. Total white cell count was 21.3×10^9/L, with neutrophils 17.8×10^9/L, lymphocytes 1.1×10^9/L and monocytes 1.5×10^9/L. The haemoglobin level was 7.5 g/dl and the platelet count was 457×10^9/L. Blood culture was performed and the patient was treated empirically with intravenous ticarcillin/clavulanate and gentamicin. A chest drain was inserted and 1500 ml of pus with air was drained. The pus was sent for gram's smear and bacterial culture. Echocardiogram did not show evidence of infective endocarditis. The patient recovered after treatment with intravenous antibiotics for 6 weeks.

On day 1 of incubation, the aerobic blood culture bottle was positive with gram-positive cocci in chains and gram-negative coccobacilli. The gram-positive cocci were identified as *Streptococcus intermedius* and *S. sanguis*. The gram-negative coccobacillus grew on chocolate agar to give colonies of 1 mm in diameter after incubation for 24 h at 37°C in air with CO_2 5%, but only to pinpoint sizes on blood agar under the same incubation conditions. The strain was factor V-dependent, but not factor X-dependent. The Vitek system (NHI) showed that it was 56% likely to be *Actinobacillus actinomycetemcomitans* and 40% *Neisseria subflava*; whereas the API system (NH) showed that it was 58% likely to be *H. aphrophilus/paraphrophilus* and 42% *H. parainfluenzae* (Table 1). The strain was β-lactamase-negative and sensitive to ampicillin, cefotaxime, imipenem, co-trimoxazole and chloramphenicol. Gram's smear of the empyema pus showed leucocytes +++, gram-negative coccobacilli and gram-positive cocci in chains. However, only a *Bacteroides* sp. was isolated on culture.

Case 2. An 82-year-old Chinese woman was admitted to hospital because of lower abdominal pain and diarrhoea for 1 day. Her past history was unremarkable except for an episode of gastro-enteritis 2 weeks previously, which was treated with intravenous cefuroxime. Upon transfer, she was afebrile. Examination revealed lower abdominal tenderness with guarding and rebound tenderness. Abdominal X-ray did not show any significant abnormality. Total white cell count was 50.2×10^9/L, with neutrophils 44.2×10^9/L, lymphocytes 4.5×10^9/L and monocytes 1.5×10^9/L. Haemoglobin level was 13.5 g/dl and platelet count 438×10^9/L. Serum urea was 15.1 mmol/L and creatinine 219 μmol/L. Liver enzymes were normal. Blood culture was performed and stool was sent for culture and *Clostridium difficile* cytotoxin detection. Empirical intravenous cefoperazone/sulbactam was administered. She rapidly went into septicaemic shock and died despite attempted resuscitation.

On day 1 of incubation, the aerobic blood culture bottle was positive with a gram-negative aerobic coccobacillus. The bacterium grew on chocolate agar to give colonies of 1 mm in diameter after incubation for 24 h at 37°C in air with CO_2 5%, but only to pinpoint sizes on blood agar under the same incubation conditions. The strain was factor V-dependent, but not factor X-dependent. The Vitek system (NHI) showed that it was 95% likely to be *H. influenzae* VIII; whereas the API system (NH) showed that it was 58% likely to be *H.*

Table 1. Biochemical profile and identification of the blood culture isolates by Vitek system (NHI) and API system (NH)

Biochemical reactions/enzymes/substrates	Case 1		Case 2	
	Vitek NHI	API NH	Vitek NHI	API NH
Phenylphosphonate	−		+	
Proline arylamidase	−	−	−	−
γ-Glutamyl-arylamidase	−	−	−	−
Glycine arylamidase	−		−	
Lysine arylamidase	+		+	
β-Galactosidase	−	−	−	−
Indole production	−	−	−	−
Phosphate choline	−		+	
Acidification of				
glucose	+	+	+	+
fructose		+		+
sucrose	−		−	
maltose	+	+	+	+
saccharose		+		+
Reduction of triphenyl tetrazolium	−		−	
Resazurin	−		−	
Ornithine decarboxylase	−	−	−	−
Lipase		−		−
Alkaline phosphatase		+		+
Urease	−	−	−	−
Penicillinase	−	−	−	−
Identification	56% *A. actino-mycetemcomitans* 40% *N. subflava*	58% *H. aphrophilus/ paraphrophilus* 42% *H. parainfluenzae*	95% *H. influenzae* VIII	58% *H. aphrophilus/ paraphrophilus* 42% *H. parainfluenzae*

aphrophilus/paraphrophilus and 42% *H. parainfluenzae* (Table 1). The strain was β-lactamase-negative, and sensitive to ampicillin, cefotaxime, imipenem, co-trimoxazole, and chloramphenicol. Stool tests for *C. difficile* culture and cytotoxin were both positive.

16S rRNA gene sequencing

PCR of the 16S rRNA genes of the two isolates showed bands at 1393 bp. There were four base differences between blood culture isolate 1 and *H. segnis* (GenBank accession no. AF300472), and two base differences between blood culture isolate 2 and *H. segnis* (GenBank accession no. AF300472), indicating that both isolates most closely resembled a strain of *H. segnis* (Fig. 1).

Discussion

H. segnis was proposed as one of the factor V-dependent *Haemophilus* species by Kilan and Schiott in 1975 and Kilan in 1976, and was formally published in the *International Journal of Systematic Bacteriology* in 1977 [23]. This organism has been isolated from dental plaque and the human oropharynx. It is rarely reported as being a pathogen, but has been associated with periodontal disease and occasionally with serious infections including infective endocarditis, acute cholecystitis, acute appendicitis and pancreatic abscess (Table 2) [1–5]. An overall review of *H. segnis* infection showed a male:female ratio of 7:4. Most patients were young (median age 29 years). Only two patients had possible predisposing factors. One was a chronic alcoholic (case 9) and the other an intravenous drug addict (case 10). Acute appendicitis was the commonest clinical condition associated with isolation of *H. segnis* (cases 3–8) and peritoneal fluid was the commonest site for isolation of this organism (cases 3–7). Only two cases of *H. segnis* bacteraemia were found in the English scientific literature (MEDLINE Search 1966–2001), one in a case of infective endocarditis (case 1) and the other in a case of pancreatic abscess (case 9) [1, 5]. Including the present two cases, the overall mortality of *H. segnis* bacteraemia was 50%, whereas infections without bacteraemia were all cured with treatment (Table 2).

The route of infection in previous reports of *H. segnis* infection was largely uncertain, although the oropharynx was proposed to be the source in all cases [1–5]. In the first case in the present study, it is likely that the patient had an aspiration pneumonia complicated by empyema and secondary bacteraemia, because the two concomitant isolates from blood culture, *S. intermedius* and *S. sanguis*, are both commensals of the oropharynx. Gram's smear of the empyema pus showed gram-negative coccobacilli and gram-positive cocci in chains, but only a *Bacteroides* sp. was isolated on culture. This can be explained by prior administration of antibiotics before the thoracocentesis. In the second case, the lady had *C. difficile* colitis and died of septicaemic shock. *H. segnis* was isolated from her blood. We speculate that the bacterium gained access to the bloodstream through the inflamed bowel mucosa. Although *H. segnis* has not been isolated from the

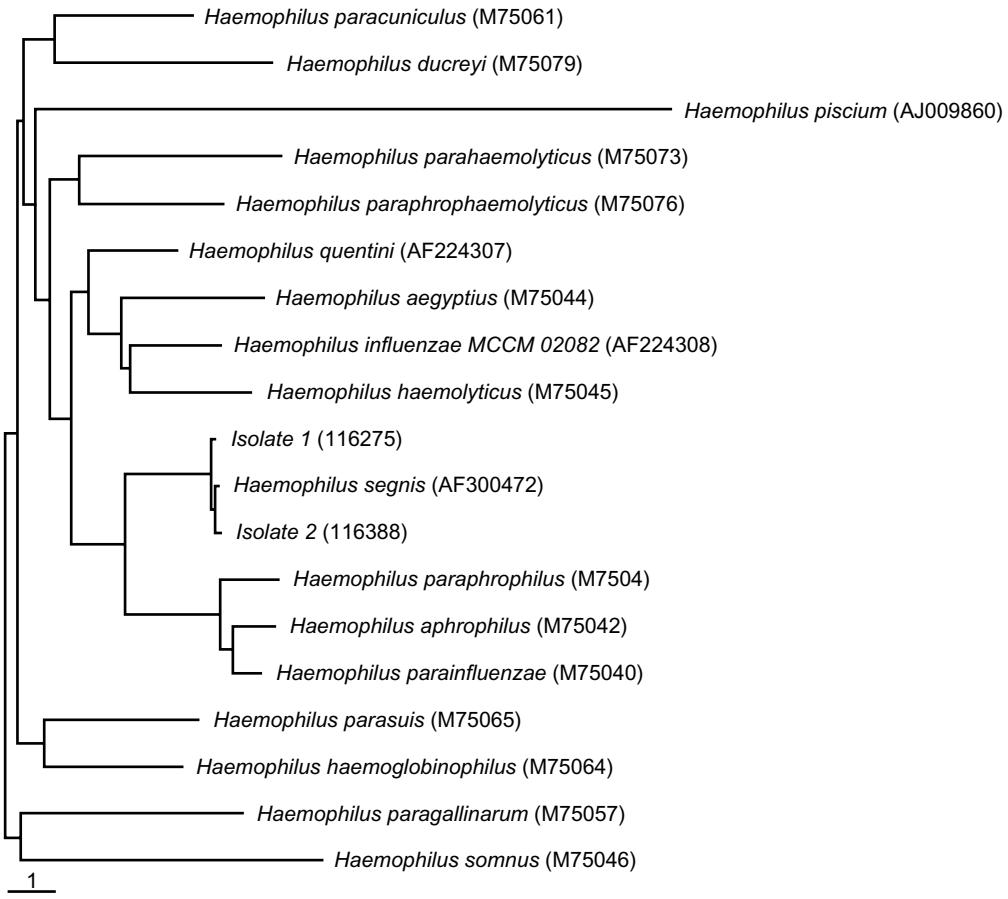

Fig. 1. Phylogenetic tree showing the relationship of isolates 1 and 2 to the other *Haemophilus* spp., *A. actinomycetemcomitans* and *N. subflava*. The tree was inferred from 16S rRNA sequence data by the neighbour-joining method. The scale bar indicates the number of substitutions per 100 bases estimated with the Jukes-Cantor correction. Names and accession nos are given as cited in the GenBank database. A total of 1393 nucleotide positions was included in the analysis.

lower gastrointestinal tract, its unexplained association with gastrointestinal tract infection suggests that it may occasionally colonise the gut. In three cases of *H. segnis* appendicitis, concomitant isolates from the peritoneal fluid were commensals of the lower gastrointestinal tract (Table 2, cases 5–7) [4] and *Haemophilus* spp. have been found to be members of the faecal flora [24, 25]. Further investigations are needed to delineate the relationship between *H. segnis* and gastrointestinal tract disease.

16S rRNA gene sequencing is useful for the identification of *H. segnis*. Identification of *H. segnis* by conventional biochemical tests has been difficult. *H. segnis* is a fastidious organism and is differentiated from other *Haemophilus* spp. primarily by its generally negative biochemical reactions (the Latin adjective segnis means sluggish) [23]. However, these tests may give inconclusive results. In the reported cases of *H. segnis* infection, all clinical isolates required confirmation of identity by reference laboratories (Table 2) [1–5]. *Haemophilus* spp. that are factor V- but not factor X-dependent include *H. segnis*, *H. paraphrophilus*, *H. parainfluenzae* and *H. parahaemolyticus*, of which *H. paraphrophilus* is CO_2-dependent and *H. parahaemolyticus* is haemolytic. *H. segnis* is phenotypically very similar to *H. parainfluenzae* and many clinical laboratories report all *Haemophilus* spp. that are factor V- but not factor X-dependent as *H. parainfluenzae* [7]. In a case of pancreatic abscess, the pathogen was reported initially as *H. parainfluenzae* and was only subsequently confirmed to be *H. segnis* by a reference laboratory (Table 2, case 9) [5]. It is possible that the true prevalence of *H. segnis* infection has been underestimated. The application of 16S rRNA gene sequencing for identification of *H. parainfluenzae* has been described in two cases of infective endocarditis, where identification by conventional techniques was difficult. In one case, the isolate was identified presumptively as *H. parainfluenzae* by its factor V dependency and biochemical reactions, but other phenotypic characteristics were atypical [6]. In the other case, the blood culture isolate was identified preliminarily by the clinical microbiological laboratory as a strain of *A. actinomycetemcomitans* [7]. Interestingly, the blood culture isolate in the first case in the present study was also identified by the Vitek (NHI) system as 56% likely to be *A. actinomycetemcomitans*, which is phylogenetically closely related to *H. parainfluenzae*, *H. paraphrophilus*, *H. aphrophilus* and *H.*

Table 2. Serious *H. segnis* infections reported in the English scientific literature

Case no.	Reference	Sex/age (y)	Underlying diseases	Site of isolation	Method of identification	Concomitant isolates	Diagnosis	Treatment	Outcome
1	1	F/76	None	Blood	Confirmed by Royal Dental College, Aarhus, Denmark	None	Infective endocarditis	Ampicillin + netilmicin	Cured
2	2	F/58	None	Gallbladder	RapID NH system, confirmed by Illinois Department of Public Health	None	Acute cholecystitis	Cholecystectomy, cephazolin	Cured
3	3	M/24	NA	Peritoneal fluid	Confirmed by CDC	None	Acute appendicitis	Appendicectomy, imipenem	Cured
4	3	M/27	NA	Peritoneal fluid	Confirmed by CDC	None	Acute appendicitis	Appendicectomy, cefoxitin	Cured
5	3	M/18	NA	Peritoneal fluid	Confirmed by CDC	*Bacteroides multiacidus*	Acute appendicitis	Appendicectomy, cefoxitin	Cured
6	3	M/22	None	Peritoneal fluid	Confirmed by CDC	*Escherichia coli* *Klebsiella pneumoniae*	Acute appendicitis	Appendicectomy, cefoxitin → Ampicillin + clindamycin + tobramycin → cefoxitin	Cured
7	3	M/32	None	Peritoneal fluid	Confirmed by CDC	*Bacteroides* sp. *Lactobacillus* sp. *Peptostreptococcus* sp. *Propionibacterium* sp.	Acute appendicitis	Appendicectomy, cefoxitin	Cured
8	4	F/18	None	Appendix tip	Confirmed by Central Public Health Laboratory, Colindale, London	None	Acute appendicitis	Appendicectomy, cephradine + gentamicin + metronidazole	Cured
9	5	M/29	Alcoholism	Blood, pancreas	Initially identified as *Haemophilus parainfluenzae*, subsequently identified as *Haemophilus segnis* by National Type Culture Collection	None	Pancreatic abscess	Surgical drainage, ampicillin + gentamicin + metronidazole	Died
10	Present study, case 1	M/32	Intravenous drug addict	Blood	16S rRNA gene sequencing	*Streptococcus milleri* *Streptococcus sanguis*	Empyema thoracis	Drainage, ticarcillin/ clavulanate + gentamicin	Cured
11	Present study, case 2	F/82	None	Blood	16S rRNA gene sequencing	None	Bacteraemia due to *Clostridium difficile* colitis	Cefoperazone/sulbactam	Died

NA, not available; CDC, Centers for Disease Control, Atlanta, GA, USA.

segnis (Fig. 1). In fact, it has been proposed that *A. actinomycetemcomitans* should probably be reclassified as a species of *Haemophilus* [26, 27].

This work was partly supported by the University Development Fund, University Research Grant Council and the Committee of Research and Conference Grants, University of Hong Kong.

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