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 septicemic 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 CO₂ 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. aphrophilus/paraphrophilus and 42% H. parainfluenzae; 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 bacteremia 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, CO₂ 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
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 (NH) (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′-AGTTTGTACCTGCGTAC-3′ (LPW55) and 5′-AGGCCGGGAAGCTATTCAC-3′ (LPW56) were used as the PCR primers and LPW55, LPW56, 5′-AGGACCGCTAATCTCGGTAC-3′ (LPW69) and 5′-TAATTCCTGTGCTGCTCCAC-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 × 10⁹/L, with neutrophils 17.8 × 10⁹/L, lymphocytes 1.1 × 10⁹/L and monocytes 1.5 × 10⁹/L. The haemoglobin level was 7.5 g/dl and the platelet count was 457 × 10⁹/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 cocacobacilli. The gram-positive cocci were identified as Streptococcus intermedius and S. sanguis. The gram-negative cocacobacillus grew on chocolate agar to give colonies of 1 mm in diameter after incubation for 24 h at 37°C in air with CO₂ 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 (NH) 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/paraaphrophilus 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 cocacobacilli and gram-positive cocci in chains. However, only a Bacteroides sp. was isolated on culture.

On day 1 of incubation, the aerobic blood culture bottle was positive with gram-positive cocci in chains and gram-negative cocacobacilli. 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 CO₂ 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 (NH) 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 (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 occasionally with serious infections including infective endocarditis, acute cholecystitis, acute appendicitis and pancreatic abscesses (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

<table>
<thead>
<tr>
<th>Biochemical reactions/enzymes/substrates</th>
<th>Vitek NH</th>
<th>API NH</th>
<th>Vitek NH</th>
<th>API NH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylphosphonate</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Proline arylamidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>γ-Glutamyl-arylaminidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glycine arylamidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lysine arylamidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Indole production</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Phosphate choline</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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</table>

Acidification of:
- glucose: + + + + +
- fructose: + + + + +
- sucrose: – – – – –
- maltose: + + + + +
- saccharose: + + + + +
- Reduction of triphenyl tetrazolium:
- Resazurin: – – – – –
- Ornithine decarboxylase: – – – – –
- Lipase: – – – – –
- Alkaline phosphatase: – + + + +
- Urease: – – – – –
- Pencilllinase: – – – – –

Identification:
- 56% A. actinomycetemcomitans
- 58% H. aphrophilus/paraphrophilus
- 40% N. subflava
- 42% H. parainfluenzae
- 42% H. parainfluenzae
- 95% H. influenzae
- 58% H. aphrophilus/paraphrophilus
- 42% H. parainfluenzae
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. 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-dependent include H. segnis, H. parahaemolyticus and H. parainfluenzae, which are laboratory reference strains. In the other case, the blood culture isolate was identified preliminarily by the clinical microbiological laboratory as a strain of A. actinomycetemcomitans [7]. 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 by the Vitek (NHI) system as 56% likely to be A. actinomycetemcomitans, which is phylogenetically closely related to H. parainfluenzae, H. parahaemolyticus, H. aegyptius and H. influenzae MCCM 02082 (M75079).}

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.
<table>
<thead>
<tr>
<th>Case no.</th>
<th>Reference</th>
<th>Sex/age (y)</th>
<th>Underlying diseases</th>
<th>Site of isolation</th>
<th>Method of identification</th>
<th>Concomitant isolates</th>
<th>Diagnosis</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>F/76</td>
<td>None</td>
<td>Blood</td>
<td>Confirmed by Royal Dental College, Aarhus, Denmark</td>
<td>None</td>
<td>None</td>
<td>Ampicillin + netilmicin</td>
<td>Cured</td>
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<tr>
<td>2</td>
<td></td>
<td>F/58</td>
<td>None</td>
<td>Gallbladder</td>
<td>RapID NH system, confirmed by Illinois Department of Public Health</td>
<td>None</td>
<td>None</td>
<td>Cholecystectomy, cephalozin</td>
<td>Cured</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>M/24</td>
<td>NA</td>
<td>Peritoneal fluid</td>
<td>Confirmed by CDC</td>
<td>None</td>
<td>Acute appendicitis</td>
<td>Appendicectomy, imipenem</td>
<td>Cured</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>M/27</td>
<td>NA</td>
<td>Peritoneal fluid</td>
<td>Confirmed by CDC</td>
<td>Bacteroides multacidus</td>
<td>Acute appendicitis</td>
<td>Appendicectomy, cefoxitin</td>
<td>Cured</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>M/18</td>
<td>NA</td>
<td>Peritoneal fluid</td>
<td>Confirmed by CDC</td>
<td>Escherichia coli</td>
<td>Acute appendicitis</td>
<td>Appendicectomy, cefoxitin</td>
<td>Cured</td>
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<tr>
<td>6</td>
<td>3</td>
<td>M/22</td>
<td>NA</td>
<td>Peritoneal fluid</td>
<td>Confirmed by CDC</td>
<td>Klebsiella pneumoniae</td>
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<td>Appendicectomy, cefoxitin</td>
<td>Cured</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>M/32</td>
<td>None</td>
<td>Peritoneal fluid</td>
<td>Confirmed by CDC</td>
<td>Bacteroides sp.</td>
<td>Acute appendicitis</td>
<td>Appendicectomy, cefoxitin</td>
<td>Cured</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>F/18</td>
<td>None</td>
<td>Appendix tip</td>
<td>Confirmed by Central Public Health Laboratory, Colindale, London</td>
<td>None</td>
<td>None</td>
<td>Appendicectomy, cephalaxe + gentamicin + metronidazole</td>
<td>Cured</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>M/29</td>
<td>Alcoholism</td>
<td>Blood, pancreas</td>
<td>Initially identified as Haemophilus parainfluenzae, subsequently identified as Haemophilus segnis by National Type Culture Collection</td>
<td>None</td>
<td>None</td>
<td>Pancreatic abscess</td>
<td>Surgical drainage, ampicillin + gentamicin + metronidazole</td>
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<tr>
<td>10</td>
<td>Present study, case 2</td>
<td>M/32</td>
<td>Intravenous drug addict</td>
<td>Blood</td>
<td>16S rRNA gene sequencing</td>
<td>Streptococcus milleri Streptococcus sanguis</td>
<td>Empyema thoracis</td>
<td>Drainage, ticarcillin/ clavulanate + gentamicin</td>
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<td>Blood</td>
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<td>Bacteraemia due to Clostridium difficile colitis</td>
<td>Cefoperazone/sulbactam</td>
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NA, not available; CDC, Centers for Disease Control, Atlanta, GA, USA.
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References