**Haemophilus haemolyticus** Isolates Causing Clinical Disease

Raydel Anderson,*a Xin Wang,*a Elizabeth C. Briere,*a Lee S. Katz,b Amanda C. Cohn,* Thomas A. Clark,* Nancy E. Messonnier,*a and Leonard W. Mayer*a

Meningitis and Vaccine Preventable Disease Branch, Division of Bacterial Diseases, National Center for Infectious and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and Enteric Diseases Laboratory Branch, Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

We report seven cases of *Haemophilus haemolyticus* invasive disease detected in the United States, which were previously misidentified as nontypeable *Haemophilus influenzae*. All cases had different symptoms and presentations. Our study suggests that a testing scheme that includes reliable PCR assays and standard microbiological methods should be used in order to improve *H. haemolyticus* identification.

*Haemophilus haemolyticus* is a human commensal bacterium that colonizes the respiratory tract (15). *H. haemolyticus* is closely related to the human pathogen *Haemophilus influenzae* but has rarely been reported to cause invasive disease.

*H. haemolyticus* and *H. influenzae* differ from other *Haemophilus* species because they require hemin (X factor) and NAD (V factor) for growth. *H. haemolyticus* can easily be distinguished from encapsulated *H. influenzae* because *H. influenzae* isolates produce one of the six structurally distinct capsules that can be easily determined by slide agglutination assay, whereas *H. haemolyticus* has never been shown to produce a capsule. However, due to the high similarity in morphology, biochemistry, and genetics between *H. haemolyticus* and nonencapsulated or nontypeable *H. influenzae*, distinguishing the two by standard microbiology methods has been challenging.

The presence of one or more of the following four features has been used for differentiating *H. haemolyticus* from nontypeable *H. influenzae*. (i) *H. haemolyticus* induces clear β-hemolysis on horse blood agar. However, the hemolytic activity may be lost due to multiple subcultures or other unknown mechanisms, and nonhemolytic *H. haemolyticus* strains are often misidentified as nontypeable *H. influenzae* (13, 15–18). (ii) More than 90% of *H. haemolyticus* strains produce hydrogen sulfide (H2S); a small proportion of *H. influenzae* bioype IV strains produce H2S (8, 10, 19). Therefore, production of H2S cannot unambiguously distinguish *H. haemolyticus* from *H. influenzae*. (ii) *H. influenzae* can be distinguished by PCR assays detecting the genes coding for protein D (hpd) and the fuculose kinase gene (fucK); a negative PCR result for both hpd and fucK genes is a strong indication of *H. haemolyticus*. (iv) 16S rRNA gene sequences of *H. haemolyticus* and *H. influenzae* form different phylogenetic clusters. Recent studies have demonstrated that use of both phenotypic and genotypic assays improves identification of *H. influenzae* and *H. haemolyticus* isolated from asymptomatic carriers (23).

To determine whether *H. haemolyticus* invasive disease has been overlooked due to its high similarity to nontypeable *H. influenzae*, we characterized isolates from cases reported to be caused by nontypeable *H. influenzae* that were submitted to the CDC through routine surveillance in 2010 or through a population-based Active Bacterial Core surveillance (ABCs) program (http://www.cdc.gov/abc/index.htm) in 1999, 2000, 2009, and 2010 in the United States.

A total of 161 isolates from 2009 to 2010 and 213 isolates from 1999 to 2000 were characterized using phenotypic assays, including Kovac’s oxidase test, API NH strips (bioMérieux; Durham, NC), hydrogen sulfide (H2S) test strips (Sigma-Aldrich, Allentown, PA), and *Haemophilus* ID Quad plates (Remel, Lenexa, KS). These isolates were further characterized using the hpd and fucK PCR assays that detect internal regions of the two genes (9, 17, 18). All isolates showed typical *H. influenzae* colony morphology, were positive for oxidase, required heme and NAD factors for growth, and produced hydrogen sulfide (H2S). The isolates from cases 1 to 5 showed hemolytic activity on *Haemophilus* ID Quad plates; other isolates (cases 6 and 7) were nonhemolytic. All seven isolates were negative for hpd and fucK by specific PCR assays.

16S rRNA gene sequencing was conducted on the seven isolates as described previously (20). Phylogenetic and molecular evolutionary analyses of 16S rRNA gene sequences were conducted using MEGA version 4 using the neighbor-joining method (22). Bootstrap numbers are from 1,000 replicates. 16S rRNA gene sequences of these isolates were compared with those of 10 *H. haemolyticus* isolates, 18 *H. influenzae* isolates, and 10 isolates representing *Haemophilus* species other than *H. haemolyticus* and *H. influenzae* that were published in GenBank and the Ribosomal Database Project (http://rdp.cme.msu.edu/) (20). As shown in Fig. 1, isolates of the same species generally formed a group. The organisms from cases 1 to 7 were grouped with other known *H. haemolyticus* but not *H. influenzae* strains. Based on the profile of these phenotypic and genotypic laboratory tests, the seven isolates from cases 1 to 7 were identified as *H. haemolyticus*. Genome sequences of isolates from case 6 (AFQ00000000) and 7 (AFQQ00000000) have recently been published (9). The fucK gene is missing, but the hpd gene is present in these two isolates, suggesting that the PCR target region of the hpd gene is species specific.

All seven patients infected with *H. haemolyticus* had different symptoms and presentations (Table 1). Two of the seven *H. hae-
molyticus cases were from California and Oregon in 1999 and five cases were from Connecticut, Georgia, Minnesota, Illinois, and Texas in 2009 to 2010. All seven patients were hospitalized with no deaths. Isolates were recovered from 5 blood, 1 synovial fluid, and 1 pancreatic specimen (Table 1). Five of the seven isolates were submitted to the CDC through the ABCs program. The other two isolates from Illinois and Texas were collected from routine surveillance and submitted for confirmation of species identification.

Haemophilus species other than H. influenzae and H. ducreyi are not common human pathogens (1, 21). Only a few bacteremia cases caused by non-H. influenzae Haemophilus species have been reported (3, 6). Two endocarditis cases caused by H. haemolyticus were reported in 1923 and in 1933 (5, 14). Our clinical and laboratory findings indicate that H. haemolyticus is associated with invasive clinical disease. Six patients had underlying medical conditions or recent surgical procedures that could have compromised their immune systems, potentially increasing their risk for invasive H. haemolyticus infection. H. haemolyticus may cause disease more as an opportunistic pathogen. Little is known about the mechanisms of H. haemolyticus pathogenicity. Capsule is considered a crucial virulence factor for many bacterial pathogens, such as H. influenzae (11); however, H. haemolyticus has not been reported to produce capsule. H. influenzae outer membrane proteins and lipopolysaccharide of H. influenzae play important roles in bacterial colonization and evasion of host defenses (7). Because of the high frequency of horizontal gene transfer among and be-

![Phylogenetic tree based on 16S rRNA gene sequences of different Haemophilus species strains. The tree was generated using neighbor joining in MEGA 4 with 1,000 replicates and shows the genetic relatedness of the isolates from the five cases to different Haemophilus species. Hi and Hhae stand for H. influenzae and H. haemolyticus, respectively. The name of each 16S rRNA gene sequence includes the bacterial species, accession number, and, for H. influenzae, the strain type number (Hi_AY613586_T9) for published sequences, as well as a case number (cases 1 to 7) for sequences in this study. All H. influenzae strains were isolated from blood with the exception of AY613466, which was from cerebrospinal fluid (CSF). The Haemophilus ducreyi strain was isolated from a chancroid ulcer. The Haemophilus parainfluenzae strains AY365452 and AY365450 were isolated from blood, M75081 and AY362908 are ATCC strains, and EU083530 is from an unpublished source. The H. haemolyticus EU185339, EU185348, and EU185340 were invasive strains from neonatal urogenital tracts. EU185345, EU185346, EU185339, EU185349, and EU185354 were isolated from sputum or nasopharyngeal swabs from chronic obstructive pulmonary disorder (COPD) patients, and M75045 is the type strain from NCTC. Haemophilus paraphrohaemolyticus is the type strain from NCTC, and Haemophilus parasuis was from an unpublished source.](http://jcm.asm.org/content/jcm/50/7/2463/F1)

FIG 1 Phylogenetic tree based on 16S rRNA gene sequences of different Haemophilus species strains. The tree was generated using neighbor joining in MEGA 4 with 1,000 replicates and shows the genetic relatedness of the isolates from the five cases to different Haemophilus species. Hi and Hhae stand for H. influenzae and H. haemolyticus, respectively. The name of each 16S rRNA gene sequence includes the bacterial species, accession number, and, for H. influenzae, the strain type number (Hi_AY613586_T9) for published sequences, as well as a case number (cases 1 to 7) for sequences in this study. All H. influenzae strains were isolated from blood with the exception of AY613466, which was from cerebrospinal fluid (CSF). The Haemophilus ducreyi strain was isolated from a chancroid ulcer. The Haemophilus parainfluenzae strains AY365452 and AY365450 were isolated from blood, M75081 and AY362908 are ATCC strains, and EU083530 is from an unpublished source. The H. haemolyticus EU185339, EU185348, and EU185340 were invasive strains from neonatal urogenital tracts. EU185345, EU185346, EU185339, EU185349, and EU185354 were isolated from sputum or nasopharyngeal swabs from chronic obstructive pulmonary disorder (COPD) patients, and M75045 is the type strain from NCTC. Haemophilus paraphrohaemolyticus is the type strain from NCTC, and Haemophilus parasuis was from an unpublished source.
between Haemophilus species, it is conceivable that the homologues of H. influenzae virulent factors may be present in H. haemolyticus and play a role in H. haemolyticus pathogenicity. Future work is necessary to define the virulent determinants of H. haemolyticus and the role of hemolysin as a virulence factor.

Our study also indicates that molecular tools are useful for discriminating H. haemolyticus from nontypeable H. influenzae when the phenotypic assays are unable to provide conclusive results (23). Detection of the two H. haemolyticus cases from 1999 suggests that disease caused by H. haemolyticus has been historically overlooked; H. haemolyticus cases have previously been misidentified as nontypeable H. influenzae cases due to the lack of proper detection methods. In addition to the hpd and fucK genes, other biomarker genes have been proposed to differentiate H. haemolyticus from H. influenzae, such as the H. influenzae adherence and penetration protein gene hpd, the [Cu, Zn]superoxide dismutase gene sodC, the immunoglobulin A protease gene iga, and the outer membrane protein P6 (4, 13, 16–18, 24). The hpd and iga genes are more reliable than the fucK, hpd, sodC, and protein P6 genes for distinguishing the two organisms (2, 4, 12–14, 23, 24). 16S rRNA gene sequencing is used for identification of bacterial species (20), but the low bootstrap value (Fig. 1) has suggested that single gene sequences may not be able to phylogenetically discriminate H. haemolyticus and H. influenzae. McCrea et al. have shown that concatenated sequences of multiple genes, including the 16S rRNA gene, adk (adenylate kinase gene), pgi (glucose-6-phosphate isomerase gene), recA (recombination protein gene), and infB (translation initiation factor 2 gene), separate the two species into distinct clusters (13). As no single trait is able to unequivocally discriminate H. haemolyticus and H. influenzae, a testing scheme that includes reliable molecular techniques such as the hpd- and iga-based PCR assays can be used in combination with standard microbiological methods in order to improve the identification of H. haemolyticus.

ACKNOWLEDGMENTS

We thank the Active Bacterial Core surveillance team, Roman Golash from the Illinois Department of Health, and the Texas Department of State Health Services for providing case information and isolates. We thank the CDC Special Bacteriology Reference Laboratory for assistance with 16S rRNA gene sequencing and Mary McCauley at the CDC for her critical review.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC.

REFERENCES


