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Detection of Cryptic Genospecies Misidentified as *Haemophilus influenzae* in Routine Clinical Samples by Assessment of Marker Genes *fucK*, *hap*, and *sodC*▽

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Clinical isolates of *Haemophilus influenzae* were assessed for the presence of *fucK*, *hap*, and *sodC* by hybridization with gene-specific probes, and isolates diverging from the expected *H. influenzae* genotype were characterized by phenotype and 16S rRNA gene sequencing. Two of 480 isolates were finally classified as variant strains ("nonhemolytic *Haemophilus haemolyticus*").

Haemophilus influenzae is an important human pathogen commonly involved in infections of the upper respiratory tract (4, 5, 21) and sometimes involved in invasive infections (2, 17). In the clinical laboratory, *H. influenzae* should be differentiated from relatives of minor pathogenic importance indigenous to the mucosal surfaces of the human respiratory tract. A challenge is the discrimination of *H. influenzae* from related species and unnamed taxa incapable of hemin biosynthesis. *Haemophilus haemolyticus* is characterized by beta-hemolysis on blood agar plates, but nonhemolytic variants of this species and other commensal, hemin-dependent taxa closely related to *H. influenzae* are difficult or impossible to discriminate by conventional phenotypic testing (3, 11, 14, 18). Recent investigations have indicated such variant strains to be more prevalent in specimens from the respiratory tract than hitherto anticipated, comprising 16 to 21% of hemin- and NAD-dependent *Haemophilus* throat isolates from healthy children and adults (13, 22) and almost 40% of presumptive *H. influenzae* sputum isolates from patients with chronic obstructive pulmonary disease (14).

Multilocus sequence cluster analysis successfully separated reference strains of *H. influenzae* from more-distantly related variant strains, and this division correlated with the presence or absence of three genes, the fuculokinase gene *fucK*, the *H. influenzae* adherence and penetration protein gene *hap*, and the [Cu,Zn]-superoxide dismutase gene *sodC* (16). In the present study, we used these marker genes to screen for variant strains among clinical isolates originally classified as *H. influenzae* based on an abbreviated identification scheme.

During a 3-month period (February through April 2007), isolates of *H. influenzae*, which were deemed clinically relevant and reported to the requesting physician, were frozen at −80°C in broth containing 10% glycerol. A total of 480 viable isolates were preserved, with 241 from the respiratory tract (including 147 sputum samples, 18 bronchoalveolar lavage [BAL] specimens, and 43 endolaryngeal suction specimens), 161 from ears (mostly effusions from suspected cases of otitis media), 68 from conjunctival swabs, and 10 isolates from other sites, including 2 from blood. The laboratory uses minimal criteria of limited specificity to identify *H. influenzae* cultured from the respiratory tract, eyes, or ears. Respiratory tract specimens examined for *H. influenzae* comprise sputa, endolaryngeal suction specimens, and BAL fluids but not throat swabs. The identification algorithm requires that the amount of hemophilic bacteria is not exceeded by other bacteria cultured from the specimen. The identification of *H. influenzae* rests on microscopic and colony morphology and dependence on NAD (1). Further characterization is required for isolates cultured from other anatomical sites. Colony blot hybridization (16) and 16S rRNA gene sequencing (9) were carried out, as described previously. Phenotypic profiles were generated with the *Neisseria-Haemophilus* card for the Vitek 2 (bioMerieux) and supplemented with selected biochemical tests (7). Capsular serotypes were confirmed by PCR (6). The bipartite population structure of *H. influenzae* suggested by multilocus sequence typing was adapted (12, 16, 19), and biotypes were defined, as described previously (7).

Representative hybridization blots obtained with probes for the three marker genes are shown in Fig. 1. Four hundred fifty-four isolates (94.6%) were positive for *fucK* and *hap* and negative for *sodC* (genotype ++0), which is the expected genotype for *H. influenzae* (16). Twenty-six isolates exhibited at least one aberrant result in genotypic testing and were further characterized. By 16S rRNA gene sequencing, these 26 isolates gave rise to 13 unique sequences (Fig. 2).

Nineteen isolates deviated by a single result in the genotyping scheme. One isolate (PN127) was negative for *fucK*, positive for *hap*, and negative for *sodC* (genotype 0+0). This isolate was recovered from the respiratory tract and was consistent with respect to phenotype with *H. influenzae* biotype II. By 16S rRNA gene sequencing, the isolate was identical to the full-genome-sequenced strain Rd, which is encompassed in *H. influenzae* phylogenetic group I (Fig. 2). Thus, the extended characterization of this isolate confirmed a classification as *H. influenzae*, despite the apparent lack of a fuculokinase gene. Twelve isolates deviated from the expected *H. influenzae* genotype solely by being negative for *hap* (genotype +00; strains PN112 and PN123 are included in Fig. 1). These isolates were all consistent with *H. influenzae* biotype II by phenotypic testing. 16S rRNA gene sequencing revealed

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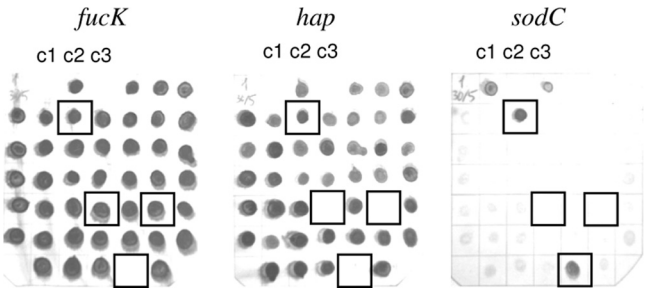

FIG. 1. Colony blot hybridization of three control strains and 43 study strains, with probes for *fucK*, *hap*, and *sodC*. c1, type strain of *H. haemolyticus*; c2, type strain of *H. influenzae*; c3, strain PN24, a porphyrin-positive variant strain related to *H. influenzae* (16). The locations (from top to left) of study strains PN122 (*H. influenzae* serotype f), PN123 (*H. influenzae* lacking *hap*), PN112 (*H. influenzae* lacking *hap*), and PN124 (unclassified variant strain) are indicated.

three separate 16S types, encompassing seven, four, and one isolate, respectively. These 16S types were located in *H. influenzae* phylogenetic group I, close to the type strain of the species (Fig. 2). Thus, the aberrant genotype characterized by the singular lack of hybridization with the *hap* probe was associated with a few, closely related clones of *H. influenzae*, as defined by 16S rRNA gene sequencing. Six isolates recovered from the respiratory tract (five) and blood (one) were positive for *sodC* as well as *fucK* and *hap* (genotype +++; strain PN122 is included in Fig. 1). By 16S rRNA gene sequencing, four isolates were identical and differed only by one nucleotide from the deposited sequence of a serotype f strain of *H. influenzae* (GenBank accession number AY613743), while two strains were 100% identical with the deposited sequence of a serotype e strain of *H. influenzae* by 16S rRNA gene sequencing (GenBank accession number AY613546). PCR capsular typing of the six isolates confirmed these *H. influenzae* serotypes. Thus, the six strains which deviated from the *H. influenzae* genotype solely by being positive for *sodC* were capsulate phylogenetic group II strains.

Seven isolates had two or three aberrant results in the genotyping scheme. These isolates were negative for *fucK* and positive for *sodC*, while five of seven isolates were negative for *hap* (genotypes 00+ and 0++). Characters pertinent to these strains are shown in Table 1. Three isolates (PN100, PN116, and PN118) were identified as *Haemophilus parainfluenzae*, while two isolates (PN129 and PN137) cultured from patients with cystic fibrosis (sputum and endolaryngeal suction specimen, respectively) clustered with *Haemophilus parahaemolyticus* by 16S rRNA gene sequencing. PN137 was nonhemolytic; otherwise, both isolates were consistent with respect to phenotype with *H. parahaemolyticus*. Three of these five porphyrin-positive isolates should have been disclosed by the original identification algorithm due to the presence of hemolysis or cultivation from unusual anatomical sites. Finally, two isolates (PN124 and PN134) were cultured from an endotracheal suction specimen from an infant suffering from recurrent aspiration and from a BAL specimen from an immunosuppressed male, respectively. By 16S rRNA gene sequencing, PN124 clustered with other mannose-fermenting strains that have been referred to as "*Haemophilus intermedius* subsp. *gazogenes*" (3, 16) (Fig. 2). PN134 fermented sucrose and xylose but not

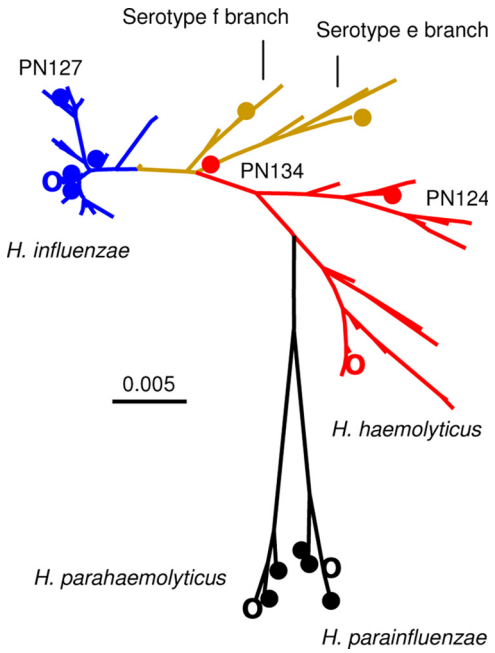

FIG. 2. Neighbor-joining tree of 13 unique study strain 16S rRNA gene sequences (filled circles) (1,361 to 1,362 nucleotides) in comparison with those of the type strains of *H. influenzae*, *H. haemolyticus*, *H. parainfluenzae*, and *H. parahaemolyticus* (open circles), plus those of 71 reference strains of *H. influenzae* and 27 variant strains excluded from *H. influenzae* (15, 16, 19). Phylogenetic group I, blue; phylogenetic group II, yellow; variant cluster and *H. haemolyticus*, red. Bar represents one substitution per 200 nucleotides.

ribose or galactose and was negative for tryptophanase, urease, and ornithine decarboxylase. The Vitek 2 system suggested identification as *Actinobacillus ureae* (probability, 93.12; confidence level, very good identification; only fermentation of xylose was contradictory to typical biopattern). By 16S rRNA gene sequencing, the strain was positioned on the stem of the serotype e branch, contiguous to both validated strains of *H. influenzae* and variant strains excluded from this species (Fig. 2); however, the comparison was obscured by the 16S rRNA gene heterogeneity of isolate PN134, which harbored 20 polymorphic positions within the sequenced fragment of the 16S rRNA gene (GenBank accession no. FJ939594).

In our collection of *H. influenzae* isolates, we found 7 of 480 strains (1.5%) were misidentified. Five of the seven mislabeled strains could have been detected by the routine use of the porphyrin test (Table 1). Only two isolates (0.4%) were finally classified as hemin- and NAD-dependent variant strains or "nonhemolytic *H. haemolyticus*." The low occurrence of such strains in our collection contrasts the much higher prevalence of those that has been reported in other settings (13, 14, 22). Our isolates were collected as a consequence of clinical examinations, and a preponderance of *H. influenzae* species over *Haemophilus* commensal species other than *H. influenzae* is to be expected in infections. Correspondingly, no variant strains were identified among the 130 isolates cultured from the middle ear after tympanocentesis (14). Furthermore, the adopted *H. influenzae* identification scheme requires dominant growth of NAD-dependent bacteria from the respiratory tract, eyes, or ears, which may be a rare scenario with bacteria of low patho-

TABLE 1. Characterization of seven clinical isolates incorrectly identified as *H. influenzae*

Strain	Origin	Results of testing for[a]:								Classification
		fucK	*hap*	*sodC*	Porph	Hem	ONPG	Man	Suc	
PN100	Urine	0	0	+	+	0	0	+	+	*H. parainfluenzae*
PN116	Ear	0	0	+	+	0	+	+	+	*H. parainfluenzae*
PN118	Skin	0	0	+	+	0	0	0	+	*H. parainfluenzae*
PN124	Trachea	0	0	+	0	0	0	+	0	Variant strain
PN129	Sputum	0	0	+	+	+	+	0	+	*H. parahaemolyticus*
PN134	BAL fluid	0	+	+	0	0	0	0	+	Variant strain
PN137	Larynx	0	+	+	+	0	+	0	+	*H. parahaemolyticus*

[a] 0, negative; +, positive; Porph, synthesis of porphyrins from δ-aminolevulinic acid; Hem, hemolysis; ONPG, hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside; Man, fermentation of D-mannose; Suc, fermentation of sucrose.

genic potential. The variant strain PN134 was actually cultured in small amounts and outnumbered by viridans group streptococci in the sample, but the isolate was identified and reported because of the invasive nature of the sampling procedure (BAL specimen). The result reported from the chronic obstructive pulmonary disease clinic, with almost 40% misidentified isolates, is unexpected (14). Apart from the selected patient material, differences in sampling methodology, particularly measures to identify bacteria present in small amounts, may in part explain the conspicuous difference in the prevalence of variant strains between the two studies.

Three marker genes identified during investigations of reference strains (16) were tested on a large collection of clinical isolates. The *hap* probe did not perform well in the latter setting, as it failed to hybridize with DNA from several putative clones of *H. influenzae* (2.6% of the isolates) and hybridized efficiently with two of seven isolates excluded from the species. The *sodC* probe successfully detected all seven misidentified strains but was positive for six (1.3%) strains of *H. influenzae*. This reactivity is expected, because *sodC* is present in capsulate phylogenetic group II strains (8, 10, 20). The best discrimination was observed with assessment of the *fucK* gene, which was undetectable in seven of seven misidentified strains and in 1 of 473 isolates of *H. influenzae*. *fucK* may be a particularly useful marker, as it is part of the multilocus sequence typing scheme (12), making primers for the amplification of *fucK* widely available. Failure to amplify the *fucK* fragment from a presumptive isolate of *H. influenzae* will be a strong indication of an incorrectly identified strain.

Nucleotide sequence accession numbers. 16S rRNA gene sequences generated in this study are deposited in GenBank under accession numbers FJ939584 to FJ939596.

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