Prevalence of *hicAB*, *lav*, *traA*, and *hifBC* among *Haemophilus influenzae* middle ear and throat strains

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Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is an important cause of illness among children. To further understand the role of laterally transferred genes in NTHi colonization and otitis media, the prevalence of *hicAB, lav*, *tna*A, and *hifBC* was determined among 44 middle ear and 35 throat NTHi isolates by dot-blot hybridization.

Bacterial colonization of the upper respiratory tract is the first step in the pathogenesis of *Haemophilus influenzae* (Hi) infection. *Haemophilus influenzae* carriage rates vary between 25% and 84% in children, and colonization is a very dynamic process characterized by rapid bacterial turnover and carriage of multiple Hi strains (Trottier *et al*., 1989; Faden *et al*., 1991; Dhooge *et al*., 2000; St Sauver *et al*., 2000; Farjo *et al*., 2004). In addition to asymptomatic colonization, NTHi are important causes of respiratory infections in humans, including acute otitis media. NTHi causes between 30% and 52% of acute otitis media in children (Eskola & Kilpi, 2000).

Haemophilus influenzae display significant strain-to-strain genetic diversity (Gilsdorf, 1998) and horizontal (lateral) gene transfer contributes significantly to maintenance of that diversity (Weiser, 2000). Munson *et al.* (2004), using *in silico* subtraction and bioinformatics techniques, reported the presence of several genes, including *hic*, *lav*, and *tnaA*, in two *H. influenzae* strains isolated from children with otitis media and absent in the fully sequenced *H. influenzae* strain Rd (Fleischmann *et al*., 1995) and suggested these genes may play a role in *H. influenzae* pathogenesis. Interestingly, *hic*, *lav*, and *tnaA* appear to have undergone lateral gene transfer between NTHi and other bacteria (Martin *et al*., 1998; Mhlanga-Mutangadura *et al*., 1998; Davis *et al*., 2001). In addition, evolutionary studies of Hi suggest that the *hif* cluster has also undergone lateral gene transfer (Mhlanga-Mutangadura *et al*., 1998). To better understand the potential role of these laterally transferred genes in otitis media pathogenesis, a population-based gene prevalence analysis was used, which has proved useful in identifying *H. influenzae* genes retained during natural selection among middle-ear isolates and not among throat isolates (Pettigrew *et al*., 2002).

The *hif* gene cluster, responsible for the biosynthesis of hemagglutinating pili, is located between *purE* and *pepN* on the *H. influenzae* chromosome and contains five genes (*hifA* through *hifE*). *hifA* encodes the major pilus structural protein, *hifB* encodes the subunit chaperone protein, *hifC* encodes an usher (assembly platform) protein, *hifD* encodes

a protein located at the pilus tip whose function remains unknown, and *hifE* encodes the tip adhesin (McCrea *et al*., 1994). The nucleotide sequences of *hifB* and *hifC* (which are contiguous in the *hif* cluster) are highly conserved among all *H. influenzae* strains, while the other genes are highly variable (McCrea *et al*., 1998). In addition, the *hif* cluster itself shows significant strain-to-strain variability in its genetic structure. The *hic* ('*hif* contiguous' DNA) genes are found within the *purE-pepN* region of the *H. influenzae* genome, adjacent to the *hif* pilus genes when this cluster is present (Mhlanga-Mutangadura *et al*., 1998). The *hic* cluster contains two conserved ORFs (*hicA* and *hicB*) and the functions of their gene products are unknown. The *hic* gene regions are highly conserved, with a DNA similarity of $>$ 97% between strains. The *tna* operon encodes tryptophanase, which catabolizes tryptophan to indole, pyruvate, and ammonia, allowing tryptophan to be used as a carbon and nitrogen source in bacteria, and contains homologs to *Escherichia coli traA* (encoding the structural tryptophanase protein), *traB* (encoding a tryptophan permease), and *traC*, encoding a termination inhibition peptide that functions in *cis* to facilitate transcription of *traA* and *B*. The production of indole has been used for biotyping *H. influenzae,* with biotypes I, II, V, and, more recently, VIII exhibiting indole production (Kilian, 1976; Doern & Chapin, 1987). *lav* is thought to encode a member of the AIDA-1/VirG/PerT family of virulence-associated autotransporters (Farjo *et al*., 2004) and contains a series of GCAA repeats, suggestive of contingency loci in other NTHi virulence genes that undergo translational phase variation, a mechanism for enhanced Hi antigenic diversity (Hood *et al*., 1996).

To determine the prevalences of *hicAB*, *lav*, *tnaA*, and *hifBC* among *H. influenzae* strains, NTHi obtained from healthy children attending daycare (35 throat strains) and from children with otitis media (44 middle ear strains) was tested. The isolates were collected from sites in Minneapolis, Minnesota; Ann Arbor, Michigan; Battle Creek, Michigan; and Bardstown, Kentucky (Pettigrew *et al*., 2002) and were grown overnight on chocolate agar plates as previously described (Ecevit *et al*., 2004). Indole production was measured using Kouvac's reagent (Boone *et al*., 2005).

Dot-blot hybridization assay was used to determine gene prevalences. Genomic DNA was isolated from *H. influenzae* strains using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol and spotted onto Hybond N+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (Pettigrew *et al*., 2002). DNA from *H. influenzae* 86-028NP (a gift from Dr Lauren Bakaletz, Ohio State University) and from *H. influenzae* Rd were used as positive and negative controls, respectively, on each membrane.

DNA probes to hybridize with the conserved regions of *hifBC and pepN* (found in single copy in all Hi strains and used to normalize the hybridization signals for DNA quantity) and probe hybridization assay conditions have been previously described (Ecevit *et al*., 2004). Additional probes were generated by PCR to detect conserved regions within *hicAB*, *tna*, and *lav*. The *hicAB* probe was made using forward primer 5′-ATTATTAGATAAGCTCGCAC-3′ and reverse primer 5′-CTTGAATCGCTTCCGTAAC-3′. The *tna* probe consisted of forward primer 5′-CATTTACCTGAA CCTTTCCGC-3′ and reverse primer 5′-CGTGCCGTAAA GTGGCGGAG-3′. The *lav* probe consisted of forward primer 5′-CCTCTCCGCTTTTGGCTGTTG-3′ and reverse primer 5′-CCCCGTTAAGTGTTCTGC-3′. All primers were synthesized at the University of Michigan Biomedical Research Core Facility and by Invitrogen (Carlsbad, CA). The probe amplicons were labeled with an alkaline phosphatase-fluorescein conjugate (Amersham Biosciences, Piscataway, NJ) and developed with an ECF detection system (Amersham).

The signal intensity of each dot on the membranes, performed in duplicate and detected using a STORM 860 Phosphor Imager (Storm System; Molecular Dynamics, Sunnyvale, CA), was expressed as a percentage of the positive controls after correcting for the background signal (Zhang *et al*., 2001). Strain samples with intermediate or discrepant hybridization intensity results with each probe were confirmed by Southern blot hybridization using the

Table 1. Prevalence of *hicAB*, *hifBC*, *tna*, and *lav* genes among NTHi isolates

Gene	NTHi strains $N = 79$ N (%)	Throat $N = 35$ N (%)	Middle ear $N = 44$ N (%)	Prevalence ratio (95% CI)	P-value
hicAB	69 (87.3)	32 (91.4)	37 (84.1)	0.919 (0.78–1.08)	0.499
lav	27 (34.2)	17 (48.6)	10 (22.7)	0.468 (0.25–0.89)	0.016
tnaA	62 (78.5)	29 (82.9)	33 (75)	0.905 (0.72–1.14)	0.399
hifBC	16 (20.3)	7 (20.0)	9 (20.5)	1.023 (0.42–2.47)	0.960
hifBC+hicAB+	15 (19)	6 (17)	9 (20)	1.193 (0.47–3.03)	0.709
hifBC − hicAB+	54 (68)	26 (74)	28 (64)	0.857 (0.64–1.15)	0.312
hifBC+hicAB −	1 (1)	1 (3)	0 (0)	0.795 (0.05–12.27)	0.870
hifBC − hicAB −	9 (11)	2 (6)	7 (16)	2.78 (0.616–12.57)	0.285

CI, confidence interval.

appropriate genes of interest, as previously described in the laboratory (Watson *et al.*, 1994; Xie *et al.*, 2006a, b).

Prevalence ratios were calculated as prevalence of the gene of interest among middle-ear strains/prevalence among throat strains. The differences in prevalence ratios of *hicAB*, *hifBC*, *tna,* and *lav* between middle ear and throat strains of NTHi were individually determined by χ^2 analysis, or Fisher's exact test when necessary, using SAS. $P < 0.05$ was considered statistically significant.

Table 1 shows the prevalences of *hicAB*, *lav, tnaA,* and *hifBC* among NTHi isolated from throat or middle-ear specimens and no statistically significant differences were seen in the prevalences of *hicAB, hifBC,* or *tnaA*. On the other hand, *lav* was more prevalent among throat NTHi (48.6%) than among ear NTHi (22.7%, $P = 0.016$). The production of indole (an indirect marker for the presence of *tna*), determined using Kouvac's reagent (Sigma-Aldrich) (Boone *et al.*, 2005), showed 100% correlation in the presence of *tnaA*.

In a previous study from the authors' laboratory (Ecevit *et al.*, 2004), *c.* 30% of NTHi strains contained the *hif* gene cluster as defined by hybridization with the same *hifBC* probe as used in the present study, with a significantly higher prevalence in throat strains (38%) compared to middle-ear strains (20%). In the present study, only 20.3% of NTHi strains contained the *hif* cluster, with no difference in prevalence between ear (20.5%) and throat (20.0%) strains. A possible explanation for this apparent discrepancy is that the throat strains in the present study were collected from healthy children while many of the strains in the previous study were collected from children with respiratory infections, suggesting that during illness, the pharyngeal population of NTHi may differ from that of healthy children, consistent with previous observations from the authors' laboratory (Pettigrew *et al.*, 2002).

Previous studies have found, as did this study, evidence for indole production (the functional equivalent of *tna* activity) in the majority of NTHi (Kilian, 1976; Oberhofer & Back 1979; Doern & Chapin, 1987; Martin *et al.*, 1998; Erwin *et al.*, 2005). Most of these studies (Kilian, 1976; Martin *et al.*, 1998) found increased prevalence of indole or *tna*-positive Hi among middle-ear strains compared to throat strains. A compilation of previously reported data (Martin *et al.*, 1998) suggested that tryptophanase activity, measured by the production of indole, is more commonly seen in pathogenic Hi strains (94–99% of otitis media isolates) than in commensals (70–75% of nonpathogenic respiratory isolates). Others (Oberhofer & Back, 1979; Erwin *et al.*, 2005) found increased prevalence among throat strains compared to ear strains, as did this study, although the difference did not reach statistical significance. The differences in the results of these prevalence studies may be related to differences in the populations of *H. influenzae* studied. In this study, for example, the strains that on routine testing appear to be *H. influenzae* but upon further testing are more closely related to *Haemophilus hemolyticus* were eliminated from testing (Xie *et al.*, 2006b).

The study by Erwin *et al.* (2005) showed the presence of *lav* in 30.2% of NTHi, in 5.9% of throat isolates, and in 57.9% of middle-ear isolates. In the present study, it was shown that *lav* was more prevalent among throat strains from healthy children (48.6%) than among middle-ear isolates (22.7%). Possible explanations for the discrepancies between results in the Erwin study and this study include the folllowing: Erwin's throat isolates may have included non-hemolytic *H. hemolyticus* strains, which can not be distinguished from *H. influenzae* by conventional laboratory testing (Fung *et al.*, 2006; Xie *et al.*, 2006a); the Erwin study used a smaller number of strains than did this study; and the Erwin study used PCR to detect the presence of *lav* while this study used dot-blot genomic hybridization and some of Erwin's PCR-negative isolates yielded a product that, by size, was interpreted to indicate the absence of the gene.

Results of this study suggest that *lav* may play a more important role in pharyngeal colonization than previous studies suggest. As an autotransporter, *lav* may promote secretion of a passenger protein that confers a survival advantage in the human pharynx. Although the family of autotransporters to which *lav* belongs to is associated with virulence activities such as adherence, invasion, and immune evasion, these same properties may also be advantageous to simple colonization, the predominant feature of commensalism. Conversely, these data suggest these properties of *lav* may not offer a selective advantage to *H. influenzae* strains that migrate to and infect the middle ear.

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