

Adhesin genes and serum resistance in *Haemophilus influenzae* type f isolates

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The incidence of invasive infections due to *Haemophilus influenzae* has decreased significantly in developed countries with high rates of vaccination against *H. influenzae* serotype b (Hib). This vaccine provides no protection against *H. influenzae* serotype f (Hif), typically associated with invasive infections in adults with chronic disease and/or immunodeficiency, and rarely in otherwise healthy adults and children. The specific properties of Hif associated with virulence remain largely uncharacterized. A panel of 26 Hif strains consisting of both invasive disease-associated and mucosal surface non-invasive disease-associated isolates was surveyed by DNA fingerprinting, biotyping and PCR detection of *hmw1*, *hmw2*, *hsf*, the *hif* fimbrial locus and the lipo-oligosaccharide (LOS) biosynthetic island, and assessment of β -lactamase expression and determination of resistance to the bactericidal activity of normal adult human serum. Repetitive sequence-based PCR fingerprinting differentiated the 26 strains into three clusters, with the majority of isolates (22/26, 84.6%) clustered into a single indistinguishable group. Most isolates (24/26, 92.3%) were of biotype I and two isolates produced β -lactamase with detection of a conjugative plasmid, and the isolates displayed a range of resistances to the bactericidal activity of human serum. All 26 isolates carried the adhesin *hsf*, 21 carried a partial *hif* fimbrial operon and 4 had the adhesin genes *hmw1/2*. A LOS biosynthetic island was detected in 20 isolates consisting of the genes *lic2BC*. It was concluded that Hif has many recognized virulence properties and comprises a relatively homogeneous group independent of the anatomical source from which it was isolated.

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INTRODUCTION

Haemophilus influenzae, a human-restricted Gram-negative coccobacillus, is a commensal of the upper respiratory mucosa and a pathogen commonly causing airway mucosal disease and occasional invasive disease. Since Margaret Pittman's original description of capsular serotypes among *H. influenzae* isolates in 1931 (Pittman, 1931), *H. influenzae* serotype b (Hib) had been the most clinically

significant strain causing invasive disease (e.g. meningitis, epiglottitis, septicaemia and osteomyelitis) in previously well infants and children (Aubrey & Tang, 2003). Since the early 1990s, routine administration of the Hib conjugate vaccines (which induce protective levels of anti-capsular antibody) has virtually eliminated Hib disease among infants and young children in developed countries (Agrawal & Murphy, 2011; Ladhani, 2012). However, the vaccine provides no protection from infection due to non-b serotypes, including *H. influenzae* serotype f (Hif), which remains a rare but significant cause of invasive infection. Given the reduction in Hib carriage among Hib conjugate vaccine recipients, concern exists as to whether invasive disease due to Hif or other non-b encapsulated strains will become more prevalent due to serotype replacement

Abbreviations: BLNAR, β -lactamase-negative ampicillin-resistant; CSF, cerebrospinal fluid; ERIC, enterobacterial repetitive intergenic consensus; Hib, *Haemophilus influenzae* serotype b; Hif, *Haemophilus influenzae* serotype f; IC₅₀, concentration of serum required to kill 50% of the inoculum; LOS, lipo-oligosaccharide; MLST, multilocus sequence typing; NHS, normal human serum; repPCR, repetitive sequence-based PCR.

(Takala *et al.*, 1991; Urwin *et al.*, 1996; Ward, 1996; Tsang, 2007). In fact, several investigations in North America and Europe in the post-conjugate vaccine era have indicated an increased incidence, albeit small, of invasive infections due to non-b *H. influenzae*, with a predominance of non-typable and Hif strains, primarily in older age groups (Urwin *et al.*, 1996; Ladhani *et al.*, 2010, 2012; MacNeil *et al.*, 2011; Resman *et al.*, 2011a; Rubach *et al.*, 2011; Ladhani, 2012).

Invasive disease associated with Hif appears to disproportionately affect individuals with underlying co-morbidities, including adults suffering from chronic obstructive pulmonary disease (COPD), ethanol abuse, chronic renal disease and human immunodeficiency virus/AIDS, and children with immunoglobulin deficiencies, severe combined immunodeficiency, human immunodeficiency virus/AIDS, malignancy and sickle-cell disease (Nitta *et al.*, 1995; Urwin *et al.*, 1996; Fickweiler *et al.*, 2004; Resman *et al.*, 2011b; Watson *et al.*, 2011). This is in contrast to Hib, which is considered a primary pathogen able to cause disease in otherwise healthy and immunocompetent individuals, predominantly children (Morris *et al.*, 2008). This discrepancy in disease epidemiology suggests that Hif and other non-b encapsulated strains may be intrinsically less virulent than Hib, a hypothesis that is supported by animal model studies of systemic infection showing decreased virulence of capsular serotype f strains relative to strains expressing capsular serotype b (Zwahlen *et al.*, 1983).

Compared with Hib, there is comparatively little knowledge and understanding of the epidemiology and virulence factors and features of serotype f strains accounting for invasive disease. In this study, a panel of 26 serotype f strains was characterized by biotyping and DNA fingerprinting, determination of β -lactamase production, PCR detection of a panel of known adhesins important for adherence and colonization, comprising *hmw1*, *hmw2*, *hsf* and the *hif* fimbriae (St Geme *et al.*, 1993, 1996, 1998; Mhlanga-Mutangadura *et al.*, 1998), PCR analysis of variability at a lipo-oligosaccharide (LOS) biosynthetic island, and assessment of their ability to resist the bactericidal activity of adult normal human serum (NHS). Importantly, we report that isolates of Hif, regardless of their anatomical source or history of causing invasive disease, comprise a relatively homogeneous group that, taken as a whole, contains a high prevalence of known virulence factors and properties.

METHODS

Bacterial strains, media and growth conditions. Reference *H. influenzae* isolates with known serotypes were obtained from the American Type Culture Collection: serotype a (ATCC 9006); Egan, a division I type b; type c (ATCC 9007); type d (ATCC 9008); type e (ATCC 8142); and type f (ATCC 9833). For certain experiments, the unencapsulated and serum-sensitive laboratory strain Rd KW20 [named R652 by our laboratory (Center for Childhood Infections,

Seattle Children's Hospital Research Institute); see Table 1] (Wilcox & Smith, 1975), non-typable and serum-resistant strain INT-1 (R2866) (Nizet *et al.*, 1996), non-typable strain 11 (R3945) (Barenkamp & St Geme, 1996), non-typable strain 12 (R2846) (Barenkamp & Leininger, 1992), fimbriated serotype b strain C54 (Pichichero *et al.*, 1982) and serum-resistant strain Eagan (E1a) (Weller *et al.*, 1977) were used as controls. Bacteria were stored at -80°C in sterile skimmed milk and cultured at 37°C in 5% CO_2 on chocolate agar supplemented with 1% (v/v) IsovitaleX (Becton Dickinson) or in Difco brain-heart infusion broth (Becton Dickinson) supplemented with haemin/HCl ($10\ \mu\text{g ml}^{-1}$; Sigma Chemical) (sBHI) and β -NAD⁺ ($10\ \mu\text{g ml}^{-1}$; Sigma) and with agar (Bacto-Agar; Difco) for solid sBHI plates. The strains are held in the collection of one of the authors (A.L.S.) consisting of isolates from clinical bacteriological laboratories where the author was located (C numbers) or strains referred to A.L.S. (R numbers). Strains isolated prior to 1982 (R1068–R1072) were a gift from Dr John Philpott-Howard, who reported on antibiotic resistance in *H. influenzae* in the UK since 1977 (Philpott-Howard & Williams, 1982).

Capsular typing by multiplex PCR. The primers used for PCR analysis are listed in Table 2. Genomic DNA was prepared using a DNeasy Tissue kit (Qiagen). Routine PCR conditions and DNA manipulations were performed as described previously (Erwin *et al.*, 2005). Initial screening of putative type f strains was with a multiplex PCR, using primers and methods reported previously (Nelson & Smith, 2010). Encapsulated control strains were obtained from the American Type Culture Collection comprising capsular types a–f. Strains with the amplicon pattern of type f in the multiplex assay were tested with primers specific for the capsule export locus *bexA* (*bexA* F and *bexA* R; Table 2) and separate specific primers (*f1* and *f3*; Table 2) for serotype f for confirmation (Falla *et al.*, 1994, 1995). All strains identified as serotype f produced a 400 bp amplicon with the confirmatory type f-specific primers identical to that produced by the Hif reference strain ATCC 9833.

Biochemical assays for biotype assignment. Biotypes were assigned by detection of indole production, ornithine decarboxylase activity and urea hydrolysis, using methods and a classification system described elsewhere (Kilian, 1976; Murray *et al.*, 2003). We originally intended to use the API NH strips (bioMérieux) for biotype assignment; however, in our experience, this assay does not yield reproducible results with these strains so the reactions were performed in test tubes as originally described by Kilian (1976).

Detection of β -lactamase activity. β -Lactamase expression was detected by testing for ampicillin hydrolysis with a modified phenol red assay (Scheifele *et al.*, 1976), adapted for a microtitre plate. Strain R2866 (INT-1) carries a plasmid-encoded β -lactamase and served as a positive control, with strain R652 (Rd KW20) as a negative control for β -lactamase expression.

Strain typing. DNA was extracted from *H. influenzae* isolates using a MoBio bacteriaemia DNA kit (MoBio Laboratories) in the laboratory of Carey-Ann Burnham (Washington University, St Louis, MO, USA). Repetitive sequence-based PCR (repPCR) was performed using ~ 100 ng DNA, and primers ERIC1 and ERIC2 (Table 2), in a final reaction volume of 25 μl using Ready-To-Go RAPD analysis beads (GE Healthcare Biosciences) (Hulton *et al.*, 1991; Kang & Dunne, 2003). Following an initial denaturation step at 95°C for 7 min, 30 cycles of PCR were performed (90°C for 15 s, 51°C for 30 s and 65°C for 7 min), with a final elongation step at 65°C for 14 min. The resultant PCR products were resolved using an Agilent 2100 (Agilent Technologies) with a Diversilab DNA chip (bioMérieux) to produce virtual gel electropherograms. Diversilab Bacterial Barcodes software (bioMérieux) was used to analyse the banding patterns and create a similarity index for the isolates (Healy *et al.*, 2005a, b).

Table 1. Panel of Hif isolates used in this study: source information and PCR analysis results

Strain*	Original designation†	Anatomical site	Year	<i>infA-ksgA</i> (<i>lic2BC</i>)‡	<i>infA-ksgA</i> (<i>losAB</i>)§	<i>purE-pepN</i> (<i>hif</i>)	<i>hmw1/2</i> ¶	<i>hsf</i> #
R232	Boston	Blood	1972	+	-	+	-	+
R543	ATCC 9833	Blood	1975	+	-	+	-	+
R1068	3	Sputum	Before 1982	+	-	+	-	+
R1069	210	Sputum	Before 1982	+	-	+	-	+
R1070	236	Nasopharynx	Before 1982	-	-	-	-	+
R1071	256	Sinus aspirate	Before 1982	+	-	+	-	+
R1073	409	Sputum	Before 1982	+	-	+	-	+
R1074	1235	Sputum	Before 1982	+	-	+	+	+
R1075	3738	Sputum	Before 1982	+	-	+	+	+
R1527	Seattle	CSF	1985	-	-	+	-	+
R1831	Seattle	Blood	1986	+	-	+	-	+
R2978	11OU35	Blood	1996	-	-	+	+	+
R3072	-	Blood	1997	+	-	+	-	+
R3073	Seattle	CSF	1991	+	-	+	-	+
R3779	2006-14	Blood	2006	+	-	-	-	+
R3788	Seattle	Sinus aspirate	2008	+	-	+	-	+
R3827	St Louis	Thigh abscess	2009	+	-	+	-	+
R3905	91:31	Blood	2010	-	-	+	-	+
R3906	106:29	Blood	2010	+	-	+	-	+
R3907	Seattle	Blood	1994	+	-	+	-	+
R3915	Seattle	Joint aspirate	2010	+	-	+	-	+
R3944	Seattle	Blood	2011	+	-	+	+	+
C10	Boston	CSF	1970	+	-	+	-	+
C581	Seattle	Sputum	1978	+	-	+	-	+
C633	13643	Sputum	1979	-	-	+	-	+
C909	Seattle	CSF	1982	-	-	+	-	+

*Strain names identify the individual who sent the strain: R1068–R1075 were from J. Philpott-Howard (Guy's, King's & St Thomas' School of Medicine, London, UK); R2978 was from L. Van Alphen (Utrecht University, Utrecht, The Netherlands); R3072 was from S. Kaplan (Baylor College of Medicine, Infectious Disease Service, Texas Children's Hospital, TX, USA); R3779 was from R. Tsang (National Microbiology Laboratory, Public Health Agency of Canada, Manitoba, Canada); R3788 was from X. Quin (University of Washington School of Medicine, Washington, DC, USA); and R3905 and R3906 were from J. Clarridge (University of Washington School of Medicine, Washington, DC, USA). Boston refers to Children's Hospital of Boston, Seattle is the Children's Hospital and Regional Medical Center of Seattle, and St Louis refers to St Louis Children's Hospital.

†The original description refers to the coding of the isolate when received or the source clinical laboratory.

‡PCR amplification of the LOS island within the *infA-ksgA* locus and subsequent PCR for *lic2BC*. +, Both amplifications were positive; -, one or other of the amplicons was not detected.

§PCR amplification of the LOS island within the *infA-ksgA* locus and subsequent PCR for *losAB*. +, Both amplifications were positive; -, one or other of the amplicons was not detected.

||PCR amplification of the *hif* fimbrial locus at *purE-pepN*. +, Amplification was positive for a ~4 kb amplicon matching that of the partial *hif* allele of ATCC 9833 (R543); -, the amplicon was not detected.

¶Amplification by PCR of *hmw*. +, Production of an ~1200 bp *hmw* amplicon, except for strain R2978, which produced a partial ~400 bp amplicon.

#Amplification by PCR of *hsf*. A '+' indicates the production of ~400 bp *hsf* amplicon.

**Partial-length amplicons at the *purE-pepN* locus of ~800 bp containing *hicaB* genes.

Isolates with a similarity index of $\geq 85\%$ were considered identical for the purpose of this analysis. A non-typable strain, R465, was included as an unrelated control and was characterized previously by enterobacterial repetitive intergenic consensus (ERIC) sequence-based PCR fingerprinting (Watson *et al.*, 2004).

Detection of adhesins by PCR. The primers used for the detection of adhesin genes are listed in Table 2. Genomic DNA was prepared using a DNeasy Tissue kit (Qiagen). Routine PCR conditions and DNA manipulations were performed as described previously (Erwin

et al., 2005). For classification purposes, the absence of an amplicon, or an amplicon of <100 bp, was considered negative for that locus. The isolates were examined for the adhesins *hmw1/2* (St Geme *et al.*, 1993) and *hsf*, including its smaller allele *hia* (St Geme *et al.*, 1996), using strain R2846 (strain 12; GenBank accession no. CP002276) as a positive control. For detection of *hsf*, strain C54 was used as a positive control and produced a ~400 bp amplicon band using the *hia_hsf* F and *hia_hsf* R primer set. The adhesin genes *hsf* and *hia* are identical at the N and C termini, such that the *hia_hsf* primer set identified both alleles. For amplification of the high-molecular-weight adhesion

Table 2. Primers used for PCR

Designation	Sequence (5'→3')	Reference
bexA F	ATGATTGCGTAAATAATGTATGTAAGAAG	Nelson & Smith (2010)
bexA R	TTGCGTCTCGTTGTAGTATTGATAC	
ERIC1	ATGTAAGCTCCTGGGGATTCAC	Hulton <i>et al.</i> (1991)
ERIC2	AAGTAAGTGACTGGGGTGAGCG	
f1	GCTACTATCAAGTCCAAATC	Falla <i>et al.</i> (1994)
f3	AATGCTGGAGTATCTGGTTC	
hia_hsf F	TGTCCGCTTGATCCGAACAATCA	This study
hia_hsf R	ACTGGTAACCAACACCTGCTGCAA	
HMWP1F	GCGTCGAAGGAGGTCAACGAACG	St Geme <i>et al.</i> (1998)
HMWP3R	GCCCCACACAATAGCGC	
infA-ksgA F	TATGTGCAGTAACCACGTGACCGT	Erwin <i>et al.</i> (2005)
infA-ksgA R	TCGACAAGTTCACCAACAGGCTCT	
lic2B-F	TAAGTATGATCCTCAAATGCAT	Erwin <i>et al.</i> (2005)
lic2BA	CAATTTAGCGATGAGTTCC	
losB-R	GGTTGAGCCATCTTGTGGAATGTC	Erwin <i>et al.</i> (2005)
purE_pepN F	GACCCATCACAACGCGAATTTGTG	Erwin <i>et al.</i> (2005)
purE_pepN R	CTGTGACCGTAAAATCTGGTTGTTTGAATC	
topoF	GAGACTCACAAGCGACAACC	Erwin <i>et al.</i> (2005)
topoR	GGCTTGAGGTGCGTCATCATCTTC	

genes *hmw1* and *hmw2* (primers HMWP1F and HMWP3R; Table 2), strain R2846 (strain 12) was used as a positive control with production of an ~1200 bp amplicon, and strain R2866 (INT-1) whose genomic sequence is available (GenBank accession no. CP002277) was used as a negative control with no amplicon detected. The fimbrial gene cluster lies between *purE* and *pepN* in strains expressing the structure, also called haemagglutinating pili. The gene cluster required for the expression of fimbriae, *hifABCDE*, is ~7.5 kb in serotype b strains, but in certain strains *hicAB* is also present, whose function is unknown (Mhlanga-Mutangadura *et al.*, 1998). The Hif reference strain ATCC 9833 (R543; Table 1) has a partial fimbrial cluster containing *hifA*, *hifC* and parts of *hifD* and *hifE*, which total ~3.7 kb (Mhlanga-Mutangadura *et al.*, 1998). For amplification of the fimbriae locus, isolates were screened using the *purE_pepN* primer set, and the fimbriated serotype b strain C54 was used as a full-length ~7 kb positive control and ATCC 9833 (R543) as a positive control for the partial ~3.7 kb allele.

Determination of the composition of the LOS biosynthetic island. Prior experiments indicated that the genes for glycosyltransferases *lic2B* and *lic2C* are between *infA* and *ksgA* in a type b genome (strain 7004; Hood *et al.*, 2004). In strains Rd KW20 (R652) and INT-1 (R2866), *infA* and *ksgA* are adjacent, whilst in strain 86-028NP, the locus contains only *lic2C*. In R2846 (strain 12) and ~20% of other non-typable strains, the locus contains the glycosyltransferases *losA* and *losB*, whose expression is phase variable due to the presence of the octameric repeat 5'-CGAGCATA-3', with the number of repeats varying between two and ten, modifying the translational reading frame; expression of these enzymes alters the structure of the surface LOS, affecting resistance to NHS (Erwin *et al.*, 2006b). Both the *lic2B-lic2C* and the *losA-losB* regions are within the 2.2 kb amplicon found with the *infA-ksgA* primers; these regions can be differentiated by subsequent PCR and/or sequencing using allele-specific primers as reported previously (Erwin *et al.*, 2005). Because of the heterogeneity of this island we examined the composition of 26 type f strains by PCR for the *infA-ksgA* locus using the *infA-ksgA* primer set (Table 2) and subsequent PCR using the *lic2B-F* and *lic2BA* primer pair to identify the *lic2BC* allele, or the *infA-ksgA F* and *losB-R* primer pair to identify the *losAB* allele.

Resistance to serum bactericidal activity. Resistance testing to human serum bactericidal activity was assayed using pooled NHS and assay conditions reported previously (Erwin *et al.*, 2005). NHS was obtained by venepuncture from healthy, adult volunteers according to an approved human studies protocol. Serial dilutions of serum in 10 mM PBS with 4 mM KCl and 0.1% (w/v) gelatine, ranging from 0.02 to 50% (v/v) serum, were tested. The reported IC₅₀ was the concentration of serum required to kill 50% of the inoculum (~2000 c.f.u.) within 30 min of incubation at 37 °C in room air. The IC₅₀ was calculated using XLfit version 4.1 software (ID Business Solutions), which unweights the lowest and highest values. The IC₅₀ values of the unencapsulated and serum-sensitive Rd KW20 strain (R652), the non-typable and serum-resistant INT-1 strain (R2866) and the serotype b encapsulated strain Eagan (E1a) are shown in Table 3 for reference.

Statistical analysis. The VassarStats website for statistical computation (<http://faculty.vassar.edu/lowry/VassarStats.html>) was used for analysis. A Mann-Whitney *U* test was used to compare mean IC₅₀ values for NHS resistance capacity between mucosal and invasive disease-associated isolates.

RESULTS

Confirmation of capsular serotype f

Initially, 36 strains previously identified as type f on the basis of serotyping in a clinical microbiology laboratory were subcultured from the collection of one of the authors (A.L.S.). These 36 isolates were examined using a multiplex PCR for capsule types a–f, and in separate reactions with primers for *bexA*, the capsule export gene conserved among all encapsulated *H. influenzae* (Nelson & Smith, 2010). Among those isolates identified as serotype f using the multiplex assay and among those that were also *bexA* positive, 25 isolates and the ATCC 9833 Hif reference

Table 3. NHS bactericidal activity for Hif isolates

Type of isolate & strain	Mean IC ₅₀ (range)	SD	n*
Controls			
R652 (Rd KW20)	1.08 (0.32–2.09)	0.48	20
R2866 (INT-1)	47.79 (39.95–50.00)	3.63	16
E1a (Eagan)	50.00 (50.00–50.00)	0.00	3
Invasive disease-associated isolates			
R232	>50.00†		5
R543	35.01 (25.04–50.00)	13.60	7
R1527	35.01 (25.00–>50.00)	13.69	5
R1831	3.37 (3.06–3.94)	0.49	3
R2978	37.32 (24.92–>50.00)	9.04	5
R3072	2.49 (1.29–3.68)	1.06	5
R3073	2.49 (1.29–2.82)	1.05	7
R3779	16.23 (11.24–24.75)	7.41	5
R3827	5.97 (4.33–6.80)	1.01	7
R3905	8.64 (7.28–10.56)	1.31	7
R3906	16.56 (14.00–18.76)	2.40	5
R3907	5.62 (3.45–8.58)	2.65	5
R3915	41.25 (23.76–>50.00)	15.15	5
R3944	24.68 (23.68–25.55)	0.94	3
C10	>50.00†		5
C909	12.79 (8.95–14.48)	2.23	5
Group‡	21.71	4.32	
Mucosal carriage and non-invasive disease-associated isolates§			
R1068	48.12 (44.37–50.00)	3.25	5
R1069	>50.00†		7
R1070	45.33 (26.63–50.00)	10.45	7
R1071	45.86 (26.63–50.00)	7.16	5
R1073	47.46 (44.33–50.00)	2.87	5
R1074	47.15 (41.45–50.00)	4.93	5
R1075	37.32 (24.93–50.00)	9.04	7
R3788	4.33 (2.15–6.89)	1.99	5
C581	45.66 (28.33–>50.00)	9.69	5
C633	46.41 (32.04–>50.00)	8.03	5
Group‡	41.76	4.29	

*Number of replicates used to calculate the reported values.

†IC₅₀ values greater than the upper limit of detection (>50.00), range and SD could not be calculated. For group mean calculations, these were considered to be 50.00.

‡Group analysis represents mean IC₅₀ and SEM.

§Mucosal isolates collected mostly from non-invasive disease specimens.

strain were subsequently confirmed to contain a specific type f capsular gene by further PCR amplification (Falla *et al.*, 1994). Eleven of the thirty-six original isolates did not test positive for *bexA* and/or for serotype f using confirmatory primers, and as these strains could not be absolutely confirmed as Hif strains, they were not used in subsequent analyses. The 25 Hif strains and the ATCC 9833 Hif strain confirmed as type f were originally isolated from diverse sources: blood ($n=10$), cerebrospinal fluid (CSF) ($n=4$), sputum ($n=7$), sinus aspirates ($n=2$), nasopharynx ($n=1$), joint aspirate ($n=1$), and thigh abscess ($n=1$) between 1972 and the 2011. The ATCC 9833 type f reference strain was isolated from blood and CSF by Pittman (1931). The site of isolation, the date isolated and

the number assigned in our laboratory to each strain is shown in Table 1.

General features and colony morphology of confirmed Hif isolates

Isolates were assayed for indole production, ornithine decarboxylase activity and urea hydrolysis for biotype assignment. Among the panel of isolates, 24/26 Hif strains (92.3%) were identified as biotype I (positive for all three of the biotyping reactions), with only 2 isolates, R1068 and R3944, identified as biotype II (ornithine decarboxylase activity was not detectable). We noted a striking variation in colony morphology on sBHI agar: the majority of the

isolates had a 'smooth' texture, whilst the CSF isolate C10 had a small flat colony and the sputum isolate C581 had a large 'mucoid' colony phenotype. One isolate, R3944, produced both a smooth colony and a wrinkled colony on subculture; each morphotype had identical results in the phenotypic analysis. β -Lactamase activity was detected in 2/26 (7.7%) Hif strains, R3788 and R3907, by an ampicillin hydrolysis assay. Consistent with the detected β -lactamase activity, strain R3907, a blood isolate, and R3788, a sinus isolate, were the only isolates determined to carry a large conjugative plasmid associated with β -lactamase activity, as they produced an ~450 bp amplicon by PCR with primers topoF and topoR (Erwin *et al.*, 2005).

Hif isolate typing by repPCR fingerprinting

DNA fingerprinting for the confirmed Hif isolates was performed using repPCR with primers detecting ERIC sequences (Healy *et al.*, 2005a), and amplicons were resolved using bioMérieux Diversilab Bacterial Barcodes software to generate a similarity index. Isolates with similarity indices >85% were considered identical in this study (Healy *et al.*, 2005a, b). A dendrogram of Hif strains with virtual electropherograms is shown in Fig. 1, illustrating three distinct and unrelated clusters of isolates. Cluster A was the largest group with 22/26 (84.6%) isolates, followed by clusters B and C with only 2/26 (7.7%) isolates each. There was no correlation between cluster assignments with respect to geographical origin, clinical disease association or biotype. Both of the β -lactamase-producing isolates, R3788 and R3907, were within cluster A. A non-typable strain, R465, was included as an unrelated control and was characterized previously by ERIC sequence-based PCR fingerprinting.

PCR detection of adhesins

The panel of Hif isolates was subjected to PCR analysis to determine the presence of genes encoding the following adhesins notable in other encapsulated and non-typable *H. influenzae* for their role in adherence and colonization: the high-molecular-weight adhesin (*hmw1* and *hmw2* genes), the Hsf adhesin (*hsf* gene) and the *hif* gene cluster (between *purE* and *pepN*) encoding surface fimbriae (St Geme *et al.*, 1993, 1998; Mhlanga-Mutangadura *et al.*, 1998). The results are shown in Table 1. Among this panel of Hif strains, all 26 isolates (100%) were PCR positive for amplification at the *hsf* adhesin locus using the *hia_hsf* primer pair. For the high-molecular-weight adhesins (*hmw1* and *hmw2*), three isolates (11.5%; R1074, R1075 and R3944) produced amplicons of ~1200 bp correlating to the presence of *hmw*, whilst one isolate, R2978, produced a ~400 bp partial *hmw* amplicon. Interestingly, the four isolates producing a full-length or partial amplicon for *hmw* were grouped into clusters B (R1075 and R2978) and C (R3944 and R1074), further distinguishing these two clusters from the majority of the isolates in cluster A testing negative for *hmw* carriage. In examination

of the Hif fimbrial locus by PCR amplifying the region between *purE* and *pepN*, all strains except R1070 and R3779 displayed an insert at this locus. Whilst most of the strains testing positive for an insert exhibited a ~4 kb amplicon consistent with the partial-length *hif* allele previously identified in ATCC 9833 (R543) containing *hifA*, *hifC* and parts of *hifD* and *hifE*, strains R3728, R3827 and R3944 all produced an 800 bp amplicon consistent with our previous work in non-typable strains in which this 800 bp amplicon was sequenced and determined to contain the *hic* genes ('*hif* contiguous' DNA) *hicAB*, two putative conserved genes of unknown function (Mhlanga-Mutangadura *et al.*, 1998). Digestion of the ~4 kb *hif* amplicon in the 21 strains with this insert with *AseI* and *DraI* yielded three identically sized fragments of ~2.7 kb among all of the positive isolates, suggesting that the *hif* sequence was relatively conserved among the isolates, although it is conceivable that sequence polymorphisms existed outside the restriction enzyme sites, masking the ability to detect differences by this method. With regard to the presence or absence of *hif* or *hicAB*, there were no correlations with the DNA fingerprinting clustering assignments, as these alleles were distributed across all three clusters (Fig. 1).

Mapping of the LOS biosynthesis island

Previous work has indicated that the LOS modification genes *lic2B* and *lic2C* are located in a genetic island between *infA* and *ksgA* in a type b genome (strain 7004; Hood *et al.*, 2004). In strains Rd KW20 (R652) and INT-1 (R2866), *infA* and *ksgA* are adjacent, whilst in the non-typable strain 86-028NP, the locus only contains *lic2C* (Harrison *et al.*, 2005). In the non-typable strain 12 (R2846), the *infA-ksgA* locus contains a different pair of glycosyltransferase genes, *losA* and *losB*, whose expression is phase variable due to the presence of the octameric repeat 5'-CGAGCATA-3' varying between two and ten copies (Erwin *et al.*, 2006b). Because of the heterogeneity of this island, we examined the 26 type f strains by PCR for the *infA-ksgA* locus. Twenty of the twenty-six isolates (76.9%) produced an amplicon of 2.2 kb at the *infA-ksgA* locus, which, when examined by a subsequent PCR using primers specific for *lic2BC*, were all positive (Table 1). Carriage of the *lic2BC* insert at the *infA-ksgA* locus did not correlate with a specific fingerprinting cluster. PCR analysis of all 26 strains for *losAB* did not yield an amplicon (Table 1), suggesting that the *losAB* allele may be absent from serotype f strains. These findings at the LOS modification locus further support the relative homogeneity of Hif strains, regardless of their clinical source.

Resistance to the bactericidal activity of NHS

The presence of a polysaccharide capsule promotes virulence by imparting resistance to the complement-mediated antibacterial properties of NHS. Unencapsulated strains, such as Rd KW20 (R652), are relatively serum sensitive compared with capsule-producing strains (e.g.

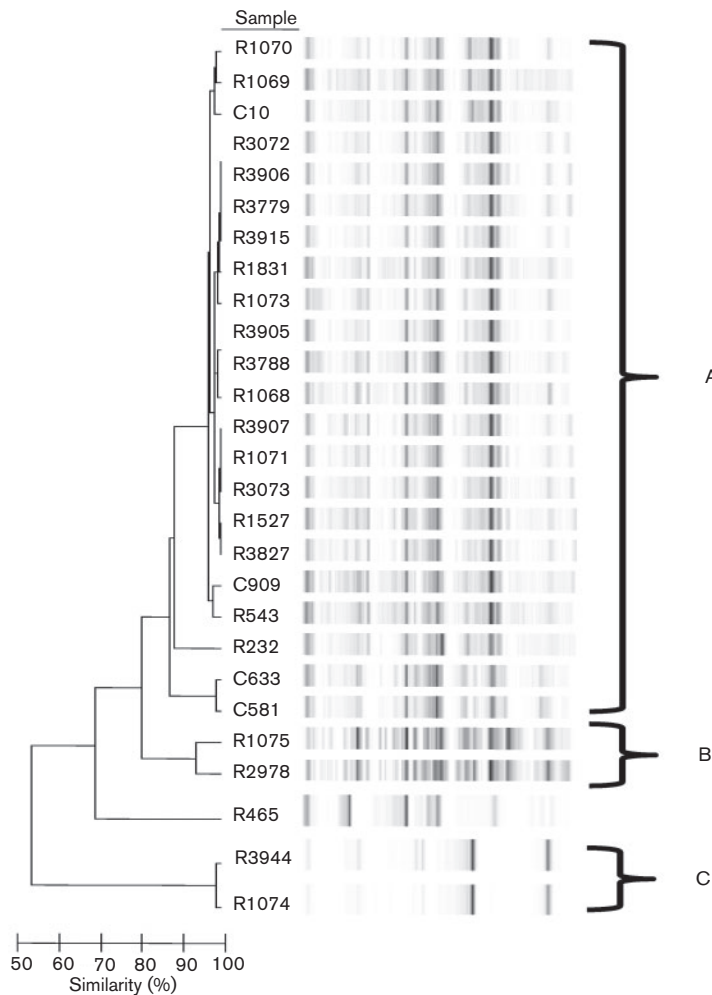


Fig. 1. Dendrogram of Hif isolates. DNA was isolated from 26 Hif isolates and used as template DNA for repPCR with primers ERIC1 and ERIC2 (Table 2). The resultant amplicons were resolved on an Agilent 2100 with Diversilab DNA chips to create virtual electropherograms and Bacterial Barcodes software was used to calculate a similarity index for the isolates. Isolates with a similarity index of $\geq 85\%$ were considered to be identical. Banding patterns are shown for the 26 isolates, organized into three unrelated clusters, A–C, with the majority of isolates (22/26, 85%) in cluster A. The non-typable strain R465, analysed previously by ERIC sequence-based PCR fingerprinting in our laboratory (Watson *et al.*, 2004), was included as a control and was confirmed to be unrelated to the other strains in the study.

Eagan), which can replicate in 50% NHS and whole blood, or the rare serum-resistant non-typable strain INT-1 (R2866) (Table 3) (Williams *et al.*, 2001). In the serum-resistance assay, the reported IC_{50} was the mean (\pm SD) concentration of serum required to kill 50% of the inoculum within a 30 min incubation period (Table 3). Five of the sixteen strains isolated from normally sterile anatomical sites had mean IC_{50} values of $<6.24\%$, the minimum value previously found with type b isolates (Table 3). Two of the sixteen invasive group isolates (the blood isolate R232 and CSF isolate C10) exhibited high-level resistance with mean IC_{50} values of $\geq 50\%$ NHS (Table 3). The mean IC_{50} value for the invasive group taken as a whole was $21.71 \pm 4.32\%$ with a wide range of resistance to NHS (Table 3). The mean IC_{50} for the ten strains isolated from mucosal airway secretions taken as a whole was $41.76 \pm 4.29\%$ (mean \pm SEM), but only one of those strains (the sputum isolate R1069) exhibited high-level resistance with a mean IC_{50} of $\geq 50\%$ NHS (Table 3). Interestingly, the mean IC_{50} values of the mucosal isolate group were significantly greater than the invasive disease-associated isolate group (Mann–Whitney *U* test, $P=0.0114$), suggesting that the ability of Hif strains to

produce invasive infections is not entirely dependent on serum-resistance capacity.

DISCUSSION

Before vaccination against Hib became widely adopted in developed countries in the mid-1990s, serotype f was the second most common capsular serotype causing invasive *H. influenzae* infection (Berndsen *et al.*, 2011; Rubach *et al.*, 2011). In the post-Hib vaccine era, invasive disease in children due to *H. influenzae* is uncommon, with the majority of these infections due to unencapsulated strains, with the remainder being non-b encapsulated strains, predominantly serotypes f and a in North America and serotypes f and e in Europe (Tsang *et al.*, 2006; Adam *et al.*, 2010; Ladhani *et al.*, 2010, 2012; Agrawal & Murphy, 2011; Resman *et al.*, 2011a; Rubach *et al.*, 2011). To understand better the ability of serotype f strains to produce invasive infection, we assembled a panel of Hif isolates from a diversity of clinical sources for factors related to pathogenesis. Importantly, we have reported here that isolates of Hif seem to comprise a relatively homogeneous group that,

taken as a whole, contain a high prevalence of known virulence factors and properties that potentially contribute to pathogenesis.

Prior investigation of Hif isolates collected from North America and Europe and typed by multilocus enzyme electrophoresis indicated that Hif strains were remarkably clonal in origin with limited genetic diversity, possibly emerging from a single original clone continuing to undergo expansion (Bruun *et al.*, 2004). Similar conclusions have been reached by studies using alternative methods including multilocus sequence typing (MLST) (Meats *et al.*, 2003), PFGE (Campos *et al.*, 2003) and ribotyping (Urwin *et al.*, 1996). There is a suggestion that most invasive infections due to non-b encapsulated strains are caused by a limited number of circulating clones that are intrinsically more pathogenic than the clones associated more often with non-invasive disease (Omikunle *et al.*, 2002). Funding for the present study was not sufficient to permit MLST of all 26 Hif strains, so DNA fingerprinting using repPCR was performed to examine the relative diversity of these isolates. The majority (~85%) of our isolates were indistinguishable by this method and clustered together, independent of the two other unrelated clusters containing two isolates each. The fact that clusters B and C contained the only four isolates carrying *hmw* alleles suggests that acquisition and carriage of *hmw* among serotype f strains is relatively uncommon and that *hmw* may be a marker for less-dominant Hif clones in circulation. Importantly, even among isolates grouping together in the same cluster, we were able to detect differences with regard to the carriage of β -lactamase-encoding conjugative plasmids, different adhesin alleles and inserts in the LOS biosynthetic island, suggesting that there may be slightly more diversity in our isolates than is suggested by DNA fingerprinting alone. In our sample, all of our isolates were biotype I except for two that were biotype II; biotype assignment did not correlate with the isolate having been an invasive or mucosal-associated isolate. A previous report found only biotype I among Hif isolates (Bruun *et al.*, 2004). This is reminiscent of serotype b strains where the majority of isolates are biotype I, especially those from invasive disease, with a smaller subset of Hib isolates of different biotypes occasionally associated with specific diseases (e.g. conjunctival Hib isolates are usually biotype III) (Kilian, 1976). Taken as a whole, the results from DNA fingerprinting and biotype assignment of our current sample of 26 Hif isolates are congruent with previous studies in showing relatively limited diversity within this serotype, despite Hif being implicated in a wide variety of clinically significant infections from mucosal surfaces and normally sterile sites.

Different protein adhesins are carried to varying extents among *H. influenzae* isolates. Most encapsulated strains contain genes for the *hsf* fibril adhesin or high-molecular-weight adhesin HMW, but rarely both, and some strains are also able to synthesize fimbriae (St Geme *et al.*, 1993, 1996, 1998). We took advantage of this to explore Hif

diversity further. All of our Hif isolates were PCR positive for the *hsf* adhesin, with 4 of the 26 isolates also PCR positive for *hmw1/hmw2* and 21 containing a partial *hif* fimbrial gene cluster. A prior report determined that only 1 of 21 type f strains contained *hmw1A/1B/1C*, and 13 of these 21 isolates were *hia* positive (Rodriguez *et al.*, 2003). The same study also found that *hifB* was absent in all type f strains; this was surprising in that 17 of the type f strains contained *hifA*, *hifC* and *hifD* detected by probes on colony blots in that study (Rodriguez *et al.*, 2003). Our finding of an ~4 kb amplicon at the *purE-pepN* locus in the majority of our isolates is consistent with our prior description of the partial fimbrial gene cluster in the type f ATCC 9833 (Mhlanga-Mutangadura *et al.*, 1998), and is in agreement with the prior study showing that serotype f strains lack *hifB* (Rodriguez *et al.*, 2003). Restriction of the ~4 kb product with *AseI* and *DraI* produced an identical pattern in the 21 strains producing an ~4 kb *hif* amplicon, suggesting sequence similarity. The complete fimbrial cluster found in the fimbriated serotype b strain C54 is 7.76 kb and contains *hicAB* in addition to *hifABCDE* (Pichichero *et al.*, 1982). The expression of haemagglutinating fimbriae only requires *hifABCDE*, so the function of *hicAB* in this strain is unknown. One of three of the type f strains in our study producing the 800 bp amplicon at the *purE-pepN* locus, previously sequenced as *hicAB* (Mhlanga-Mutangadura *et al.*, 1998), exhibited a wrinkled colonial morphology (blood isolate R3944), suggesting that *hicAB* alone was not sufficient for this phenotype, and therefore the function of the *hicAB* allele remains undefined. A limitation of DNA PCR surveys for virulence genes, as performed in this study, is the inability to determine whether carriage of a particular gene correlates with protein expression, as this method does not consider transcriptional or translational regulatory features potentially influencing expression of a certain gene. Verification of protein expression from these genes was beyond the scope of this study but would be important for future investigations to characterize further the virulence factors expressed in serotype f strains.

The prevalence of β -lactamase expression in this panel at 7.7% of 26 isolates was less than that reported by two prior investigations of Hif samples, where activity was detected in 20.9% of 43 isolates and in 24.5% of 49 isolates (Urwin *et al.*, 1996; Campos *et al.*, 2003); both of these studies had larger sample sizes and assayed for β -lactamase activity using a nitrocefin-based assay for detection. We have had inconsistent results with the nitrocefin disk and therefore measured the hydrolysis of ampicillin colorimetrically, which may account for some differences. A side-by-side comparison of these two methods has not been performed. An obvious limitation of this method is the inability to detect β -lactamase-negative ampicillin-resistant (BLNAR) isolates encoding altered penicillin-binding proteins through mutation in the *ftsI* gene conferring resistance (Ubukata *et al.*, 2001). A review of the literature failed to find reports of BLNAR isolates among Hif isolates, and the

BLNAR phenotype is rare in North America in general, but evidence does suggest that, in areas of Europe and Asia, there is increasing prevalence of BLNAR isolates among non-typable and serotype b isolates, suggesting that future studies on antimicrobial susceptibility patterns should include methods to capture these strains as well (Hasegawa *et al.*, 2006; Dabernat & Delmas, 2012).

Serum resistance varied greatly across our sample group of Hif isolates and did not correlate with potential virulence as predicted by specimen source. For example, some blood isolates were relatively serum sensitive, whilst some isolates from sputum were equally serum resistant. Surprisingly, the mean IC₅₀ value of the mucosal surface-isolated group was significantly greater than that of the invasive isolate group. Previously, we have shown that low-level serum resistance of oropharyngeal non-typable *H. influenzae* isolates permits them to establish a lower respiratory tract infection (Nakamura *et al.*, 2011), indicating that low-level serum resistance in some sputum isolates is not surprising. Serum resistance of an encapsulated strain is imparted largely by the presence of the polysaccharide capsule (Moxon & Kroll, 1988), as evidenced by the serum resistance of a *bexA*⁻ and phenotypically unencapsulated strain Eagan with an IC₅₀ value of 1.20% (K. L. Nelson and A. L. Smith, unpublished data). In an invasive unencapsulated strain, such as R2866 (INT-1), serum resistance results from phase-variable expression of the *lgtC*-mediated α Gal1-4 β Gal epitope, which mimics the p^K blood group, with the resulting phase variant not being recognized as foreign (Weiser & Pan, 1998; Erwin *et al.*, 2006a). The *lic2B* and *lic2C* LOS biosynthetic genes flanked by the *infA* and *ksgA* genes are associated with a serum-resistance phenotype in a serotype b strain (High *et al.*, 1996; Hood *et al.*, 2004). Approximately 20% of non-typable *H. influenzae* isolates contain phase-variable *losA* and *losB* genes, which, when expressed, mediate low-level serum resistance (Erwin *et al.*, 2006b). None of our panel of isolates was positive for *losAB*, and only 20/26 isolates were positive for *lic2BC*. Interestingly, several of the *lic2BC*-negative Hif isolates were highly serum resistant in our assay, suggesting that other mechanisms may account for their phenotype *in vitro*. Phase variation of other surface structures, such as LOS, may compete with the binding by bactericidal antibody and complement, decreasing the level of serum resistance. It is entirely possible that some strains in our panel that tested serum sensitive may be so due to selection of a phase variant exhibiting that phenotype, and thus serum resistance as assayed *in vitro* may not always reflect the true serum-resistance potential of an isolate *in vivo*. We demonstrated previously a lack of correspondence between *in vitro* serum resistance and the isolation of non-typable *H. influenzae* strains from normally sterile sites (Erwin *et al.*, 2005). These data suggest that serum resistance is multifactorial and is not a specific requirement for a particular strain to produce an invasive infection. Rather, an invasive infection is probably due to a combination of factors (e.g. impaired host defences or bacterial strains with

a slight competitive advantage) all occurring simultaneously to result in disease.

In summary, Hif isolates have multiple traits and known virulence factors associated with pathogenesis, and as a whole remain a rare but significant cause of morbidity and mortality, which may be slowly increasing in incidence worldwide in the post-Hib-conjugate-vaccine era. The finding that Hif strains seem to have many properties in common with other non-b serotypes, in addition to some non-typable strains, suggests that future vaccine strategies could be developed potentially to target multiple different serotypes of *H. influenzae* simultaneously.

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