Absence of high molecular weight proteins 1 and/or 2 is associated with decreased adherence among non-typeable *Haemophilus influenzae* clinical isolates

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High molecular weight (Hmw) proteins 1 and 2, type IV pilin protein (PilA), outer-membrane protein P5 (OmpP5), *Haemophilus* protein D (Hpd) and *Haemophilus* adhesive protein (Hap) are surface proteins involved in the adherence of non-typeable *Haemophilus influenzae*. One hundred clinical isolates were evaluated for the presence of the genes encoding these proteins by PCR and for their adherence capacity (AC) to Detroit 562 nasopharyngeal cells (D562). The majority of isolates were from blood (77/100); other sites were also represented. Confluent D562 monolayers (1.2×10⁶ cells per well) were inoculated with standardized minimal infective doses (m.o.i.) of 10², 10³ or 10⁴ c.f.u. per well. The AC was categorized as low (<10%) or high (≥10%) depending on the percentage of c.f.u. adhering per well. All the isolates evaluated showed adherence: 69/100 (69%) demonstrated high adherence, while 31/100 (31%) showed low adherence. Of all the genes evaluated, *hmw1A* and/or *hmw2A* were detected in 69/100 (69%) of isolates. The presence of *hmw1A* and/or *hmw2A* was associated with increased adherence to D562 cells (P≤0.001). Dot immunoblots were performed to detect protein expression using mAbs 3D6, AD6 and 10C5. Among the high-adherence isolates (n=69), 72% reacted with 3D6 and 21% with 10C5. Our data indicate that the absence of Hmw1 and/or Hmw2 was associated with decreased adherence to D562 cells.

INTRODUCTION

*Haemophilus influenzae* is a Gram-negative bacillus commonly found in the human nasopharynx, and is capable of causing a variety of diseases. Encapsulated *H. influenzae* produces one of six chemically distinct polysaccharide capsules defined as serotypes a–f based on their reactivity with immune specific antisera used for serotyping (Pittman, 1931). *H. influenzae* not agglutinating with the specific antisera are considered non-typeable *H. influenzae* (NTHi). Others have used the lack of the *cap* locus to confirm complete deletion of the capsule in these non-typeable isolates (Davis et al., 2011).

*H. influenzae* infection can occur by coming into direct contact with respiratory droplets containing this organism. The bacterial adhesins and pilins allow NTHi to adhere and colonize the human nasopharynx. NTHi is more commonly associated with non-invasive diseases such as otitis media, pharyngitis, tonsillitis and sinusitis. However, NTHi can also cause invasive diseases such as bacteraemia and meningitis (Murphy et al., 2009; Hardy et al., 2003; Rubach et al., 2011). Based on data from the Active Bacterial Core Surveillance (ABCs) programme, the majority of invasive *H. influenzae* disease in the United States is currently caused by NTHi, with the largest burden of disease occurring in those >65 years old and <5 years of age, particularly in pre-term and low birth weight infants (MacNeil et al., 2011). While overall rates of *H. influenzae* invasive disease have decreased and rates have remained...
steady at ~4 cases per 100,000 population in the >65 year olds, a considerable burden of the disease still affects these two cohorts disproportionately (MacNeil et al., 2011).

Several surface proteins play a role in adhesion: high molecular weight proteins 1 and 2 (Hmw1 and Hmw2), type IV pilin protein (PilA), outer-membrane protein P5 (OmpP5), Haemophilus protein D (Hpd) and Haemophilus adhesive protein (Hap). Hmw1 (125 kDa) and Hmw2 (120 kDa) are surface-exposed proteins with 80% similarity, and have been shown to mediate attachment to Chang epithelial cells (St Gme et al., 1993). PilA, the main subunit of type IV pilis, is involved in adherence and biofilm formation (Jurcisek et al., 2007). OmpP5 is a fimbrial protein that mediates bacterial binding to nasopharyngeal mucin (Reddy et al., 1996), intercellular adhesion molecule 1 (ICAM-1) (Avadhanula et al., 2006) and carciinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) (Bookwalter et al., 2008). Hpd is an outer-membrane lipoprotein with adhesive capacity and ciliotoxicity effects (Ahren et al., 2001). Hap is an autotransporter protein that mediates adherence and microcolony formation (Hendrixson & St Gme, 1998).

NTHi is very adept at using its adhesins to adhere and colonize the human nasopharynx. In addition, it has the ability to evade host immune responses and cause invasive disease. Targeting outer-membrane proteins as potential vaccine candidates could potentially reduce carriage and disease. While research has elucidated many different mechanisms involved in adherence, few studies have characterized clinical isolates for the presence of multiple adhesins. In this study, we evaluated the phenotype of NTHi clinical isolates for their adherence capacity (AC) to Detroit 562 nasopharyngeal carcinoma cells (D562) to determine the primary adherence factor(s) that are clinically important. Detroit 562 cells have been extensively used as an in vitro model to study the adherence of other respiratory pathogens (Kimaro Mlacha et al., 2013; Romero-Steiner et al., 2003) and could potentially offer a good model for screening surveillance isolates in terms of AC.

METHODS

**Bacterial isolates.** One hundred invasive and non-invasive clinical isolates, including 11 from Universidad Nacional Autonoma de Mexico (UNAM), Mexico (1990–2006), and 89 from the ABCs programme, United States (2005–2008), were used in this study. Of these, 50 were randomly chosen from the ABCs group. The isolates were evaluated for their adherence to Detroit 562 nasopharyngeal carcinoma cells (D562). Of the total 100 isolates, 5 were from nasopharynx, 11 from ear, 77 from blood, 3 from pleural fluid, 1 from submental aspirate, 1 from CSF and 2 were of unknown source. We confirmed the identity of the isolates using conventional microbiological procedures: Haemophilus ID Quad plates (Remel), slide agglutination with H. influenzae serotype-specific rabbit antisera (Becton Dickinson) and RapidNH (bioMérieux). Eleven out of 100 isolates (UNAM) were also confirmed for the absence of the bexA gene.

NTHi isolates were grown on chocolate II agar (CAP) supplemented with haemoglobin and IsoVitalex (Becton Dickinson) and incubated overnight at 37 °C with 5% CO₂. Stock cultures were prepared by harvesting the bacteria and transferring them into brain heart infusion broth (Becton Dickinson) supplemented with 2% Fildef enrichment (FE; Becton Dickinson), and incubating until an OD₆₀₀ of 0.4 was reached. Aliquots of bacterial stocks were stored at −70 °C in 15% (v/v) glycerol.

**Study design.** All isolates were evaluated for their AC to D562 cells. The target-gene screening was conducted in two stages. In the first stage, 50 isolates that were not randomized (11 from UNAM and 39 from the ABCs programme) were screened for all 6 gene targets (pila, hap, hmw1A, hmw2A, hpd and ompP5). In the second stage, 50 randomly selected isolates from the ABCs system were tested for hmw1A, hmw2A, hpd and ompP5 based on the preliminary statistical analysis of the previous non-randomized isolates. These 50 isolates represent approximately 8% of the total collection available from the ABCs 2007–2008 programme (http://www.cdc.gov/abcs). The study design is summarized in Fig. S1 (available in JMM Online).

**Adherence assay.** The adherence assay was based on the method described for *Streptococcus pneumoniae* by Romero-Steiner et al. (2003) with modifications. D562 cells (American Type Culture Collection, Manassas, VA, USA) were seeded in 96-well microtitre plates (Costar) and incubated at 37 °C with 5% CO₂ until there was confluent growth (approx. 6–7 days, 1.2 × 10⁵ cells per well). The monolayers were washed with 125 µl per well of Eagle’s minimum essential media (MEM; Gibco-Invitrogen) supplemented with 7% fetal bovine serum and without antibiotics. Next, 80 µl per well of fresh MEM media were added, followed by 20 µl per well of standardized minimal infective doses (m.o.i.) of 10⁶, 10⁷ or 10⁸ c.f.u. per well. Bacterial frozen stocks were thawed and gently diluted in Hanks’ balanced salt solution with Ca²⁺ and Mg²⁺ buffer (Life Technologies) supplemented with 2% FE to the corresponding m.o.i. Each m.o.i. was tested in 10–20 replicate wells. After 2 h incubation at 37 °C with 5% CO₂, the monolayers were washed five times with 120 µl per well of 10 mM PBS (pH 7.2; Gibco-Invitrogen) supplemented with 0.2% BSA (Sigma-Aldrich) and overlaid with 100 µl per well of BHI containing 1% agar supplement with 2% FE enrichment. Microtitre plates were incubated at 37 °C with 5% CO₂ for 18 h and the numbers of c.f.u. were enumerated using an automated colony counter (AlphaInnotech; Alpha Innotech) retro-fitted with an ELISPOT adaptor. Adherence was calculated by dividing the number of c.f.u. per well by the standardized bacterial inoculum and multiplying by 100 to normalize results for ease of comparison. Any m.o.i. yielding <10 colonies was considered too low to count. Any m.o.i. yielding >150 colonies was considered too numerous to reliably count.

**PCR.** DNA lysates were prepared by picking a few colonies of bacteria grown overnight on CAP and making a heavy suspension in 1.0 ml of 10 mM Tris-Cl at pH 8. The lysates were boiled for 10 min at 99 °C and stored at −20 °C. Conventional PCR was performed to detect ompP5, pilA, hap, hmw1A and hmw2A, while in-house real-time PCR was performed to detect hpd. Primers for conventional PCR were designed based on the alignment of all available sequences published in GenBank for the aforementioned genes, with the exception of ompP5, which had a primer designed according to a research article published by Duim et al. (1997). MEGALIGN (DNASTAR Lasergene 8) software was used to design the primers based on conserved regions. The designed primers shown in Table S1 were synthesized at the Centers for Disease Control and Prevention Biotechnology Core Facility (Atlanta, United States). PCR amplification reactions consisted of 50 µl reaction volumes containing: 5 µl template DNA; 55.5 µl dH₂O; 5 µl 10× buffer; 1.75 µl 10 mM dNTPs; 1.0 µl each of 20 µM working stock primers; and 0.75 µl Taq polymerase enzyme (Roche). PCR conditions were optimized for each gene target as follows: denaturation at 94 °C for 5 min; 35 cycles of annealing and extension, which varied for the primers [ompP5 (45 °C; 30 s; 72 °C, 1.5 min), hmw1A and hmw2A];
Lack of Hmw1/Hmw2 results in decreased NTHi adherence

(50°C, 30 s; 72°C, 1 min), hap and pilA (50°C, 30 s; 72°C, 1.5 min); extension at 72°C for 5 min; and a 4°C hold. PCR products were analysed using agarose gels containing SYBR-Green (Invitrogen). Several PCR amplicons were selected for further sequencing using a BigDye terminator kit (Applied Biosystems) and a 3730 DNA analyser. The resulting sequences were analysed using BLAST (http://blast.ncbi.nlm.nih.gov) to confirm gene identity.

Real-time PCR for hpd detection was performed as described previously (Wang et al., 2011). Each PCR included 2 µl template DNA, 4.5 µl dH2O, 12.5 µl master mix (Applied Biosystems), 2 µl each primer and 2 µl probe. The cut-off cycle threshold value <35 was considered positive.

Hmw1 and Hmw2 expression. Dot immunoblots were used to screen isolates for expression based on the technique described by Tondella et al. (2000). Western blotting (WB) was performed to resolve isolates that had inconsistent results or were weak in reactivity by dot immunoblot. Growth from an overnight culture was harvested in 5 ml PBS and 0.2% sodium azide. The bacteria were suspended to obtain an OD600 of 1.0, before being heat treated at 70°C for 1 h, group definition equal to 4, and number of resamplings for bootstrapping equal to 1000 (Table S2).

A total of 20 isolates (15 with 3D6 and AD6, and 5 with 10C5) were chosen for MLST analysis to represent the range of bacterial stocks were made by adding 2.5 µl appropriate stock, 7.5 µl PBS and 10 µl 10% SDS. Only 10 µl working stock lysate was subjected to SDS-PAGE using 7.5% polyacrylamide gels. Proteins were transferred to a PVDF membrane (Bio-Rad). All incubations, dilutions and washes were performed using 2% casein. Primary incubation of primary antibody incubation, blots were washed four times with PBS and incubated for 2 h with goat anti-mouse Ig-horseradish peroxidase conjugate (Sigma-Aldrich). The blots were subjected to a final 10 min incubation. All reactions with visible dots (compared to positive-control dots) were scored as positive.

WB. A total of 20 isolates (15 with 3D6 and AD6, and 5 with 10C5) were confirmed by WB according to the methodology described by Crook et al. (1998), with a few modifications. Briefly, crude extracts were prepared from culture plates incubated overnight. Working stocks were made by adding 2.5 µl appropriate stock, 7.5 µl PBS and 10 µl 10% SDS. Only 10 µl working stock lysate was subjected to SDS-PAGE using 7.5% polyacrylamide gels. Proteins were transferred to a PVDF membrane (Bio-Rad). All incubations, dilutions and washes were performed using 2% casein. Primary incubation of mAbs 3D6 and AD6 (1:25 dilution) and mAb 10C5 (1:2 dilution) was performed for 1 h. The goat anti-mouse horseradish peroxidase conjugate detection antibody (Bio-Rad) was used at a dilution of 1:1000. Hmw1 and Hmw2 can vary in size; therefore, the presence of a band(s) between 116 and 200 kDa was scored as positive. The sequencing data were analysed using the web-based software MGIP (http://mgip.biology.gatech.edu/home.php) to obtain allelic profiles. This information was then entered into the H. influenzae database (http://haemophilus.mlst.net/) to obtain the sequence type (ST). eBURST was used to assess phylogenetic relationships (http://eburst.mlst.net; version 3.8). The parameters were kept at the following default conditions: number of loci per isolate set at 7, group definition equal to 4, and number of resamplings for bootstrapping equal to 1000 (Table S2).

Statistical analysis. The dichotomous nature of the statistical analysis software program required data to be separated into two categories. The cut-off was set based on the spread of all data observed and after the number of c.f.u. per well was normalized according to the corresponding m.o.i. As a result, we set an arbitrary cut-off point of <10% to differentiate low from high adherence. Analysis of categorical data was then used to compare numbers of isolates with high or low adherence with the presence or absence of each gene target. This test was performed using Mantel–Haenszel or Fisher’s exact test in 2 × 2 tables (Mantel & Haenszel, 1959). A second clustering analysis was used to analyse interactions between multiple genes. The data gathered from this test did not provide any additional information and therefore were not included.

RESULTS

Adherence

A total of 100 isolates were tested for their AC (Table 1). AC was categorized as low (<10%) or high (≥10%) depending on the normalized percentage of c.f.u. adhering per well. All isolates adhered to D562 cells in a dose–response manner. Using the 10% arbitrary cut-off, 69/100 (69%) had high adherence, while 31/100 (31%) had low adherence (Table 2).

Genotype and adherence

Initially, pilA was detected in 49/50 (98%) of isolates and hap in 48/50 (96%) of isolates. hpd was detected in 49/50 (98%), hmw1A/2A in 33/50 (66%) and ompP5 in 26/50 (52%) of isolates. We also determined whether the presence of a given gene was correlated with high adherence. Of the genes evaluated by parametric analysis (n=50), hmw1A and/or hmw2A were significantly associated with high adherence (P<0.0001). To confirm whether these initial observations applied to randomly selected isolates from the US ABCs data, an additional set of 50 isolates was amplified for hmw1A, hmw2A, hap and

<p>| Table 1. AC of NTHi isolates based on a normalized standard inoculum of 100 c.f.u per well |
|-----------------------------------|-----------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Adherence range (%)</th>
<th>No. of isolates (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–4.9</td>
<td>24</td>
</tr>
<tr>
<td>5–9.9</td>
<td>7</td>
</tr>
<tr>
<td>10–14.9</td>
<td>20</td>
</tr>
<tr>
<td>15–19.9</td>
<td>13</td>
</tr>
<tr>
<td>20–24.9</td>
<td>10</td>
</tr>
<tr>
<td>25–29.9</td>
<td>7</td>
</tr>
<tr>
<td>30–34.9</td>
<td>3</td>
</tr>
<tr>
<td>35–39.9</td>
<td>7</td>
</tr>
<tr>
<td>40–44.9</td>
<td>2</td>
</tr>
<tr>
<td>45–49.9</td>
<td>2</td>
</tr>
<tr>
<td>50–54.9</td>
<td>2</td>
</tr>
<tr>
<td>55–59.9</td>
<td>0</td>
</tr>
<tr>
<td>60–65.2</td>
<td>3</td>
</tr>
</tbody>
</table>

http://jmm.sgmjournals.org
ompP5 genes. pilA and hap were excluded from the analysis because they were found in the majority of the initial 51 isolates. All 100 isolates were evaluated for the following targets: 69/100 (69%) were hmw1A and/or hmw2A positive, 97/100 (97%) were hpd positive, 47/100 (47%) were ompP5 positive. A similar categorical analysis involving the larger isolate collection (n=100) demonstrated a positive association between hmw1A and hmw2A and adherence (P<0.0001). Using a larger sample size, hpd was also positively associated with adherence (P=0.03); however, the association was not as strong as with hmw1A and hmw2A. Therefore, additional expression analyses (i.e. dot immunoblotting and WB) were limited to hmw1A and hmw2A genes.

Among the high adherence isolates, 66/69 (96%) were PCR positive for hmw1A and/or hmw2A. There were a few exceptions. Among the high adherence isolates, 3/69 (4%) were PCR negative for both hmw1A and hmw2A; among the low adherence isolates, 3/31 (10%) were positive for hmw1A and/or hmw2A by PCR.

PCRs for hmw1A and hmw2A were performed independently. However, due to the high homology between hmw1A and hmw2A, we were unable to discriminate these genes by sequencing of PCR amplicons. As a result, the analysis was reported as aggregate data.

When tested with all 100 isolates, 3 isolates tested negative for hpd and 28 isolates that tested positive for hpd were characterized to have low adherence. While there was a significant association with this target, the association with hpd was not as strong as for hmw1A and hmw2A genes. There was no significant association found for ompP5.

**Analysis by source**

There was a significant difference between the adherence of invasive and non-invasive isolates (P=0.005 by Mantel–Haenszel test) with invasive isolates being more adherent than non-invasive isolates. In addition, a higher proportion of invasive isolates were hmw1A and/or hmw2A positive compared to non-invasive isolates (P=0.003). It is worth noting that all five nasopharyngeal isolates had low adherence to D562 cells, and were negative for hmw1A and hmw2A by PCR.

**Genotype and expression**

Dot immunoblots were performed to assess expression, and to determine whether genotype was correlated with expression of Hmw1 and Hmw2. All isolates, regardless of their PCR results, were tested by dot immunoblots using mAbs. When the dot immunoblot results from mAbs 3D6 and AD6 were compared, there were nine discrepant reactions where 3D6 was positive and AD6 was negative. Since 3D6 was more sensitive than AD6 and they both detect the same protein, we chose to represent analysis based on the results obtained with 3D6. Among the 31 isolates that were hmw1A and hmw2A negative by PCR, only 2/31 (6%) reacted with mAb 3D6 and none reacted with mAb 10C5. Of the hmw1A and/or hmw2A positive isolates, 51/69 (74%) reacted with 3D6 and 15/69 (22%) with 10C5. Isolates with inconsistent results compared to PCR or adherence (n=6) or with weak reactivity by dot immunoblot (n=14) were confirmed by WB. Fig. 1 is an example of the reactivity of selected NTHi isolates generating bands of varying sizes in the desired molecular mass range on a Western blot. Some bands stained more intensely than others, indicating differences in expression levels. Differences in staining intensity were also observed in dot immunoblots where a standardized inoculum was used. Only 1 sample out of 20 sent for WB confirmation had discordant results with the dot immunoblot.

**Adherence and expression**

Adherence and expression data were compared. Of the isolates with high adherence, 50/69 (72%) reacted with

**Table 2. Distribution of positive gene target amplifications and AC among NTHi clinical isolates**

<table>
<thead>
<tr>
<th>Source</th>
<th>PCR reactivity for each gene*</th>
<th>High adherence (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hmw1A/2A (n=100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pilA (n=50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ompP5 (n=50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hap (n=50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hpd (n=50)</td>
<td></td>
</tr>
<tr>
<td>Nasopharyngeal</td>
<td>0/5 (0%)</td>
<td>0/5 (0%)</td>
</tr>
<tr>
<td>Ear</td>
<td>6/11 (55%)</td>
<td>6/11 (55%)</td>
</tr>
<tr>
<td>Blood</td>
<td>56/77 (73%)</td>
<td>57/77 (74%)</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>3/3 (100%)</td>
<td>2/3 (67%)</td>
</tr>
<tr>
<td>Submental aspirate</td>
<td>1/1 (100%)</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>CSF</td>
<td>1/1 (100%)</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2/2 (100%)</td>
<td>2/2 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>69/100 (69%)</td>
<td>69/100 (69%)</td>
</tr>
</tbody>
</table>

*The number of samples tested for each gene target is indicated.

ND, Not done.
and none reacted with mAb 3D6. Low adherence was with low adherence, 3/31 (10 %) reacted with mAb 3D6 and 15/69 (22 %) with mAb 10C5 (Fig. 2). Of the isolates previously reported with this mAb (Barenkamp, 1996). It is worth mentioning that the proteins were not of uniform size.

(a) Reactivity with mAb AD6 for whole-cell lysates of NTHi isolates. Lanes were loaded as follows: 1, protein ladder marker; 2, M07393 positive control (high adherence, positive by PCR, positive for expression by dot immunoblot); 3, M20617 negative control (low adherence, negative by PCR, negative expression); 4, M08922 (high adherence, negative by PCR, positive expression); 5, M09107 (low adherence, positive by PCR, positive expression); 6, M09762 (high adherence, negative by PCR, positive expression); 7, M09822 (high adherence, positive by PCR, weak dot immunoblot); 8, M10762 (high adherence, positive by PCR, weak dot immunoblot); 9, M11299 (low adherence, negative by PCR, positive expression); 10, M15900 (high adherence, positive by PCR, weak dot immunoblot).

(b) Reactivity with mAb 3D6. Lanes were loaded as follows: 1, protein ladder marker; 2, M07393 positive control (high adherence, positive by PCR, positive for expression by dot immunoblot); 3, M20617 negative control (low adherence, negative by PCR, negative expression); 4, M08922 (high adherence, positive by PCR, positive expression); 5, M09107 (low adherence, negative by PCR, negative expression); 6, M09762 (high adherence, negative by PCR, positive expression); 7, M09822 (high adherence, positive by PCR, weak dot immunoblot); 8, M10762 (high adherence, positive by PCR, weak dot immunoblot); 9, M11299 (low adherence, negative by PCR, weak dot immunoblot). 10, M15900 (high adherence, positive by PCR, weak dot immunoblot).

MLST

Isolates were chosen for MLST analysis. A total of 27 isolates were tested resulting in 19 different STs. All STs from the 27 isolates were analysed in eBURST to assess phylogenetic relationships. A group was defined as having multilocus genotypes sharing four loci in common. The analysis yielded four groups: group 1, ST-139, ST-103 and ST-423; group 2, ST-107 and ST-155; group 3, ST-349; and group 4, ST-105 and ST-149. The most common type was ST-107 found among 4/27 isolates (Table S2). Each of the 7 different loci had 10–13 different alleles, indicating that there was high DNA sequence heterogeneity.

Fig. 1. Confirmation of questionable immunoblot results by WB with mAbs. mAbs AD6 and 3D6 recognize both Hmw1 and Hmw2 epitopes. (a) Reactivity with mAb AD6 for whole-cell lysates of NTHi isolates. Lanes were loaded as follows: 1, protein ladder marker; 2, M07393 positive control (high adherence, positive by PCR, positive for expression by dot immunoblot); 3, M20617 negative control (low adherence, negative by PCR, negative expression); 4, M08922 (high adherence, negative by PCR, positive expression); 5, M09107 (low adherence, positive by PCR, positive expression); 6, M09762 (high adherence, negative by PCR, positive expression); 7, M09822 (high adherence, positive by PCR, weak dot immunoblot); 8, M10762 (high adherence, positive by PCR, weak dot immunoblot); 9, M11299 (low adherence, negative by PCR, positive expression); 10, M15900 (high adherence, positive by PCR, weak dot immunoblot). (b) Reactivity with mAb 3D6. Lanes were loaded as follows: 1, protein ladder marker; 2, M07393 positive control (high adherence, positive by PCR, positive for expression by dot immunoblot); 3, M20617 negative control (low adherence, negative by PCR, negative expression); 4, M08922 (high adherence, positive by PCR, positive expression); 5, M09107 (low adherence, negative by PCR, negative expression); 6, M09762 (high adherence, negative by PCR, positive expression); 7, M09822 (high adherence, positive by PCR, weak dot immunoblot); 8, M10762 (high adherence, positive by PCR, weak dot immunoblot); 9, M11299 (low adherence, negative by PCR, weak dot immunoblot). 10, M15900 (high adherence, positive by PCR, weak dot immunoblot).

DISCUSSION

The incredible fluidity of NTHi indicates that this pathogen has the capacity to vary its surface-exposed adhesins as a response to the host. Identifying which of these adhesins is essential depends on the biological system under evaluation. NTHi adherence has been evaluated in both immortalized and primary cell cultures (Janson et al., 1999; Jurcisek et al., 2007; St Geme et al., 1993), as well as in vivo systems like the chinchilla otitis media model (Johnson et al., 2011). The adherence of NTHi may vary depending of the type of target cell used, the target organ tissue and/or the assay conditions. Efforts to identify the key components of adherence and initiation of a focal point of infection are worthwhile, and contribute to the understanding of the biology of this bacterium and its interaction with the host. Previous reports have included a limited number of adhesins (Ecevit et al., 2004; Rodriguez et al., 2003; St Geme et al., 1998). To our knowledge, this is the first report of a large number of invasive isolates from a nationwide population-based surveillance programme (recently in circulation) for the evaluation of genes encoding adhesins.

In this study, we have demonstrated that NTHi have various adherence capacities to D562 nasopharyngeal cells, and that the absence of Hmw1 and/or Hmw2 is associated with decreased adherence to this cell line. Hmw1 and Hmw2 have been shown to function as adhesins, and to be highly immunogenic during NTHi infection (Winter & Barenkamp, 2006). St Geme et al. (1998) had shown that hmw1A and/or hmw2A genes are present in 80 % of NTHi isolates. The presence or absence of the surface proteins encoded by these genes can be a result of variable expression of Hmw1 and Hmw2 as demonstrated by Dawid et al. (1999). In addition, the quantity of proteins can also be regulated through a transcriptional regulatory mechanism involving 7 bp repeats present in the promoter region of hmw1A (Giufre et al., 2008). Our data indicate that this number is slightly lower (69 %), perhaps due to

3D6 and 15/69 (22 %) with 10C5 (Fig. 2). Of the isolates with low adherence, 3/31 (10 %) reacted with mAb 3D6 and none reacted with 10C5. Low adherence was significantly associated with the absence of expression of Hmw1 and/or Hmw2 (P<0.001). However, 18/69 (26 %) isolates with high adherence lacked Hmw1 and Hmw2.
our larger sampling population, differences in isolation dates or the age of the populations the isolates were from. Two isolates had PCR negative reactions, yet were positive by dot immunoblot for protein expression. We suspect that there were minor nucleotide substitutions or indels occurring that may have prevented the primers from annealing to the selected target. It is worth mentioning that the isolates were highly diverse (not clonal) as shown by the 27/100 isolates with different MLST profiles.

We tested a large number of isolates for the expression of Hmw1 and Hmw2 using mAbs. To increase our throughput, we utilized dot immunoblots as a screening method for expression of Hmw1 and Hmw2. We have demonstrated that the dot immunoblot is a simple, rapid and reliable method for screening large numbers of isolates for expression of Hmw1 and Hmw2. Isolates with inconsistent results, compared to adherence or PCR data, or weak reactivity to mAbs 3D6 and 10C5 by dot immunoblot, were confirmed by WB. Out of the 20 confirmed isolates, only 1 produced discrepant results between the dot immunoblotting and WB, indicating good agreement between the two assays. mAb 3D6 was shown to be more sensitive in detecting Hmw1 and Hmw2 than mAb AD6 by dot immunoblot. Dot immunoblots and Western blots with mAb AD6 had higher disagreement than with mAb 3D6. Therefore the results with mAb 3D6 were selected for analysis.

Our WB data show proof of phenotypic diversity. Protein expression varied among the isolates tested. There were various band sizes and intensities observed when reacted with the mAbs. This heterogeneity in the size of the banding pattern has also been observed in another study (Barenkamp & St Geme, 1996). The presence of multiple reacting protein bands indicates antigenic heterogeneity. We speculate that truncated protein products could be a possible explanation as to why ~25% of the high adherence isolates that were PCR positive were negative for expression. The various intensities in the banding patterns observed by WB, and also by dot immunoblots, have been experimentally shown to be attributed to the number of repeats in the promoter region (Dawid et al., 1999; Giufre` et al., 2008). We also speculate that these strains are able to upregulate and downregulate expression of Hmw1 and Hmw2 to avoid the immune response and survive in different environments (Dawid et al., 1999; Giufre` et al., 2008).

Although our study sampled more invasive isolates, it appears that invasive isolates tend to be more adherent than non-invasive isolates. Based on the comparison, non-invasive isolates (i.e. nasopharyngeal isolates, n=5) lacked the hmw1A and hmw2A genes compared to invasive isolates (P=0.005) leading to a lower AC to nasopharyngeal cells. While this observation suggests that Hmw1 and Hmw2 may have an important role in invasive disease, our study did not look into whether the strains actually invaded the D562 cells. It is interesting to note that we were unable to detect hmw genes in all isolates from nasopharyngeal sources (n=5). It is possible that these isolates have mutations that prevented the primers from annealing to the target region. Lack of these genes may also indicate that nasopharyngeal isolates utilize other adhesins and/or pathogenic mechanisms to cause disease in vivo (Jurcisek et al., 2007; Johnson et al., 2011). Additional research involving a larger number of nasopharyngeal isolates is needed to further understand this observation.

In our in vitro model, all NTHi isolates evaluated had the capacity to adhere in a dose–response manner. The
increased adherence of some NTHi isolates was correlated with expression of Hmw1 and Hmw2. However, this was not true for all NTHi isolates. There were ~25% with high adherence that did not express the Hmw proteins under our assay conditions. This highlights the role of other adhesins, such as Protein D, OMP P5 and Hia, as has been documented in other in vitro and in vivo systems (Johnson et al., 2011; Duim et al., 1997; Cardines et al., 2007), in the attachment of NTHi. The contribution of Hpd as an adhesin to D562 can be noted by the fact that three of our isolates lacked hpd and also demonstrated lower adherence to D562 cells. The presence of hpd was also positively associated with adherence to D562. This finding is in agreement with a report by Johnson et al. (2011) of a mutant lacking Protein D (Hpd/GlpQ⁻), which exhibited reduced adherence to respiratory epithelial cells (A549 and normal bronchial epithelial cells).

Regarding the presence of ompP5, a total of 13/100 isolates were positive for ompP5 but negative for hmw1/2 (data not shown). All of these isolates had low adherence with the exception of two. Although the association between ompP5 and adherence was not significant, this finding demonstrates that the presence of ompP5 contributes to adherence in this model. The overall positivity rate for ompP5 was lower than previously reported, even though we used the same primer sequences as Duim et al. (1997). These investigators reported that 1/5 strains (20%) from clinical isolates was found to be negative for the expression of Omp P5; however, all strains were positive for the gene. These investigators demonstrated transcriptional regulation for ompP5 with an identified ribosome-binding site sequence (AGGA). Additional characterization (whole genome sequencing) of the isolates is needed to confirm the presence or absence of ompP5.

Although we only evaluated the expression Hmw1/2, there is no question that the presence of other adhesins contributes to the adherence of NTHi in our model. Additional research is needed to elucidate the regulatory mechanisms involved in the expression of these and other adhesins in the adherence to D562 cells.

There were several limitations to our study. Our experimental design was limited by the fact that we conducted studies in vitro with immortalized nasopharyngeal cells that do not express cilia. It is worth mentioning that the use of an immortalized cell line offers advantages for the screening in the laboratory of high numbers of isolates as is often needed during surveillance studies. Our study design tested for adherence only and invasion was not evaluated. Only 11/100 isolates were characterized for the presence of the cap locus (bexA; data not shown). We were unable to discriminate between hmw1A and hmw2A despite the design of separate primers for each gene target and the performance of independent PCRs. Therefore, all PCR data regarding these two genes were analysed as aggregate data. We did not evaluate the presence of Hia, as we only had one confirmed meningitis isolate from CSF and this particular adhesin had been reported by Cardines et al. (2007) in nine NTHi isolates causing meningitis.

In summary, we evaluated the distribution of six genes encoding adhesins in clinical isolates. We have shown that all NTHi isolates adhered to Detroit 562 nasopharyngeal carcinoma cells, although various adherence capacities were demonstrated. Moreover, we also demonstrated that the absence of Hmw1 and/or Hmw2 correlated with decreased adherence. This study contributes to the understanding of NTHi pathogenesis and the presence of biomarkers that may be used for future strain characterization and/or surveillance.

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