Intercellular passage of epithelial cell layers: a pathogenic mechanism for Haemophilus Influenzae infections
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CHARACTERIZATION OF ADHERENCE OF NONTYPEABLE *HAEMOPHILUS INFLUENZAE* TO HUMAN EPITHELIAL CELLS
Muriel van Schilgaarde, Peter van Ulsen, Paul Eijk, Michiel Brand, Martin Stam, Jamal Kouame, Loek van Alphen and Jacob Dankert.

Submitted for publication.
**H. influenzae Adherence**

**Abstract**

The adherence of 58 nontypeable *H. influenzae* isolates obtained from otitis media, from the lower respiratory tract of patients with chronic obstructive pulmonary disease (COPD), and from the throat of healthy individuals was compared. In total, 32 isolates adhered to either Chang epithelial cells or NCI-H292 lung epithelial cells. Otitis media isolates, but not the isolates obtained from COPD patients, adhered significantly more to Chang cells (p<0.01) and NCI-H292 cells (p<0.05) than throat isolates. Since high molecular weight (HMW) proteins are considered major adhesins involved in the adherence of nontypeable *H. influenzae*, the correlation between adherence and HMW expression was determined. PCR with *hmw* specific primers and immunoblotting with 2 polyclonal sera showed that 23 (72%) of the 32 adhering isolates were *hmw* or HMW positive and only one of the 26 non-adherent strains. Based on the inhibiting effect of dextran sulfate, 5 different adherence patterns to the 2 cell lines were distinguished among the 32 adherent isolates. Using *H. influenzae* strain 12 expressing two well defined HMW proteins (HMW1 and HMW2) and its isogenic mutants as a reference we observed HMW1-like adherence to both cell lines for 16 of the 32 adherent isolates. Four others showed HMW2-like adherence to NCI-H292. Of the 3 other patterns of adherence one probably also involved HMW proteins. Screening of the isolates with 6 HMW-specific monoclonal antibodies in whole cell ELISA showed that the HMW proteins of COPD isolates and carrier isolates were more distinct from the HMW proteins from strain 12 than those from otitis media isolates. Characterization of the HMW protein of a COPD isolate by adherence and DNA sequence analysis showed that despite large sequence diversity in the *hmwA*, probably resulting in the antigenic differences, the HMW protein mediated the HMW2-type of adherence of this strain.

**Introduction**

The gram-negative bacterium *H. influenzae* is a commensal of the human upper respiratory tract. Encapsulated strains, in particular those with a serotype b polysaccharide, are important pathogens causing systemic disease such as meningitis, epiglottitis, cellulitis, arthritis, sepsis and pneumonia. Nonencapsulated (nontypeable) *H. influenzae* is a frequent cause of respiratory tract infections including otitis media, sinusitis, and lower respiratory tract infections in patients with chronic obstructive pulmonary disease (COPD) (van Alphen, 1992; Moxon and Wilson, 1991; Turk, 1984).

Adherence of *H. influenzae* to the respiratory epithelial cells is considered an important step in the colonization of the respiratory mucosa. Several adhesins of *H. influenzae* have been determined each with different adherence specificities (Stephens and Farley, 1991; Wilson et al., 1992; St.Geme and Cutter, 1996). Adherence of *H. influenzae* by a fimbriae-mediated as well as a fimbriae-independent mechanism has been described. Fimbriae-mediated adherence seems to be especially relevant for the
adherence of encapsulated *H. influenzae* isolates to different cells (Farley et al., 1990; Loeb et al., 1988; Sterk et al., 1991; St.Geme and Cutter, 1995), since this type of adherence is not hampered by capsule expression (St.Geme, 1996; St.Geme and Cutter, 1996). Only a minority of nontypeable *H. influenzae* isolates from different sources contained a fimbriae gene cluster (Geluk et al., 1998; Gilsdorf et al., 1992; Krasan et al., 1999). Attachment of nontypeable *H. influenzae* to different cell lines is mediated by various non-fimbrial proteins (Barenkamp and St.Geme, 1996a; St.Geme et al., 1994; St Gmc et al., 1993). The most common of these are two immunogenic high molecular weight (HMW) proteins designated HMW1 and HMW2 (St Gmc et al., 1993; Barenkamp and Leininger, 1992), that are detected in 75-80% of unrelated nontypeable *H. influenzae* strains (Barenkamp and St.Geme, 1996b; St.Geme, 1996; Krasan et al., 1999). Despite significant sequence similarity of the predicted amino acid sequences of HMW1 and HMW2, these proteins mediate binding to distinct human epithelial cells indicating different receptor specificity (St.Geme et al., 1998) (St.Geme, 1996; Hultgren et al., 1993; St.Geme and Grass, 1998). HMW1 recognizes a sialylated glycoprotein and HMW1-mediated adherence is inhibited in the presence of heparin or dextran sulfate (St.Geme, 1994; Noel et al., 1994). The receptor for HMW2 is currently unknown.

We were interested in adherence characteristics of nontypeable *H. influenzae* isolates from carriers compared to those isolated from COPD patients and from otitis media patients. Adherence of 58 nontypeable *H. influenzae* isolates was determined to two human epithelial cell lines, the Chang conjunctiva epithelial cell line and the bronchial epithelial cell line NCI-H292. The association between the presence of HMW and adherence was analyzed by PCR with *hmw*-primers and whole cell ELISA and western blotting with polyclonal sera. Among the *H. influenzae* isolates five different adherence patterns were present, including the patterns for HMW1 and HMW2 mediated adherence. The HMW proteins showed very different reactivity patterns with 6 monoclonal HMW antibodies, irrespective of the adherence patterns of the isolates. The *hmw*-gene of a COPD isolate was cloned and sequenced. It appeared that the HMW2-type of adherence of this isolate was associated with the HMW protein encoded by this gene, and that this protein differed antigenically from the HMW protein of the prototype *H. influenzae* strain 12 due to sequence diversity of the *hmwA* gene.

### Materials and Methods

**Bacterial isolates and plasmids.** In total 58 nonencapsulated (nontypeable) *H. influenzae* isolates were used. Nineteen isolates were isolated from sputum samples of 17 patients with COPD with an age ranging from 30-85 years, 9 from middle ear fluid samples of 9 children with otitis media, and 30 from throat swabs of healthy carriers. Of these throat isolates, 21 isolates were cultured from 20 healthy children visiting the health care center for routine checks, 6 isolates from 2 children (Lomholt et al., 1993), and 3 from 2 students at our department. All isolates were determined as nontypeable based on the absence of agglutination with antisera for *H. influenzae* capsule type a to type f.
COPD isolates that were derived from the same patient have been characterized genotypically as different strains by random amplified polymeric DNA analysis (Moller et al., 1995). Nontypeable H. influenzae strain 12, originally obtained from a child with acute otitis media, is the prototype isolate of which the genes encoding the HMW1 and HMW2 proteins were originally isolated and sequenced (Barenkamp and Leininger, 1992). Strain 12 mutants expressing HMW1 but not HMW2 (strain 12-2), HMW2 but not HMW1 (12-10), or neither HMW1 nor HMW2 (strain 12-4) have been described previously (St.Gem et al., 1993) and were kindly provided by dr. S.J. Barenkamp. Plasmid pT1-17 (St.Gem et al., 1993) contains the hmw1 gene cluster with an insertion of a 1.3 kb kanamycin resistance gene (hmw::kan) and was also provided by dr. S.J. Barenkamp. Plasmid pGJB103 is a shuttle vector that replicates in both H. influenzae and E. coli (Tomb et al., 1989). It was obtained from the recombinant plasmid pEJH39-1 that was provided by dr. E. J. Hansen (Hansen et al., 1988).

**Culture conditions.** All H. influenzae isolates were grown overnight on chocolate agar plates at 37°C in 5% CO₂. E. coli DH5α was grown on LB agar plates at 37°C. H. influenzae transformants expressing the kanamycin marker were grown on chocolate agar plates supplemented with 20 µg/ml kanamycin. Transformants expressing pGJB103 were grown in the presence of tetracycline in the concentration of 5 µg/ml for H. influenzae or 12.5 µg/ml for E. coli DH5α. All isolates were stored at −70°C in broth containing 20% glycerol.

**Adherence assays.** NCI-H292 epithelial cells, derived from a human lung mucoepidermoid carcinoma (ATTC CRL1848) (Banks-Schlegel et al., 1985; van Schilfgaarde et al., 1995) and Chang epithelial cells, originating from human conjunctiva (ATTC CCL20.2) were grown to near confluency or confluency on 12 mm glass coverslips (Menzel-gläser, Germany) in 24-wells tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes NJ., USA) in 1 ml RPMI (Gibco, Life Technologies, Breda, The Netherlands) + 10% fetal calf serum (FCS). Bacterial isolates were cultured overnight on chocolate agar plates and suspended in Dulbecco’s phosphate buffered saline (DPBS, Gibco) to an OD₆₀₀ of 1.0 (equivalent to 10⁸ CFU/ml). Epithelial cells were incubated with 50 µl bacterial suspension added to 450 µl fresh RPMI, supplemented with 25 mM HEPES buffer (Gibco) and 10% FCS for 6 h. Inhibition of HMW1-mediated adherence was performed by addition of dextran sulfate to a final concentration of 0.1 mg/ml. The end volume of each well was kept constant at 500 µl. After incubation the unbound bacteria were removed from the cells by washing three times with DPBS. Cells were fixed to the coverslips by addition of 1 ml fixative (1% glutaraldehyde, 4% paraformaldehyde; Merck, Darmstadt, Germany), and stained with 0.007% crystal violet solution. Bacterial adherence was determined by counting the number of bacteria per cell on ten cells of at least three experiments performed in duplicate, as described before (van Schilfgaarde et al., 1995).

**Detection of HMW proteins in whole cell ELISA.** Expression of HMW proteins was determined by whole cell ELISA as described before (van Alphen et al., 1991), using
3D6, 1D5, 2G3, 4G4, AD6 and 10C5 mouse IgG monoclonal antibodies (Mabs) in a 1:250 dilution, or the polyclonal antiserum 25D/E.coli-absorbed against the recombinant HMW1 protein (Barenkamp and Leininger, 1992) in a 1:50 dilution. The Mabs and antiserum were provided by dr. S.J. Barenkamp. Rabbit anti-mouse horse peroxidase (HRP) was used as a conjugate. The reactivity of the antibodies was determined by measuring the optical density using an ELISA reader at 405 nm. The reactivity was expressed as $-$ = OD < 0.5, + = OD 0.5-1.0, ++ = OD 1.0-1.5, +++ = OD >1.5. All isolates were tested two times.

**Detection of HMW by Western blot.** Whole-cell lysates were prepared from bacteria grown overnight on chocolate agar plates. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters followed by western blot analysis. Rabbit polyclonal serum 25D/E.coli-absorbed was used in a 1:50 dilution, and rabbit polyclonal serum K1-050, raised against nontypeable *H. influenzae* strain 1482, in a 1:75 dilution.

**Detection of hmw genes by polymerase chain reaction.** On the basis of the sequence analysis of the *hmw* gene clusters (Barenkamp and Leininger, 1992) two primers were designed, recognizing sequences in the part of the *hmwA* gene that is identical in both *hmw1* and *hmw2*. The first primer HMWP1HI (5'-CGTGCAGGAGGATGACGACG-3') recognizes nucleotide 288-304 and the second primer HMWP3RHI (5'-GGCCCAACTACAAATAGCGACG-3') recognizes nucleotide 1515-1532. This primer combination gives rise to an expected PCR product of 1.24 kb. Non-adhering isolates were screened by PCR with this combination one time, and most adhering isolates at least two times. PCR was performed on chromosomal DNA isolated by the phenol-chloroform method (Langenberg et al., 1986), or with bacterial lysates obtained by heating a *H. influenzae* colony suspended in 1 ml H2O for 5 min at 100°C. PCR was performed in a TRIO-thermoblock (Biometra), with an initial denaturation step of 5 min at 95°C followed by 30 cycles of 1 min 95°C, 1 min 55°C and 3 min 72°C. The program was finished with 10 min at 72°C. The reaction products were determined by agarose gel electrophoresis. Strain 12 was included as a positive control.

**Molecular cloning of the hmw gene.** Chromosomal DNA was isolated by the phenol-chloroform method (Langenberg et al., 1986). Sau3A partial restriction digests of chromosomal DNA were separated by agarose gel electrophoresis, and DNA fragments in the 8- to 12- kb range were used to construct a library by ligation into the unique BglIII-site of pGJB103. The ligation mixture was transformed to *E. coli* DH5α by electroporation. Approximately 1000 clones were obtained and selected for *hmw*-positives clones by two subsequent adherence assays on NCI-H292 cells.

**Construction of hmw-knock out mutants of *H. influenzae* isolates.** Knockout mutants of *H. influenzae* isolates were constructed by insertion of a kanamycin resistance gene into the *hmw* genes by homologous recombination. *H. influenzae* isolates were made competent and transformed according to the method of Herriott et al. (Herriott et al., 1970). Competent cells were used fresh, or stored at -70°C after addition of 15%
H. influenzae Adherence

glycerol. Plasmid pT1-17 was linearized with XbaI, and 0.5 µg was used per transformation.

**DNA sequence analysis.** DNA sequence analysis was performed using the big dye terminator cycle sequencing ready reaction kit (Applied Biosystems) as suggested by the manufacturer. DNA analysis was performed with an automated fluorescent DNA sequencer, model 310 (Applied Biosystems). Data were analyzed with Auto assembler 2.0, ABI Prism, and aligned with known hmw sequences using Clone Manager 4.1. Two primers near the Bgl II site of pGJB103 were designed to sequence the fragment in the pGJB103 clone. (PGJB-P1: CCGCTCATGAGACAATAACCCTGAT; PGJB-P2: GGGAATAAGGGCGACACGGAAATGTT). The sequences of several subclones in pUC19 were determined using the M13 and M13 reversed primers. Several oligonucleotide primers were generated as necessary to complete the sequences. The sequences of the complete hmwA and hmwC genes have been submitted to GenBank, accession numbers AF180944 and AF180945 respectively.

**Statistics.** Data were evaluated with Fisher's exact or the chi-square test with Yate's corrections for sample sizes greater than 50. P-values of <0.05 were considered statistically significant.

**Results**

**Adherence of H. influenzae isolates to two human epithelial cell lines.** The ability of the 30 nontypeable H. influenzae isolates from the throat of healthy individuals, 9 otitis media isolates and 19 COPD isolates to adhere to the Chang and NCI-H292 epithelial cell lines was compared (Table 1). In total, 32 isolates (55%) adhered to either the Chang or NCI-H292 cells. Significantly more otitis media isolates adhered to Chang cells (89%) compared to isolates from healthy individuals (33%) (p<0.01) and isolates from COPD patients (42%) (p<0.05). Also significantly more isolates from patients with otitis media (78%) than from healthy carriers (33%) adhered to NCI-H292 (p<0.05). Although more COPD isolates adhered to the NCI-H292 compared to isolates of healthy individuals, the difference was not significant.

Since the HMW proteins are major adhesins, a PCR assay was designed to determine the presence of the hmwA gene cluster among the isolates, using primers annealing to a conserved part of the hmw1A and hmw2A genes. With chromosomal DNA of strain 12 as template, an expected PCR product of 1.24 kb was amplified. Of the 58 nontypeable H. influenzae isolates, 23 (40%) gave a PCR product of the expected length, being 22 of the 32 adherent isolates and one nonadherent isolate (Table 2). Whole cell ELISA and western blotting with the polyclonal rabbit serum 25D, performed to identify HMW expression, showed the presence of HMW proteins in 21 (36%) isolates. There was a large variation in the molecular weight of the HMW proteins, ranging from 100 kD to 150 kD, confirming data as described before for other isolates (St.Geme et al., 1998; Barenkamp and Leininger, 1992). All the 21 HMW positive isolates were also positive.
with the *hmw*-PCR, except one. Of the in total 23 *hmw*-PCR positive isolates, 3 isolates did not react with the 25D polyclonal serum. Since HMW proteins vary strongly, we reasoned that this negative result may be due to lack of cross reactivity of the antiserum 25D. Therefore, another rabbit serum (K1-050) was used. Western blotting with this serum showed a strong positive reaction with both the HMW1 and the HMW2 protein of the prototype *H. influenzae* strain 12. Also, HMW proteins of 2 of the three 25D-negative isolates were recognized. So, of the 23 *hmw*-PCR positive strains, 22 showed an HMW reaction in western blots (Table 2). In addition, one of the 35 PCR-negative isolates expressed HMW proteins.

**TABLE 1. Adherence of 58 *H. influenzae* isolates from different sources to Chang or NCI-H292 epithelial cells.**

<table>
<thead>
<tr>
<th>Isolates from</th>
<th>Total No.</th>
<th>No. (%) of isolates adherent to</th>
<th>No. (%) non-adherent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chang cells</td>
<td>NCI-H292 cells</td>
<td>Chang and NCI-H292</td>
</tr>
<tr>
<td>Carriers</td>
<td>30</td>
<td>10 (33)</td>
<td>10 (33)</td>
</tr>
<tr>
<td>COPD</td>
<td>19</td>
<td>8 (42)</td>
<td>11 (58)</td>
</tr>
<tr>
<td>total</td>
<td>58</td>
<td>27 (45)</td>
<td>28 (47)</td>
</tr>
</tbody>
</table>

* Compared to carrier isolates p<0.01 and compared to COPD isolates p<0.05
** Compared to carrier isolates p<0.05

Taken these results together, we found that detection of *hmw* with PCR as well as HMW with western blotting gave false negative results, which were probably due to sequence diversity and antigenic diversity, respectively. We therefore considered isolates that were positive in either the PCR or the western blotting with the polyclonal sera as *hmw/HMW* positive. Thus, in total 24 (41%) of the 58 isolates were *hmw/HMW* positive. Of the 32 adhering *H. influenzae* isolates, 23 isolates (72%) were *hmw/HMW* positive. All 26 nonadherent isolates were *hmw/HMW* negative, except one otitis media isolate (Table 2). These data show that there is a strong correlation between *hmw/HMW* and adherence to the two cell lines used. There was a significant difference in the total number of *hmw/HMW* positive otitis media isolates (67%) (p<0.05) and COPD isolates (57%) (p<0.05) compared to throat isolates from healthy individuals (23%) (Table 2).

Using whole cell ELISA with a panel of 6 HMW-specific Mabs to detect HMW expression by these isolates we found only 15 isolates positive with at least one of the Mabs, indicating lack of cross reactivity of the Mabs (Table 2). The 6 *hmw/HMW* positive otitis media isolates were Mab positive, but only 3 (43%) of the 7 *hmw/HMW* positive isolates obtained from healthy carriers, and 6 (55%) of the 11 *hmw/HMW* positive isolates from COPD patients were positive with one of the Mabs (Table 2). These results showed that the HMW proteins expressed by isolates obtained from otitis...
media patients differed less from the prototype HMW proteins than the HMW proteins expressed by isolates from healthy carriers or COPD patients.

**Adherence patterns of the nontypeable* H. influenzae* isolates.** For *H. influenzae* up till now two HMW proteins have been characterized that display distinct cellular binding specificities (St.Geme and Grass, 1998). The adherence patterns of the 3 isogenic mutants of strain 12, expressing HMW1, HMW2, or none of these HMW proteins were determined to establish the specificity of HMW1 and HMW2 mediated adherence on these cell lines. The mutant strain 12-4, deficient in both HMW proteins did not adhere to either cell line, indicating that strain 12 did not express adhesins for these cell lines other than HMW1 and HMW2. The mutant strain 12-2 (HMW1-positive) adhered efficiently to both cell lines. This HMW1-mediated adherence to both cell lines was inhibited in the presence of dextran sulfate, as expected (Hultgren et al., 1993). Using the mutant strain 12-10 (HMW2-positive), less than 5 bacteria per cell bound to Chang cells indicating that HMW2 had low affinity for Chang epithelial cells. This is in agreement with data described before (St.Geme et al., 1998; Hultgren et al., 1993; St.Geme, 1996; St.Geme et al., 1993). However, this strain adhered efficiently to NCI-H292 cells, irrespective of the presence of dextran sulfate.

**TABLE 2.** Association of *hmw/HMW* with adherence of 58 *H. influenzae* isolates from different sources.

<table>
<thead>
<tr>
<th>Isolates from</th>
<th>No. of adherent isolates</th>
<th>No. of non-adherent isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td><em>hmw</em></td>
</tr>
<tr>
<td>Carriers</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Otitis media</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>COPD</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>22</td>
</tr>
</tbody>
</table>

*hmw* was detected by PCR.

Expression of HMW protein(s) recognized by the polyclonal sera in western blot

Expression of HMW protein(s) recognized by at least 1 of the 6 Mabs against HMW in whole cell ELISA

To discriminate between HMW1 and HMW2 types of adherence of the isolates we determined adherence of the 32 adhering isolates to the two cell lines in the presence and absence of dextran sulfate (Table 3). Of the 32 adhering isolates, 16 isolates adhered to both cell lines in the absence of dextran sulfate, while in the presence of dextran sulfate no adherence occurred indicating an HMW1-mediated adherence (Group I, HMW1). Four isolates adhered only to NCI-H292 cells, irrespective of the presence of dextran sulfate, which is indicative for HMW2-mediated adherence (Group II, HMW2). Twelve isolates adhering to Chang epithelial cells showed different adherence patterns to NCI-H292. Four isolates did not adhere to NCI-H292 cells (group III). 4 isolates adhered to
NCI-H292 cells but not in the presence of dextran sulfate (Group IV), and 4 adhered to NCI-H292 in the presence as well as the absence of dextran sulfate (Group V). The distribution of the 32 adhering isolates over the 5 adherence patterns did not seem to be associated with the source of the isolates.

**TABLE 3. Grouping of 32 adherent *H. influenzae* isolates, based on adherence properties.**

<table>
<thead>
<tr>
<th>Adherence² to</th>
<th>Chang cells No. of isolates</th>
<th>Adherence pattern</th>
<th>hmw Mabs</th>
<th>HMW Mabs</th>
<th>HMW Mabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>without dextran sulfate</td>
<td>NCI-H292 cells without dextran sulfate</td>
<td>++ -</td>
<td>+++ -</td>
<td>16²</td>
<td>I (HMW1)³</td>
</tr>
<tr>
<td>- -</td>
<td>+++ -</td>
<td>4</td>
<td>II (HMW2)³</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>++ +</td>
<td>- -</td>
<td>4</td>
<td>III</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>++ +</td>
<td>+++ -</td>
<td>4</td>
<td>IV</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>++ +</td>
<td>++ ++</td>
<td>4</td>
<td>V</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>22</td>
</tr>
</tbody>
</table>

¹Adherence is given as: - less than 5 bacteria per cell, ++ 10-50 bacteria per cell, +++ more than 50 bacteria per cell.

²Two isolates did not adhere to Chang epithelial cells.

³This adherence pattern correlated with HMW1 or HMW2 mediated adherence of strain 12, as indicated.

⁴One isolate was *hmw+*/HMW-, another was *hmw-/HMW+*.

HMW expression was associated with 3 adherence patterns: 14 of the 16 group I (HMW1) isolates (88%), and the 4 isolates of group II (HMW2) were *hmw*/HMW positive as well as the 4 isolates of group IV (Table 3). The differences in adherence pattern of the 4 group IV strains may be explained by expression of an HMW adhesin with another specificity than HMW1 and HMW2, or an HMW protein involved in the HMW1-like adherence pattern to NCI-H292 cells, together with another adhesin. The 4 isolates from group III, and 3 of the 4 isolates within group V were *hmw*/HMW negative. Although the involvement of HMW in adherence of these isolates to Chang or NCI-H292 cells can not be ruled out, adherence to these cells due to another adhesin is more likely.

The HMW proteins of the isolates were further studied using six HMW-specific Mabs in whole cell ELISA. Using the prototype strain 12 and its HMW1 and HMW2 isogenic mutants, it was observed that Mab 3D6 recognized HMW1 and HMW2, Mab 2G3 and Mab 10C5 were specific for HMW1 and Mab AD6 was specific for HMW2. The Mabs 1D5 and 4G4 did not react with the reference strains in whole cell ELISA although they reacted in western blot. Apparently, these Mabs recognized only the denatured proteins. Of the 16 isolates of group I, exhibiting a HMW1 type of adherence, 7 did not react with any Mab. Of the 8 isolates reacting with the HMW1 specific Mabs (2G3 and 10C5), 7 isolates showed HMW1-type of adherence. Of the 10 isolates reacting with the HMW2
specific Mab (AD6), 9 showed HMW1-type adherence and 1 did not adhere at all. None of the 4 isolates of group II correlating with the strain 12 HMW2-mediated adherence pattern showed reactivity with Mab AD6 or with any other Mab (Table 3). Fourteen of the 15 isolates that reacted with at least one Mab reacted also with Mab 3D6, indicating that the epitope recognized by this Mab was indeed a common epitope. The hmw/HMW positive isolate of the 26 non-adherent isolates reacted with Mab AD6 but not with Mab 3D6.

In conclusion, detection of hmw or HMW was strongly related to adherence but we found no correlation between the HMW1 and HMW2 adherence pattern and reactivity with the HMW1 or HMW2 specific Mabs.

**Cloning and sequencing of the hmw gene of a COPD isolate.** The 11 COPD isolates adhering to NCI-H292 cells expressed HMW. However, the HMW proteins of COPD isolates differed antigenically from HMW proteins expressed by strain 12. Only 6 reacted with the HMW specific Mabs. Of the 4 isolates of group II (HMW2) none was recognized by the Mabs in ELISA. Among these 4 isolates, the COPD isolate A950006 was also negative with the 25D polyclonal serum in whole cell ELISA and western blot. A HMW-knock out mutant of this isolate, constructed by kanamycin box insertion in the hmw gene, showed reduced adherence compared to the parent isolate, indicating that the HMW protein was the major adhesin of this isolate (Fig.1).

![Graph showing adherence of different isolates](image)

*Figure 1.* Adherence of strain A950006 and clones to NCI-H292 cells shown as the number of bacteria per cell. White bars, adherence without dextran sulfate. Black bars, adherence in the presence of dextran sulfate.
To characterize the HMW adhesin of this strain A950006, a chromosomal library was made with 8-12 kb Sau3A digested chromosomal fragments of A950006. The fragments were ligated into the BglII site of the plasmid pGJB103, and this was transformed to E. coli DH5α. After selection for clones adhering to NCI-H292 cells, 8 different clones were obtained that adhered to NCI-H292. All these clones were positive for hmw by PCR, indicating that the hmw gene product of A950006 was likely mediating the adherence to NCI-H292 cells. The plasmid of clone 6 containing a 10 kb chromosomal fragment was transformed to the hmw-mutant of strain A950006 resulting in wild type adherence pattern of this clone (Fig.1). Transformation of this plasmid to H. influenzae strain Rd also resulted in adherence of the Rd clone to NCI-H292 cells (data not shown).

![Figure 2](image)

**Figure 2.** Western blot of whole cell lysates of strain A950006 and clones. The lysates were probed with *E. coli* absorbed rabbit serum 25D. Lanes: 1, *E. coli* DH5α containing pGJB103; 2, *E. coli* DH5α clone 6 containing hmw; 3, *H. influenzae* Rdrec1 containing pGJB103; 4, *H. influenzae* Rdrec1 clone 6 containing hmw; 5, strain A950006; 6, A950006 (hmw::kana); 7, strain 12-2 (HMW1+/HMW2-); 8, strain 12-10(HMW1-/HMW2+)

The results of the analysis of the clones with the polyclonal rabbit serum 25D in western blot are summarized in Fig.2. The serum did not react with whole cell lysates of strain A950006, suggesting a low affinity of this polyclonal serum for the A950006 HMW. In contrast, the *E. coli* and Rd clones containing the hmw-gene on a plasmid, did react with the 25D serum. Also when the expression of an HMW was restored in the A950006 hmw-knock out mutant by complementation of the cloned gene, a protein was recognized (not shown). When western blotting was performed with the polyclonal rabbit serum K1-050, an HMW protein of A950006 was recognized which disappeared after hmw::kana insertion (Fig.3). The expression was restored in this A950006 hmw-knock out mutant by complementation with the plasmid of clone 6. Combining the results of adherence assays and the immunoblotting with the polyclonal sera, it is suggested that strain A950006 expressed an HMW protein with HMW2-type adherence specificity, although this protein was not detected by serum 25D.
Sequencing analysis of the \textit{hmw} gene of strain A950006. The \textit{hmw} gene of strain A950006 was further characterized by DNA sequencing analysis of the cloned fragment. The fragment contained a complete \textit{hmwABC} gene cluster as judged from the length of the chromosomal insert. This was confirmed by aligning sequences obtained from subclones, which aligned to parts of all three ORF’s of the gene cluster (not shown). An additional ORF in front of the \textit{hmwA} gene showed homology to ORF HI1598 of \textit{H. influenzae} Rd. Sequence analysis of the complete \textit{hmwC} gene revealed that the nucleotide sequence of the \textit{hmwC} gene of strain A950006 is 97% and 96% identical to the \textit{hmw1C} and \textit{hmw2C} genes of strain 12 respectively, and the deduced amino acid sequence is 98% and 96% identical to HMW1C and HMW2C. Similar to the upstream region of the \textit{hmw1C} gene, the 5’-flanking region of the A950006 \textit{hmwC} gene contains a series of direct tandem repeats with a 9-bp sequence repeated multiple times. However, the \textit{hmwC} gene of strain A950006 contained 19 AAAACTAAG repeats, which differed from the repeated sequence from \textit{hmw1C}, which is CAAACCCAAG. The sequence of the \textit{hmwA} gene of A950006 consisted of a 4671-bp ORF with 75% homology to \textit{hmw1A} and 76% homology to \textit{hmw2A} of strain 12, while the deduced amino acid sequence was 70% homologous to HMW1A and 68% homologous to HMW2A (Fig. 4.). The first 1255-bp of the \textit{hmwA} gene are more conserved showing 92% homology to both \textit{hmw1A} and \textit{hmw2A}. The upstream region of the \textit{hmwA} gene contained 22 copies of the 7-bp repeat ATCTTTC, which was also apparent in the \textit{hmw1A} and \textit{hmw2A} flanking regions.

In conclusion, the \textit{hmwA} gene of \textit{H. influenzae} A950006, encoding a HMW protein mediating HMW2-like adherence, differed considerably from \textit{hmw1A} as well as \textit{hmw2A}.
The RGD sequence of the A950006 HMW and of HMW2 is indicated.

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Identical amino acids are indicated with •. Gaps are indicated with –.

The RGD sequence of the A950006 HMW and of HMW2 is indicated.
Discussion

In this study we analyzed adherence patterns of nontypeable *H. influenzae* from 9 otitis media and 19 COPD patients in comparison with those of 30 isolates from the throat of healthy individuals to two human epithelial cell lines. Of the 58 isolates tested 32 (55%) adhered to one or both of the epithelial cell lines used. The majority (72%) of the adherent isolates expressed HMW proteins as determined by PCR with *hmw* specific primers and western blotting with two polyclonal sera, indicating that these adhesins were involved in the adherence.

Different adherence patterns of the isolates to the two human epithelial cell types were observed. We showed that 20 isolates had HMW1-like or HMW2-like adherence. In addition, 3 other patterns of adherence were detected among the other 12 adherent isolates. The presence of the *hmw* gene or HMW proteins correlated with the HMW-like adherence patterns in most cases. Only two isolates with an HMW-like adherence pattern were *hmw/HMW* negative. All 12 strains with adherence patterns different from HMW-like adherence (groups III, IV and V), adhered to Chang epithelial cells, irrespective of the presence of dextran sulfate. Since the Hia protein mediates efficient *in vitro* adherence of nontypeable *H. influenzae* to Chang epithelial cells (Barenkamp and St.Geme, 1996a), the Hia protein or other unknown adhesins may play a role in the adherence patterns of these isolates. Five of the 12 isolates, the 4 isolates of group IV and one isolate of group V, expressed HMW proteins. Therefore, these 5 isolates may express HMW proteins that give rise to a different adherence pattern than HMW1 and HMW2-type adherence, or that they may express HMW adhesins in combination with Hia or an unknown adhesin. The combined presence of *hia* and *hmw* has been reported earlier for an otitis media isolate (Krasan et al., 1999), but among 59 nontypeable *H. influenzae* strains in another study, none harbored both *hmw* and *hia* (St.Geme et al., 1998).

One isolate expressing an HMW protein did not adhere, indicating that HMW proteins may not always function in adherence. In addition, the *hmw* knock out mutant of strain A950006 showed residual adherence, indicating that besides the HMW protein another adhesin was also involved in the adherence of this strain. However, loss of adherence by knock out mutation of the *hmw* of 3 representative isolates from groups I, II and IV confirmed that the HMW proteins were the major adhesins of these strains (data not shown). Although HMW-expressing strains may also adhere through other adhesins, the strong correlation of adherence with HMW and the inhibitory effect of dextran sulfate in most HMW positive isolates, indicate that HMW proteins were relevant adhesins for these isolates.

Since significantly more otitis media isolates and COPD isolates were *hmw/HMW* positive than isolates from healthy carriers, HMW expression may be important for the onset of infections such as otitis media as well as for COPD. A similar high frequency of HMW positive nontypeable *H. influenzae* isolates from otitis media has been reported in
other studies, that showed that 75-80% of these isolates expressed HMW proteins (Barenkamp and St.Geme, 1996b; St.Geme et al., 1998; Krasan et al., 1999). Otitis media isolates also adhered significantly more to the two cell lines than carrier isolates. However, the proportion of COPD isolates and that of carrier isolates adhering to the cell lines was similar, suggesting that carrier isolates adhered more often by adhesins other than HMW. It may be that non-adhering isolates express adherence factors for which no receptors are available on these two cell lines as shown for fimbriae-mediated adherence of *H. influenzae* (van Schillgaarde et al., 1995; St.Geme and Cutter, 1996). For non-adherent isolates an alternative adherence mechanism may be important in COPD patients. In the lower respiratory tract of these patients neutrophil defensins are present continuously due to the low-level inflammation of the bronchial tree and it has been shown that non-adherent *H. influenzae* are able to adhere to epithelial cells in the presence of neutrophil defensins (Gorter et al., 1998).

Screening of the isolates with a panel of 6 HMW-specific monoclonal antibodies in whole cell ELISA showed that the HMW proteins of COPD isolates and carrier isolates were more distinct from the HMW proteins from strain 12 than those from otitis media isolates. In another study using electrophoretic typing of *H. influenzae* isolates from different sources it was found that otitis media isolates were more clonal than isolates from COPD isolates (van Alphen et al., 1997). Since infections in COPD are chronic and antibody mediated defense mechanisms are active in these patients, the antigenic heterogeneity may be the consequence of accumulation of mutations as observed in other immunogenic outer membrane proteins during persistent infections of these patients (Duim et al., 1997; Duim et al., 1994).

Adherence characteristics and the results of western blotting of strain A950006 and its *hmw*-knockout mutants, showed that this strain expressed an HMW adhesin that was not recognized by serum 25D, nor by any of the Mabs. Sequence analysis of the *hmwA* gene of this strain showed that it was 75% homologous to the *hmw1A* and 76% homologous to *hmw2A*, as published by Barenkamp and Leininger (Barenkamp and Leininger, 1992). Also, 22 copies of a 7-bp repeat were found in front of the *hmwA* gene of strain A950006. Since it was shown that the presence of 17 copies or more of this sequence leads to a low level of expression of the HMW proteins (Dawid et al., 1999), the high number of these copies in strain A950006 may lead to a low level of HMW expression in this strain, and the diversity in the *hmwA* gene to antigenic differences. A low expression level in conjunction with antigenic differences can explain the lack of the reactivity of the anti-HMW polyclonal serum 25D with strain A950006. Reactivity of this polyclonal serum with the A950006 HMW expressed by the *E. coli* and *H. influenzae* clones may be due to overexpression of the HMW from the plasmid by these clones.

We found no association between the HMW1 and HMW2 like adherence patterns of the isolates and reactivity with the HMW1 specific Mabs or the HMW2 specific Mab respectively, indicating that antigenic sites and adherence sites are different. The predicted amino acid sequence of the HMWA of strain A950006 contained the sequence...
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RGD from amino acids 460 to 462. The RGD sequence of the *Bordetella pertussis* filamentous hemagglutinin, which is related to the HMW proteins, has been implied in adherence to the integrin CR3 (Relman et al., 1990). HMW2A also contains the RGD sequence but at a different position, namely from amino acids 785 to 787. Since the HMW protein of strain A950006 mediated adherence similar to HMW2-mediated adherence, this RGD sequence may be involved in adherence of group II isolates.

In conclusion, HMW is strongly related to the ability of nontypeable *H. influenzae* isolates to adhere to the Chang and NCI-H292 epithelial cell lines. Probably as a result of the variability in *hmwa* sequence, HMW proteins show a large antigenic diversity, although their HMW1 or HMW2 like adherence patterns are conserved.

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