The isolation and characterization of non-typeable *Haemophilus influenzae* from the sputum of adult cystic fibrosis patients


**ABSTRACT:** The role of non-typeable *Haemophilus influenzae* in cystic fibrosis (CF) remains unclear. We wanted, therefore, to determine the presence and characteristics of non-typeable *H. influenzae* in sputum samples from patients with CF.

In order to do this, we have assessed sputum samples from 55 consecutive clinically stable patients seen routinely at an adult CF out-patient clinic. Quantitative bacterial culture was performed using a selective media containing cefsulodin, and isolates were characterized by biotyping and outer membrane protein profile analysis.

In 17 (30%) of these samples, non-typeable *H. influenzae* was isolated and was present in similar viable numbers (mean $7.7 \times 10^8$ colony-forming units (cfu)·mL$^{-1}$; SEM 3.1) to *Pseudomonas aeruginosa* (mean $8.0 \times 10^9$ cfu·mL$^{-1}$; SEM 24). All non-typeable *H. influenzae* isolates recovered were beta-lactamase negative and sensitive to a range of antibiotics. Several biotypes and outer membrane protein profiles were observed, with no apparent association between these two phenotypic characteristics.

The study showed that large numbers of non-typeable *H. influenzae* are often present in sputum from adult patients with CF. Further longitudinal studies of outer-membrane protein profile analysis are required to determine the dynamics of non-typeable *H. influenzae* colonization in individual patients and the clinical significance.


Chronic pulmonary infection remains the major cause of morbidity and mortality in patients with cystic fibrosis (CF) [1]. *Staphylococcus aureus* is the predominant respiratory pathogen in childhood, but chronic *Pseudomonas aeruginosa* infection supervenes in the majority of patients by adolescence [2]. The presence of other *Pseudomonas* species and Gram-negative bacteria, such as non-typeable *Haemophilus influenzae* (NTHI), has also been reported [2–5]. This persistent microbial load is thought to perpetuate a vigorous host response, leading to pulmonary inflammation and tissue destruction [6].

*Pseudomonas aeruginosa* infection in CF has been extensively investigated [5, 7–9]. In contrast, the role of NTHI in CF lung secretions has been less well-characterized. Although NTHI is isolated during childhood [10, 11], its role in the pathology of CF lung disease remains unresolved, even though aggressive antibiotic regimens directed against this organism have been recommended [12]. A point prevalence rate of only 10% has been reported for NTHI in CF patients over 15 yrs of age [13], but this low figure may reflect difficulties in isolating this fastidious microorganism in the presence of *Pseudomonas* spp. [14]. This possibility is supported by observations that many patients improve when $\beta$-lactam antibiotics are given, despite the absence of NTHI on sputum culture [12], and the presence of high titres of serum antibodies to NTHI [11, 15]. Thus, it is likely that the presence and importance of NTHI has been underestimated and should be reviewed.

Characterization of NTHI strains present in CF lung secretions has been limited to biotyping, where temporal changes in distribution have been observed [16]. In a recent study, the distribution of biotypes in children with CF and age-matched children with asthma were shown to be similar [17], suggesting that biotype is not a critical factor. Analysis of outer membrane proteins may be more relevant, since NTHI expresses several outer membrane proteins which demonstrate marked antigenic heterogeneity [18], and are thought to be important targets of the human immune response [19]. Longitudinal studies in chronic obstructive lung disease and patients with non-CF bronchiectasis have demonstrated changes in the outer membrane protein P2 [20, 21] which may allow NTHI to evade normal clearance mechanisms in
a similar manner to that postulated for *Pseudomonas aeruginosa* [22, 23].

The current study was undertaken to determine the presence and numbers of NTHI in the sputum of stable adult CF patients using quantitative culture techniques with selective media. In addition we wished to characterize the NTHI isolates by phenotypic methods for comparison with previous studies.

**Patients and methods**

Sputum samples were obtained with the aid of physiotherapy from 55 out-patients (22 males and 33 females) seen at the Adult Cystic Fibrosis Centre in Manchester. The mean (SEM) age of the patients was 21 yrs (range 16–32 yrs). The mean forced expiratory volume in one second (FEV1) was 61% (SEM 4%) and the mean forced vital capacity (FVC) was 78% (SEM 4%) of the value predicted for age, sex and height. All patients were clinically stable and had no recent evidence of an acute exacerbation (characterized by an increase in cough and sputum production associated with a fall in FEV1), as defined by Elborn et al. [24]. In addition, in the 4 weeks prior to collection of the sample, none of the patients had received intravenous antibiotics or had experienced symptoms requiring any change to regular therapy. Forty eight of the patients were receiving maintenance antibiotic therapy, which included oral flucloxacillin alone (n=18), nebulized colomycin and oral flucloxacillin (n=26), and nebulized gentamycin (n=4). All patients were taking pancreatic supplements.

Sputum samples were collected into sterile plastic universal containers and shaken manually before being divided into two aliquots. One aliquot was sent to the local CF centre microbiology laboratory and the other was sent by post to the laboratory in Birmingham, UK.

**Sputum bacteriology**

All aliquots of sputum were subjected to primary sputum culture by removing a standard 5 µL loopful of the sputum and inoculating aerobic culture plates, together with a chocolate agar plate containing bacitracin (300 mg·L⁻¹) for anaerobic culture, as previously recommended for the isolation of NTHI in CF lung secretions [5]. Identification of the organisms present were performed using standard bacteriological methods. At the laboratory in Birmingham, the sputum aliquot was also subjected to quantitative bacterial culture as previously described [25]. Briefly, the sputum sample was homogenized with an equal volume of Sputasol (Oxoid, Basingstoke, UK) and a dilutional series from 1 in 10 to 1 in 100,000 was prepared in sterile saline. A fixed volume (10 µL) of each of these dilutions was dispensed by precision pipette and spread onto the surface of MacConkey, blood, chocolate, and chocolate agar plates containing 20 mg·L⁻¹ cefsuladin (based on the reported sensitivity of over 90% of Pseudomonas strains to this antibiotic) for aerobic culture (37°C in 5% CO₂ for 24 h), and onto chocolate agar plates containing 300 mg·L⁻¹ bacitracin for anaerobic culture. Following overnight incubation, the colony forming units (cfu) of predominant organisms present were counted on plates yielding between 30 and 300 colonies, and the results were expressed as mean cfu·mL⁻¹ of original sputum (±SEM). Viable numbers of the resulting bacterial species present were compared using an unpaired Student t-test (two-tailed).

**Characterization of NTHI isolates**

The identity of each NTHI isolate recovered was confirmed by colonial morphology, appearance on Gram stain, and the requirement for nicotinamide-adenine dinucleotide and haemin. To determine whether isolates were non-capsulated, serotyping was performed using type specific antiserum to capsular serotypes a–f [26]. All NTHI isolates identified were stored at -70°C until required, (Protect Preservers, Technical Service Consultant Ltd, Bury, Lancashire, UK). The following investigations were performed:

**Biotyping**. Biotyping was performed by the method of Kilian [27] to test for the production of indole, ornithine decarboxylase and urease. Isolates were classified as belonging to one of the eight recognized biotypes [27].

**Outer membrane protein profiles.** Whole cell lysates were prepared using a modification of the method described by Murphy et al. [28]. Briefly, the stored isolates of NTHI were regrown to purity on chocolate agar plates, incubated overnight, and harvested into 10 mL 0.01 M hydroxyethylpiperazine ethanesulphonic acid (HEPES) buffer (pH 7.4). After centrifugation (5,000×g for 15 min) and resuspension, the cells were subjected to ultrasonication (2×15 s at 20 W) using a Lucas Dawe Ultrasonics Soniprobe (Middlesex, UK). The protein concentration of the resulting whole cell lysate preparation was determined by the method of Lowry et al. [29], and diluted to 1 mg·mL⁻¹ protein. Outer membrane protein profiles were obtained by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the whole cell lysate preparations, using 12.5% acrylamide separating gels in the Tall Mighty Small vertical slab gel electrophoresis unit (Hoefer Scientific Instruments, Newcastle-under-Lyme, UK).

Gels were stained in Fast Stain solution (Arrow Scientific Ltd, Berkshire, UK) and destained in 10% acetic acid in distilled water. The resulting outer membrane profiles of each of the recovered NTHI isolates were compared by visual inspection of the resulting bands, and the size of the P2 and P5 outer membrane proteins were obtained from standard molecular weight markers run on the same gel.

**Beta-lactamase production.** Any beta-lactamase activity produced by each NTHI isolate was measured using the chromogenic substrate, nitrocefin, in microtitre plates, as described previously [30]. For calibration purposes a standard beta lactamase was used from *Bacillus cereus* type 1. The presence of beta-lactamase activity was confirmed...
by a colour change of nitrocefin substrate from yellow to red, measured spectrophotometrically at 492 nm (Dynatech MR 5000, Fisons, UK).

**Antibiotic sensitivity profiles.** The minimal inhibitory concentration (MIC) of NTHI isolates at an inoculum of $5 \times 10^5$ cfu mL$^{-1}$ was determined to a panel of antibiotic agents (amikacin, ampicillin, amoxicillin, cefoxitin, cef-tazidime, cefuroxime, chloramphenicol, ciprofloxacin, gentamycin, imipenem, tetracycline, tobramycin, trimethoprim/sulphamethoxole), using commercially available microtitre plates containing a range of concentrations of the lyophilized antibiotic agents (Sensititre R Plates, Sensititre Ltd, UK). Following incubation at 37°C in air for 18–24 h, the wells of the microtitre plates were examined for evidence of turbidity. The MIC of the antibiotic for the NTHI isolates was determined as the lowest concentration where no visible growth was observed. The MIC of the antibiotic for the NTHI isolates was determined as the lowest concentration where no visible growth was observed. The MIC of the antibiotic for the NTHI isolates was determined as the lowest concentration where no visible growth was observed. The minimal concentration causing 50 and 90% inhibition (MIC50 and MIC90) of the 17 NTHI isolates obtained in this study was calculated and expressed in mg L$^{-1}$.

All reagents, unless stated otherwise, were obtained from Sigma Chemical Co., Poole, Dorset, UK.

**Results**

The bacterial isolates recovered from the CF sputum samples at both the local CF centre and the Birmingham (UK) laboratory are shown in figure 1. In both laboratories, *Pseudomonas aeruginosa* was recovered from 35 patients (60%), other *Pseudomonas* spp. from 10 patients (18%), and *Staphylococcus aureus* from 12 patients (22%). In contrast, the Birmingham laboratory isolated NTHI from 17 patients (30%) and *Branhamella catarrhalis* from 4 (7%); whereas, the local CF centre only isolated NTHI from 1 patient (where it was the sole isolate) but did not isolate *Branhamella catarrhalis* from any sputum samples (fig. 1).

The distribution of bacterial isolates recovered from the sputum of the 55 patients by the Birmingham (UK) laboratory are shown in table 1. Of the 17 patients where NTHI was isolated, it was the sole pathogen in only two patients, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (n=2). The isolates had low MICs to the aminoglycosides tested (table 3).

![Fig. 1. – The incidence of bacterial isolates recovered at the local Cystic Fibrosis Centre and the Birmingham (UK) Laboratory from sputum samples taken from 55 consecutive cystic fibrosis patients.](image)

**Table 1. – Distribution of bacterial isolates obtained from the 55 cystic fibrosis (CF) sputum samples, based on the results of the Birmingham (UK) Laboratory.**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Pts</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTHI alone</td>
<td>2</td>
</tr>
<tr>
<td>NTHI + <em>Staphylococcus aureus</em></td>
<td>4</td>
</tr>
<tr>
<td>NTHI + <em>Pseudomonas aeruginosa</em></td>
<td>5</td>
</tr>
<tr>
<td>NTHI + <em>Pseudomonas spp.</em></td>
<td>1</td>
</tr>
<tr>
<td>NTHI + <em>P. aeruginosa + S. aureus</em></td>
<td>2</td>
</tr>
<tr>
<td>NTHI + <em>Pseudomonas spp. + S. aureus</em></td>
<td>2</td>
</tr>
<tr>
<td>NTHI + <em>Branhamella catarrhalis</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> alone</td>
<td>24</td>
</tr>
<tr>
<td><em>Pseudomonas spp.</em></td>
<td>7</td>
</tr>
<tr>
<td><em>P. aeruginosa + S. aureus</em></td>
<td>2</td>
</tr>
<tr>
<td><em>P. aeruginosa + B. catarrhalis</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> alone</td>
<td>2</td>
</tr>
<tr>
<td><em>Branhamella catarrhalis</em></td>
<td>1</td>
</tr>
</tbody>
</table>

NTHI: non-typeable *Haemophilus influenzae*; Pts: patients.

**Table 2. – Quantitation of bacterial species isolated in the Birmingham (UK) laboratory, expressed as colony forming units per mL (cfu·mL$^{-1}$).**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Bacterial species cfu·mL$^{-1}$×10$^8$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTHI (n=17)</td>
<td>7.7±4.3</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (n=35)</td>
<td>8.0±2.4</td>
</tr>
<tr>
<td>Other <em>pseudomonas</em> spp. (n=10)</td>
<td>4.9±2.2</td>
</tr>
<tr>
<td><em>Branhamella catarrhalis</em> (n=4)</td>
<td>10.7±4.9</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (n=12)</td>
<td>7.1±3.7</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM. NTHI: non-typeable *Haemophilus influenzae*.

NTHI was isolated, it was the sole pathogen in only two patients but it was isolated in association with other organisms from the remaining 15 patients. In 10 of these 15 patients, *Pseudomonas aeruginosa* or *Pseudomonas* spp. were also recovered. In the 38 patients where NTHI was not isolated, the predominant organism was *Pseudomonas aeruginosa*. Interestingly, *Branhamella catarrhalis* was recovered as the sole isolate in one patient, but also associated with *Pseudomonas aeruginosa* in two patients, and NTHI in one.

Quantitative bacterial culture revealed no significant difference in the viable numbers of each bacterial species recovered, as shown in table 2. There was no difference between viable numbers of NTHI or *Pseudomonas* spp. present. Furthermore, the numbers of NTHI isolated were similar when present as the sole organism or when recovered in association with *Pseudomonas* spp. None of the 17 NTHI isolates produced β-lactamase activity. The sensitivities of the NTHI isolates to a range of antibiotics are shown in table 3. The isolates had low MICs to the β-lactam antibiotics, which included amoxicillin and cef-tazidime, and the other drugs commonly used in the treatment of respiratory exacerbations at the CF centre, including ciprofloxacin and the carbacephem, imipenem. The NTHI isolates had high MICs to the aminoglycosides tested (table 3).

When the 17 NTHI isolates were characterized further, eight were found to be biotype III, four biotype II, four biotype I and one isolate was biotype V (fig. 2). Seven different outer membrane protein profiles were observed...
in the 17 NTHI isolates (fig. 2). Low molecular weight markers, seen in lane 1, allow determination of the size of the major protein bands P2 (between 36–42 kDa) and P5 (32–36 kDa), which form the basis of a subtyping system allowing isolates to be compared [28]. For the purpose of description, isolates were grouped on the basis of the position of P2 and P5. In 8 of the 17 isolates (Group A), the P2 protein was in the same position approximately 38 kDa. Four of the remaining isolates (Group B) had a different OMP profile to Group A, with the P2 protein having a molecular weight of approximately 37 kDa and P5 at approximately 35 kDa. The remaining five

Table 3. – Sensitivity of the 17 NTHI isolates recovered in the Birmingham (UK) laboratory

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC50 mg·L⁻¹</th>
<th>MIC90 mg·L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>8.0</td>
<td>160</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>0.6</td>
<td>1.25</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&lt;4.0</td>
<td>&lt;4.0</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&lt;1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.6</td>
<td>1.25</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Imipenem</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>4.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Trimithoprim/sulphamethoxazole</td>
<td>&lt;0.5/9.5</td>
<td>&lt;0.5/9.5</td>
</tr>
</tbody>
</table>

All isolates were β-lactamase negative. NTHI: non-typeable Haemophilus influenzae; MIC50 and MIC90: minimal concentration causing 50 and 90% inhibition.

Fig. 2. – Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell lysate preparations of the 17 non-typeable H. influenzae (OMP) isolates. The biotype of each isolate is displayed beneath each OMP profile. a) Group A - molecular weight markers are shown in lane 1 and the 8 isolates in lanes 2–9 showing the P2 protein in the same position. b) Group B – molecular weight markers are shown in lane 10. The four isolates in Group B with the same OMP profile but different to the profile in Group A are shown in lanes 11–14. c) Group C - molecular weight markers are shown in lane 15. The five isolates in this group, shown in lanes 16–20, have five different OMP profiles.
isolates, designated Group C, each had a different OMP profile with P2 and P5 in different positions to either Group A, Group B or to other isolates within Group C.

Discussion

Employing quantitative bacterial culture techniques and a selective media incorporating cefsulodin, we have isolated non-typeable *Haemophilus influenzae* from the sputum of 17 clinically stable adult cystic fibrosis patients (30% of the patients studied). This is in contrast to a much lower isolation rate quoted in previous studies. A screening study of 102 patients reported the incidence of NTHI as 11% [31], whereas in an early well characterized study, the incidence of persistent colonization of NTHI was only 1% [10]. Recently, a point prevalence rate of only 10% has been calculated for NTHI in CF patients over 15 years of age [13]. Such low rates may reflect the use of routine methods rather than the precise quantitative procedures which were employed in our particular study. This would be supported by the results of the routine methods employed at the CF centre, where only one sputum culture was positive for NTHI (a patient in whom NTHI was the sole isolate) compared to the 17 isolates obtained in the Birmingham (UK) laboratory. Of the 17 patients from whom NTHI was isolated in our study, 10 also had *Pseudomonas aeruginosa* or other *Pseudomonas* spp. present (table 2). On routine testing, Pseudomonas might have been assumed to be the important pathogen, however, quantitative culture in the Birmingham (UK) laboratory revealed that NTHI was present in similar viable numbers. Furthermore, *Branhamella catarrhalis*, which is increasingly recognized as a pathogen in chronic lung disease [19], was isolated in sputum from four patients when quantitative techniques were used, but was not isolated using routine methods. This observation suggests that the prevalence of this organism may also be greater than currently recognized in CF and warrants further study.

The results obtained in the Birmingham laboratory (fig. 1) suggest that under or over-reporting of NTHI does not occur as a result of posting samples, particularly since the identical isolation rates in both centres for *Pseudomonas* spp. and *Staphylococcus aureus* suggest that there is no significant loss of bacterial species. Furthermore, in separate experiments, the Birmingham laboratory has demonstrated no differences in the viable numbers of NTHI and other bacterial species present in CF samples collected locally, when an aliquot was cultured immediately and a further aliquot was placed in the post and cultured on receipt 24 h later (unpublished observations).

From a therapeutic point of view, the isolates that we obtained were all β-lactamase negative and, despite the aggressive antibiotic regimes employed within the CF centre, remained sensitive to a spectrum of the agents used routinely, including ceftazidime, ciprofloxacin and imipenem. In addition, all the isolates were sensitive to ampicillin, amoxycillin and chloramphenicol. The aminoglycosides were the only agents which would not have been adequate for treatment of NTHI. Our findings may explain why some patients experience clinical improvement following routine intravenous therapy, with agents such as ceftazidime, when there is no change in the *Pseudomonas* spp. cultured from the patients sputum.

Previous studies to characterize the strains of NTHI present in lung secretions in CF patients have been limited to biotyping, and it was reported initially that the majority of strains in CF were biotype I [32]. However, a subsequent study demonstrated biotype V to be more common than biotype I, although biotype I organisms were more commonly isolated in association with exacerbations [17]. In contrast, studies in non-CF chronic adult lung disease have revealed a predominance of isolates of biotypes II and III [33]. In CF, rapid temporal changes in biotype distribution have been described [16], and the predominance of biotype III in our study of stable adult patients may represent a bias to this particular well-characterized "adult" group, so that the results are similar to non-CF adult chronic lung disease or reflect the small number of samples collected over a short time-period. Further longitudinal studies with this group of patients studied when stable and during exacerbations may help clarify the results.

Analysis of outer membrane protein profiles allows further subtyping of organisms [28]. Longitudinal studies in chronic obstructive lung disease and bronchiectasis have demonstrated size changes in the outer membrane proteins P2 and/or P5 [20, 21]. A longitudinal study by our group in patients with non-CF bronchiectasis has shown marked size polymorphism in the P2 protein from NTHI isolates. In some cases, these changes were observed during persistent colonization in clinically stable patients, and in others they were associated with the start of an infective exacerbation [21]. In addition, detailed studies have shown antigenic drift in immunodominant epitopes of P2 caused by small changes in amino acid composition [34]. This is particularly important, since P2 is an important target of human bactericidal antibodies [35], and this protein has been shown to express a highly strain-specific immunodominant epitope [36]. Thus, regular changes in P2 or colonization by a new strain with a different P2 protein may enable the organism to evade host defences or cause an acute exacerbation of the disease.

In our study, seven different outer membrane protein profiles were demonstrated (fig. 2). Six of these corresponded to profiles observed by MURPHY et al. [28], but one isolate (lane 18, fig. 2c) did not correspond to any OMP profile previously recognized by MURPHY et al. [28] and has not been previously observed in our own studies of patients with non-CF bronchiectasis [21].

There was no apparent association between OMP profile and biotype. In particular, in Group C (fig. 2), biotype III was associated with four different OMP profiles. Conversely, in Group A, eight isolates with the same OMP profile were associated with three different biotypes. The significance of this with regards to "strain" characteristics remains unclear. The variety of profiles obtained in this small study suggests that a longitudinal study of NTHI isolates in CF patients with a larger number of isolates is warranted. Such studies would establish whether antigenic drift is occurring in NTHI isolates in CF, and whether this organism plays a role in exacerbations of CF lung disease.
The current study has shown that NTHI is present in sputum samples from 30% of clinically stable patients with CF. Furthermore, NTHI is present in similar viable numbers to those of Pseudomonas aeruginosa and other important bacterial species in CF, suggesting a significant role in the pathogenesis of airways inflammation. We have demonstrated a variety of outer membrane protein profiles in this small number of isolates. Although the study has not investigated longitudinal changes in outer membrane profiles, the frequent isolation of NTHI suggests that a larger longitudinal study is justified to elucidate the role of NTHI in adult CF lung disease.

References

13. Pederson SS. Lung infection with alginate-producing, mucoid Pseudomonas aeruginosa in cystic fibrosis. APMS 1992; 100: (Suppl. 28).