Flagellar expression in clinical isolates of non-typeable

*Haemophilus influenzae*

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**INTRODUCTION**

*Haemophilus influenzae* is a Gram-negative coccobacillus that naturally colonizes the nasopharynx of healthy humans [1]. It belongs to the *Pasteurellaceae* family and is thought to be a non-flagellated bacterium that causes systemic disease and sporadic infection of the respiratory mucosa. Human isolates of *H. influenzae* can be subdivided into encapsulated (typeable) and non-encapsulated (non-typeable) forms. Encapsulated strains express one of six different capsular polysaccharides, designated as serotypes a–f. *H. influenzae* type b (Hib) is the most common serotype associated with invasive disease. In contrast, *H. influenzae* strains lacking capsular polysaccharides are referred to as non-typeable (NTHi) and are generally associated with moderate diseases of the upper respiratory tract and may cause acute sinusitis and/or acute otitis media in children [2]. In adults, NTHi is a common cause of community-acquired respiratory tract infections and can cause chronic bronchitis and pneumonia in patients with chronic obstructive pulmonary disease [2–5].

Several studies have identified NTHi surface components, such as adhesive pili, outer membrane proteins (OMP) and lipo oligosaccharides (LOS), which contribute to mucosal attachment and colonization [6–8]. Moreover, many NTHi strains express type IV pili (T4P), which are involved in biofilm formation, competence for DNA uptake, twitching motility and long-term colonization of the mammalian nasopharynx [9–13]. However, the presence of flagella, important for motility, adherence and biofilm formation, has not been detected.

Studies of related bacteria have shown that flagellar motility is important during animal infection. In experiments with chickens and gnotobiotic piglets infected with non-motile or mixed-motile and non-motile populations of *Helicobacter*...
**METHODS**

**Bacterial strains and growing conditions**

Descriptions of the bacterial strains used in this study are listed in Table 1. *H. influenzae* and *A. pleuropneumoniae* strains were routinely cultured in brain heart infusion (BHI) agar medium (Bionox de México) supplemented with 5 % Fildes [peptic digest of sheep blood that supplies the X (haemin) and V (nicotinamide adenine dinucleotide, NAD) factors necessary for the adequate growth of *H. influenzae*]. The strains were incubated at 37 °C, 24 h, 5 % CO₂. The *E. coli* strains E2348/69, JPN15 and AGT01 were grown in tryptone soya agar (TSA) (Bionox de México) and incubated at 37 °C for 24 h under aerobic conditions.

**Identification of *H. influenzae* strains**

*H. influenzae* strains were identified and selected according to their morphology and microscopic characteristics by Gram staining (Gram-negative coccobacilli, small, pleomorphic and 0.3×1.0 µm size), as well as according to their growth requirements for the *V* and *X* factors, serology and biotyping. Finally, a 16S confirmatory PCR was done using the following oligonucleotides: 16S HiF 5′-CGTATTA TCGGAAGATGAAAGTGC-3′ and 16S HiR 5′-AGTACTC TAGTTACCAGTCTGA-3′ [20, 21].

**Swarming motility assays**

Bacteria were placed in the centre of BHI plates using a sterile inoculating loop (10 µl). Several growth conditions were tested, including agar 0.3–1.5 % (w/v), Fildes 1–1.5 % and CO₂ 5 %, temperatures of 23, 30, 37 and 42 °C, and incubation times from 24–120 h. Bacterial motility was determined by measuring the radial growth from the centre of the plate. All motility assays were performed in triplicate. *A. pleuropneumoniae* was grown under the same conditions as a positive control, and *E. coli* E2348/69 and *E. coli* JPN15 strains were grown in aerobic conditions for 24 h at 37 °C as motility-positive controls. The *E. coli* AGT01 strain was grown under the same conditions as the negative control.

**Detection of *fliC* and *flgH* genes**

*fliC* and *flgH* gene amplification and subsequent sequencing was performed using the following oligonucleotides, for *fliC*: D2 primer 5′-GCACAAGTCATTAGTACCAACAGGCTCT-3′ and R2: 5′-GGCCTGCTGGATGATCTGCG-3′; for *flgH*: flgH forward 5′-gcatactgacctccagcttggt3′ and flgH reverse 5′-CATTGGCGACAGGGTTAAGGAAGAAC-3′ [22].

These degenerate specific oligonucleotides were designed according to the nucleotide sequences reported in the GenBank database for the *fliC* gene of *E. coli* K12 (ID 949101), *Shigella flexneri* (ID 1078378) and *Salmonella enterica* subsp. *enterica* serovar Typhi (ID 1248507). The PCR products were purified using the Zymo Gel Extraction Kit (Zymo Research) and sequenced at the Institute of Biotechnology, IBT, Cuernavaca, Morelos, Mexico.

**Chemotaxis-like assays**

Chemotaxis-like tests were performed on trypticase soy broth (TSB) supplemented with 0.3 % agar (Bionox de México) and one of the essential growth factors for *H. influenzae* (haemin or NAD). The other factor, haemin (10 µg ml⁻¹) or NAD (2 µg ml⁻¹), was supplemented in a disk

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**Table 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><em>E. coli</em> E2348/69</td>
<td>Wild-type EPEC O127:H6, Na⁺</td>
<td>[41]</td>
</tr>
<tr>
<td>AGT01</td>
<td><em>fliC</em> mutant of E2348/69 (EPEC <em>fliC</em>-cat)</td>
<td>[33]</td>
</tr>
<tr>
<td>JPN15</td>
<td>Strain E2348/69 spontaneously cured of the large plasmid encoding the <em>bfp</em> cluster</td>
<td>[42]</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em> BC5235</td>
<td>Strain isolated from the lung abscess of a pig with acute porcine contagious pleuropneumonia</td>
<td>[43]</td>
</tr>
<tr>
<td><em>H. influenzae</em> Rd KW20</td>
<td>Non-capsulated strain, considered avirulent, derived from serotype d. Non-pathogenic and lacking adhesins</td>
<td>[2, 44]</td>
</tr>
<tr>
<td>33930</td>
<td>Hib strain, isolated from cerebrospinal fluid</td>
<td>ATCC</td>
</tr>
<tr>
<td>BUAP96</td>
<td>Non-typeable strain, isolated from the middle ear from a case of paediatric otitis media</td>
<td>This study</td>
</tr>
<tr>
<td>BUAPNAN</td>
<td>Type b strain, isolated from cerebrospinal fluid from a case of paediatric meningit</td>
<td>This study</td>
</tr>
<tr>
<td>BUAPPAU</td>
<td>Non-typeable strain, isolated from the nasopharynx</td>
<td>This study</td>
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close to the inoculum and was used as the chemoattractant agent. Motile bacteria were inoculated in the centre of the plate. Plates were incubated at 37°C with CO2 5%, and observed every 24 h for 5 days to determine the chemoattraction.

**Transmission electron microscopy (TEM)**

Bacteria were harvested from swarming plates and were resuspended in 100 µl PBS. Diluted cultures (10 µl) were applied to carbon-coated copper grids (Electron Microscopy Sciences) and fixed with 1 % glutaraldehyde for 1 min, rinsed with one drop of distilled water and negatively stained with 0.05 % uranyl formate and 1 % uranyl acetate. Samples were visualized and photographed using a JEM-2000EX transmission electron microscope at 80 keV (JEOL, Tokyo, Japan) on Kodak Electron Image Film (Eastman Kodak, Rochester, NY).

**Ryu stain**

Flagella were stained using the Ryu staining protocol according to Kodaka et al. [23]. Briefly, the dye was prepared by mixing 10 parts mordant solution (2 g tannic acid, 10 ml phenol 5 % and 10 ml saturated aluminium potassium sulfate 12-hydrate) with one part staining solution (crystal violet 12 % in ethanol).

Bacterial colonies (NTHi BUAP96, E. coli E2348/69 and AGT01 strains) were recovered from the swarm border in motility assays and transferred to a drop of water on a microscopy slide.

Slides were dried at room temperature, stained using the Ryu staining for 5 min and washed with running water for 2 min. The preparations were observed using differential interference contrast (Nikon Eclipse Ti-E microscope) and the images were captured using NIS-Elements software version 4.20.

**Purification of flagellin-like H. influenzae**

NTHi BUAP96 and NTHi BUAPPAU strains were grown on plates under motility conditions. The growing bacteria were removed and resuspended in 500 µl PBS (pH 7.4). The bacterial suspension was then centrifuged twice at 8000 g for 20 min and the supernatant was recovered. The outer membranes and bacterial debris were removed by centrifugation at 10 000 g for 30 min. Proteins, including putative flagellin protein, contained in the supernatant were precipitated by adding cold acetone (4°C) and centrifuged at 8000 g for 30 min. Proteins were dialysed three times against PBS [24], and protein concentration was determined by Bradford assay.

**Flagellin-like immunodetection**

Ten micrograms of the flagellin preparation were heated in a boiling water bath for 5 min in Laemmli sample buffer (Bio-Rad) and analysed by SDS-PAGE (12 % separating gel). The gel was transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked with 5 % skim milk and incubated with anti-flagellin rabbit polyclonal antibodies (1 : 2000) from A. pleuropneumoniae, Salmonella spp. or Azospirillum brasilense. After incubation, membranes were washed three times with TPBS (PBS plus 0.05 % Tween 20) and then incubated with a 1 : 5000 dilution of goat anti-rabbit IgG alkaline phosphatase-conjugated (H+L) antibodies (Zymed, San Francisco, CA).
CA) and revealed with BCIP/nitro-blue tetrazolium chloride single reagent (Millipore, Billerica, MA).

**Statistical analysis**

All motile experiments were performed twice in triplicate. Mean values are represented with the corresponding standard deviations. Data were analysed using a one-way ANOVA test followed by Tukey’s test (Prism version 5, Graphpad Software, San Diego, CA). Differences were considered significant at $P<0.001$.

**RESULTS**

**Motility in *H. influenzae* strains**

Fifty strains of capsulated *H. influenzae* (24 clinical strains and 26 isolated from healthy carriers) and 46 strains of non-capsulated *H. influenzae* (15 clinical strains and 31 isolated from healthy carriers) were analysed for motility (Tables S1, S2, S3 and S4, available in the online Supplementary Material). We assessed different agar and Fildes concentrations on plates, and different incubation times until we observed the optimal condition in which the *H. influenzae* strains showed radial movement from the inoculation site (Fig. 1 and Tables S1, S2, S3 and S4). The best growing conditions where *H. influenzae* clinical isolates exhibited motility were on 0.3 % BHI agar supplemented with 10 % Fildes, during 72 h at 37 °C in a CO$_2$ 5 % atmosphere. The motility phenotype started showing a ring after 24 h of incubation and reached its maximum at 72 h. The strain displaying the greatest motility was NTHi BUAP96 (18.0 mm), followed by Hib BUAPNAN (14.5 mm), Hib 33 930 (12.7 mm) and Hib Rd KW20 (12.0 mm) relative to NTHi BUAPPAU that displayed a limited (5.0 mm) motility halo (Fig. 1 and Table 2). The NTHi BUAP96 strain displayed greater motility relative to both the NTHi BUAPPAU and non-flagellated *E. coli* AGT01 strains ($P<0.001$).

The motility observed in NTHi BUAP96 was similar to that of *A. pleuropneumoniae* and *E. coli* JPN15. However, the motility was lower than that displayed by the enteropathogenic *E. coli* (EPEC) strain E2348/69.

**NTHi BUAP96 strain possesses fliC and flgH genes**

We used PCR to determine whether NTHi BUAP96 possesses genes required to express flagella. Using specific

<table>
<thead>
<tr>
<th>Strain</th>
<th>Swarming diameter in (mm)</th>
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<tbody>
<tr>
<td><em>E. coli</em> E2348/69</td>
<td>37.0±1.4</td>
</tr>
<tr>
<td><em>E. coli</em> JPN15</td>
<td>24.5±0.7</td>
</tr>
<tr>
<td><em>E. coli</em> AGT01</td>
<td>3.0±0.8</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>20.0±1.4</td>
</tr>
<tr>
<td>NTHi BUAP96</td>
<td>18.0±1.2*</td>
</tr>
<tr>
<td>Hib BUAPNAN</td>
<td>14.5±0.7</td>
</tr>
<tr>
<td>Hib 33930</td>
<td>12.7±1.7</td>
</tr>
<tr>
<td>Hib Rd KW20</td>
<td>12.0±2.8</td>
</tr>
<tr>
<td>NTHi BUAPPAU</td>
<td>5.0±0.3</td>
</tr>
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</table>

*P<0.001 as determined by one-way ANOVA.

![Fig. 2. PCR amplification of fliC and flgH genes from NTHi BUAP96. Panel A. fliC gene: lane M, marker φ174; lane 1, *E. coli* E2348/69 (motile); lane 2, *E. coli* AGT01 (non-motile); lane 3, NTHi BUAP96. The asterisk indicates that the PCR product for the fliC gene was interrupted with a chloramphenicol acetyl transferase cassette (2159 bp). Panel B. flgH gene: lane M, marker GeneRuler 1 kb plus DNA ladder; lane 1, *E. coli* E2348/69 (motile); lane 2: *K. pneumoniae* ATCC 7603 (non-motile); lane 3: *H. influenzae* Rd KW20; lane 4: *A. pleuropneumoniae*; lane 5: NTHi BUAP96. PCR products corresponding to the amplified genes are indicated with an arrowhead.](image-url)
primers, we identified the flagellin gene \( fliC \). Our PCR amplification of \( fliC \) resulted in a weak 1490 bp band (Fig. 2a), which was partially sequenced at the 5' and 3' ends, showing 93% (5' end) and 97% (3' end) identity with the \( fliC \) gene from \( E. coli \); 98 and 91% with \( A. pleuropneumoniae \); and 61 and 76% identity with \( fliC \) from \( S. flexneri \). The sequence of the 700 pb \( flgH \) PCR product from NTHi BUAP96 (Fig. 2b), showed 96% identity relative to the \( S. flexneri \) and \( E. coli \) TW14359 \( flgH \) gene.

**H. influenzae express lateral flagella**

To determine the presence of flagella in the motile strains, we used TEM to analyse samples from the NTHi BUAP96 motility assay plates. These results revealed that this strain displayed one or two flagella on the surface with characteristic waving morphology (Fig. 3a, b). Interestingly, flagella were mainly observed in bacteria that were in fission (Fig. 3c). Flagella were present on bacteria presenting bacillary, coccobacillary and coccoid morphology (Fig. 3d). Moreover, some coccoid-form bacteria displayed more than one flagellum (Fig. 3e). The average length of flagella was 5 \( \mu \)m and the average width was 30 nm. The observed flagella appeared to be laterally expressed.

Flagella were also revealed using Ryu staining in NTHi BUAP96 (Fig. 4c). Although the number of flagellated bacteria in the preparation was low, the flagella produced by NTHi BUAP96 were detected by conventional optical microscopy. Fig. 4a, b show the positive control \( E. coli \) E2348/69 and the negative control \( E. coli \) AGT01, respectively.

**Chemotaxis in H. influenzae**

It is known that flagella play an important role in chemotaxis [25]. In the present study, we investigated whether the NTHi BUAP96 strain displaying flagella could perform chemotaxis. To test this hypothesis, we grew the NTHi BUAP96 strain on soft BHI agar plates (0.3 %) supplemented with only one of the two factors required for its

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*Fig. 3.* Identification of flagella by TEM. (a) and (b) TEM micrographs showing two flagella on the bacterial surface of NTHi BUAP96. (c) and (d) The presence of flagella was observed in dividing cells in NTHi BUAP96. (e) Coccoid-shaped bacteria displayed more flagella than those with bacillary morphology. Flagella are indicated with arrows. Scale bars are 200 nm (b), 500 nm (a, c and e) and 1 \( \mu \)m (d).
growth (V or X); the other essential factor was supplemented on an impregnated disk that was placed on the plate surface (Fig. 5a). The NTHi BUAP96 strain, in both cases, exhibited motility towards the chemoattractant on the disk (Fig. 5b, c). The NTHi BUAPPAU (the less motile strain) showed minimal migration towards the disk with the chemoattractant (non-motile tactic bacteria; data not shown).

**H. influenzae** flagella are formed by putative flagellin

The presence of a flagellin-like protein in NTHi BUAP96 was examined by Western blotting using heterologous polyclonal antibodies against flagellin proteins (Fig. 6a, b). Our results revealed that the *A. pleuropneumoniae* anti-flagellin antibody cross-reacted with proteins from our *H. influenzae* preparation, showing a band of approximately 55 kDa in the NTHi BUAP96 strain. Additionally, when using anti-flagellin antibodies from *Salmonella* spp. and *A. brasilense* (Fig. 6c), we also observed a band in the NTHi BUAP96 of 55 kDa. In contrast, no flagellin band was detected when using the *A. pleuropneumoniae* anti-flagellin antibody with the non-motile NTHi BUAPPAU strain (Fig. 6d).

**DISCUSSION**

Flagella and flagellum-mediated motility play an important role in the virulence of several gastrointestinal bacterial pathogens such as *Salmonella typhimurium* [26], *E. coli* [27] and *Vibrio cholera* [28]. Flagella induce the movement of pathogenic bacteria towards their colonization site and in *Pseudomonas aeruginosa, Listeria monocytogenes* and *Vibrio anguillarum* are also involved in the adherence and invasion of host cells [29–31]. Furthermore, flagellum-mediated motility is considered a main virulence factor for *Heliobacter pylori* stomach colonization [32]. Although *H. influenzae* has been considered a non-motile organism lacking flagella, the NTHi BUAP96 strain of *H. influenzae* in the present work, a clinical paediatric isolate from a middle ear infection (Table 1), might use flagella for their pathogenic migration from the nasopharynx.

The expression of surface appendages has been related to specific micro-environments or nutritional growth conditions *in vitro*. For example, in EPEC, flagellum expression is induced in the presence of eukaryotic cells and by unknown signals secreted by them [33]. In the present study, we found that, similar to *A. pleuropneumoniae* which is phylogenetically related to *H. influenzae*, and where flagella are expressed in 0.3 % BHI with agar at 37°C, 72 h, 10 % CO₂ [19], clinical isolates of *H. influenzae* become mobile at 37°C and 5 % CO₂ in 0.3 % BHI agar supplemented with 10 % Fildes. We incubated for 72 h because *H. influenzae* is a slow-growing organism, with a 103–107 min generation time [34].

The *H. influenzae* strains analysed in this study showed a swarming motility similar to that displayed by *E. coli* E2348/69, *A. pleuropneumoniae* [19] and *E. coli* JPN15 (atypical EPEC). We used atypical EPEC as a negative control for T4P, since *H. influenzae* also express T4P that could mediate

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**Fig. 4.** Flagellum detection with Ryu stain photomicrographs of flagellated bacteria grown on motility agar observed with Ryu stain. Images were taken under light microscopy at ×100 magnification. The photographs are representative of three experiments in which several fields were observed. (a) *E. coli* E2349/68 was used as positive control for flagellum expression. (b) *E. coli* AGT01 was used as negative control for flagellum expression. (c) NTHi BUAP96 bacteria with flagella. A flagellum is indicated with an arrowhead.
twitching motility on agar plates, although at different agar concentrations (1%) [35, 36]. Furthermore, our TEM microscopy analysis revealed that the flagellar morphology expressed by NTHi BUAP96 is similar to that of flagella of other bacteria. The size and form correspond to a flagellar filament and not to T4P, which are thinner filaments (Table S5) and expressed under different growing conditions [12].

As we have shown, the motility of NTHi BUAP96 is similar to that of A. pleuropneumoniae, a slow flagellum-mediated motility strain [19]. Therefore, we suggest that the swarming motility observed in our clinical H. influenzae isolate may also be slow. Moreover, our TEM observations revealed that NTHi BUAP96 displayed one or two lateral flagella, similar to A. pleuropneumoniae [19], and it is known that swarming motility in semi-solid media requires functional lateral flagella [37].

Is known that bacteria use flagella and chemotaxis to move towards a nutritional attractant [38]. Although the chemotactic mechanisms in Haemophilus are unknown, we used a model of chemotaxis to reveal that NTHi BUAP96 is capable of moving towards a required growth factor (V or X) and, although small molecules such as NAD or haemin could diffuse in soft agar plates, the bacterial migration was observed toward the site with the highest growth factor concentration on the disk, suggesting that flagella may be involved in this movement.

Our results from PCR assays revealed that NTHi BUAP96 possesses the genes for fliC and flgH that could be involved in the structure of the flagellar machinery. However, other genes need to be investigated to complete the set of genes involved in this flagellar assembly [e.g. flgF (ring MS), fik (ring P) and fliA (regulation)].

Our results from immunodetection assays, using heterologous anti-flagellin polyclonal antibodies from several bacteria against the supernatant proteins extracted from NTHi BUAP96, revealed that there is a cross-reaction between these strains. Therefore, the NTHi BUAP96 55 KDa band is similar in size to the flagellins of P. aeruginosa strains a-type 5933 and 5939, which have molecular masses of 51 and 52 kDa, respectively [39], and to the flagellin from an atypical EPEC of 50 kDa described recently [40].

![Chemotaxis assay](https://example.com/chemotaxis-assay.png)

**Fig. 5.** H. influenzae chemotaxis-like assays. Chemotaxis-like assay for NTHi BUAP96 in soft agar. (a) Graphic representation of the test. A colony taken from motility assays was inoculated at the centre of the plate; tactic and motile bacteria were able to migrate toward the disk impregnated with the growth factor necessary for growth. (b) Assay in soft agar supplemented with NAD (2 µg ml⁻¹) showing the motility of NTHi BUAP96 to the haemin disk (dark disk). (c) Assay in soft agar supplemented with haemin (10 µg ml⁻¹) showing the motility of NTHi BUAP96 to the NAD (white disk). The images are representative of at least three independent experiments. An arrowhead indicates the inoculation site.
Further studies are required to determine the exact role of flagella in these bacteria and how they contribute to their pathogenic potential. In conclusion, we presented both direct and indirect evidence indicating that NTHi BUAP96 is motile in vitro and capable of expressing flagella. These results highlight the biological role for flagella as an important motility factor that could contribute to the interaction of *H. influenzae* with their host cells. Flagella expressed by NTHi BUAP96 are a very important finding in the pathogenesis of this organism, which is currently considered non-motile and non-flagellated.

**Acknowledgements**

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


