Title: Improvement rate of acute otitis media caused by *Haemophilus influenzae* at one week is significantly associated with the time to recovery

Running Title: Treatment failure and relapse of acute otitis media

Keywords: acute otitis media; *Haemophilus influenzae*; biofilm; cellular invasion

Authors: Hisakazu Yano,a Yoshitaka Yamazaki,b Liang Qin,c Naohiro Okitsu,d Koji Yahara,e Mihoko Irimada, d Yoichi Hirakata, a Mitsuo Kaku, a Toshimitsu Kobayashi, f and Hiroshi Watanabe c

Affiliations:

Department of Infection Control and Laboratory Diagnostics, Tohoku University Graduate School of Medicine, Sendai, Japan a; Department of Infectious Diseases, Suzaka Hospital, Suzaka, Japan b; Division of Infectious Diseases, Department of Infectious Medicine, Kurume University School of Medicine, Kurume, Japan c; Department of Otolaryngology, Tohoku Rosai Hospital, Sendai, Japan d; Graduate School of Frontier Sciences & Institute of Medical
18 Science, University of Tokyo, Tokyo, Japan; Department of Otolaryngology, Head and Neck Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan

19 Address correspondence to H. Yano, yanohisa@med.tohoku.ac.jp
ABSTRACT

Acute otitis media (AOM) is the commonest upper respiratory tract infection in childhood. Children with AOM were enrolled at Tohoku Rosai Hospital between July 2006 and June 2011 if middle ear fluid culture after tympanocentesis yielded only *Haemophilus influenzae*. Susceptibility to ampicillin was determined, and a microtiter biofilm assay and invasion assay using BEAS-2B cells were performed. The association between these bacterial characteristics and clinical relapse and treatment failure of AOM was evaluated. Seventy-four children (39 boys and 35 girls) with median age of 1 year (IQR: 0.25-2) were enrolled. Among 74 *H. influenzae* isolates, 37 showed intermediate resistance or resistance to ampicillin (MIC≥2 µg/ml). In the microtiter biofilm assay, median OD600 was 0.68 (IQR: 0.24-1.02) and 70 isolates formed biofilms. The median invasion rate was 15% (IQR: 0-10%) and 46 isolates invaded BEAS-2B cells. Relapse and treatment failure occurred in 19 and 6 children, respectively. There was no significant difference of the invasion rate between patients with/without relapse or treatment failure. Also, there was no significant association between biofilm formation and relapse or treatment failure. The improvement of the severity score after 1 week was significantly associated with the recovery time (P<0.0001). We did not identify any significant association between relapse or treatment failure and bacterial factors.
AOM has a multifactorial etiology, and this may explain why we could not find a significant association. Improvement of the severity score after 1 week of treatment may be a useful predictor of the outcome of AOM.
INTRODUCTION

Acute otitis media (AOM) is the most common disease of the upper respiratory tract in childhood and treatment of AOM is the most frequent reason that children take antibiotics in the USA (1). In Japan, the incidence of AOM has increased recently and many children now need hospitalization to receive intravenous antibiotics for the treatment of intractable AOM with persistent purulent otorrhea (2).

We previously reported that Haemophilus influenzae can form a biofilm both in vitro (3) and in vivo (4). Bacterial biofilms are recognized as having an important role in various human infections and bacteria in a biofilm are more resistant to antibiotic therapy than are planktonic micro-organisms, suggesting that biofilms might play an important role in the pathogenesis and chronicity of otitis media (5). In addition, investigation of the mechanism of airway epithelium invasion by H. influenzae has revealed that the bacteria are internalized by the adenoid cells of children (6). We also previously demonstrated that H. influenzae isolated from clinical samples could invade and destroy human bronchial epithelial cells (BEAS-2B cells) (7), suggesting that such activity might delay the resolution of AOM. However, the association between biofilm formation or invasion of bronchial epithelial cells and the clinical course and outcome of AOM due to H. influenzae has been unclear.
Accordingly, we measured biofilm formation and invasion of bronchial epithelial cells by *H. influenzae* isolated from children with AOM, and we evaluated the association between these bacterial characteristics and the clinical course and outcome of AOM.
MATERIALS AND METHODS

Patients and study design. Children who attended the Department of Otolaryngology of Tohoku Rosai Hospital between July 2006 and June 2011 were enrolled if they fitted the following criteria: (i) age of less than 6 years; (ii) diagnosis of AOM by an otolaryngologist on the basis of symptoms (fever, irritability, and tugging of the ear) and signs (redness and bulging of the tympanic membrane); (iii) acute illness lasting less than 7 days; (iv) no spontaneous perforation and no tympanostomy tubes; and (v) follow-up until at least Day 10 ± 2 of the study (the 3rd visit). In addition, culture of middle ear fluid (MEF) after tympanocentesis only yielded *H. influenzae* in all patients. The age, sex, and attendance at a day care center were recorded.

MEF specimens by tympanocentesis were immediately placed in a sterile cotton swab (Seed swab γ No. 2, Eiken Chemical Co. Ltd., Tokyo, Japan). Each swab was plated onto chocolate and sheep blood agar plates, which were incubated at 35°C with 5% CO₂ for 18 to 24 h. *H. influenzae* strains were identified and confirmed by colony morphology, Gram staining, growth in chocolate agar but not in blood agar, the catalase test, and X and V factor requirements.

The first follow-up visit was scheduled for Day 4 or 5 (the day of enrollment was July 11, 2017 by guest http://jcm.asm.org/ Downloaded from
defined as Day 1), with additional follow-up visits for clinical evaluation on Day 10 ± 2 and Day 17 ± 2. All patients were followed until the recovery score equaled zero at 7 to 10 days intervals. In addition, unscheduled visits were allowed at any time if the patient's condition deteriorated.

Scores were assigned for the temperature (0 = less than 38°C, 1 = 38.0 to 38.5°C, 2 = 38.6 to 39°C, 3 = above 39°C) and for irritability, ear tugging, and redness and bulging of the tympanic membrane (0 = absent, 1 = mild, 2 = moderate, 3 = severe) (8).

Criteria for clinical failure and relapse were as follows. By definition at enrollment, initial examination of the MEF always detected purulent, mucopurulent, or seropurulent fluid. Persistence of MEF for more than 2 weeks after the first visit was defined as treatment failure. Relapse was defined as occurring when an ear that had responded previously developed new MEF at any time during follow-up.

After examination and tympanocentesis at the first visit, each child was treated with either amoxicillin (60 mg/kg/day), amoxicillin/clavulanic acid (90/6.4 mg/kg/day), or cefditoren pivoxil (18 mg/kg/day). When the clinical findings of the children treated with amoxicillin or amoxicillin/clavulanic acid did not improve at the first follow-up visit, the children were treated with cefditoren pivoxil.
This study was approved by the Human Ethics Review Boards of Tohoku Rosai Hospital (No. Oki-1), Tohoku University Graduate School of Medicine (No. 11518), and Kurume University (No. 07002).

Antimicrobial susceptibility testing, detection of β-lactamase, and serotypes. The minimum inhibitory concentration (MIC) of ampicillin (AMP), amoxicillin-clavulanic acid, piperacillin, cefaclor, ceftriaxone, cefditoren, meropenem, and levofloxacin was determined by the broth dilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (9). *H. influenzae* ATCC 49247 was used as the reference strain. β-lactamase production was assessed by the Nitrocefin test. Among the β-lactamase-non-producing isolates, strains with an MIC ≥ 2 μg/ml for AMP were defined as β-lactamase-non-producing AMP-resistant (BLNAR) *H. influenzae*, while MIC = 1 μg/ml meant low-BLNAR, and MIC ≤ 0.5 μg/ml meant β-lactamase-non-producing AMP-susceptible (BLNAS) *H. influenzae*. Among the β-lactamase-producing isolates (β-lactamase-producing AMP-resistant: BLPAR), strains that were resistant to amoxicillin-clavulanic acid (≥ 4/2 μg/ml) were classified as β-lactamase-producing amoxicillin-clavulanic acid resistant (BLPACR) *H. influenzae*. *H. influenzae* isolates were serotyped by slide agglutination with antisera purchased from DENKA SEIKEN Co., Ltd.
Microtiter biofilm assay. Biofilm formation by all *H. influenzae* isolates was assessed using a 96-well microplate, as described previously (4). The culture medium containing planktonic cells was stained with 1% crystal violet at room temperature. After rinsing three times with water, the dye bound to the biofilm was extracted with 230 μl of 95% ethanol for 15 min and the amounts of dye extracted was quantified by measuring the absorbance at 600 nm (OD600) with a microplate reader. The strains were tested in quadruplicate for each experiment and representative results from three different experiments are reported.

Invasion assay with BEAS-2B cells. An invasion assay using BEAS-2B cells was done with all isolates of *H. influenzae* as described previously (7). Bacterial suspensions of *H. influenzae* (about $6 \times 10^6$ cfu/ml) were added at 10 μl/well to the cell monolayers (MOI=0.6) and incubated for 3 h at 37°C under a 5% CO2 atmosphere, followed by washing 3 times with phosphate-buffered saline (PBS) and treatment with gentamicin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 200 μg/ml for 2 h. Then the monolayers were washed 3 more times with PBS and viable intracellular bacteria were released by treatment with 0.5 ml of 1% Triton X-100 (Sigma-Aldrich) in PBS for 15 min, after which samples were harvested and vortexed for 1 min to lyse the cells. The resultant suspensions were plated in serial
dilutions on chocolate agar plates (Nissui Pharmaceutical Co., Tokyo, Japan) at 35°C, and the colonies were counted after overnight incubation. *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* RDEC-1 were used as the positive and negative control strains for invasion assay. In the preliminary experiment, we confirmed that 1% Triton X-100 in PBS does not lyse *H. influenzae* isolates. Experiments were repeated at least 3 times with each strain. The bacterial invasion rate was calculated as follows: bacteria recovered from BEAS-2B cells (cfu/ml)/inoculated bacteria (cfu/ml) × 100 (%).

**Statistical analysis.** Statistical analyses were performed as follows. First, we examined differences of the time to recovery (when the severity score declined to zero) among patients with different severity scores at the initial examination by creating a box and whisker plot and performing Kruskal-Wallis test. We also examined differences of the relapse rate using Fisher’s exact test. Next, potential factors influencing the time to recovery were examined by multiple regression analysis, including the age, drug resistance, severity score at the initial examination, biofilm formation (average of 9 measurements), invasion rate (average of 3 measurements), and improvement of the severity score at 1 week after the initial examination. A forward-backward procedure was employed to construct a regression model. We confirmed significance of each factor selected by controlling for the influence of sex and age as...
covariates. A significance level of 0.05 was used in the regression analysis. R statistical environment software (version 2.14.2) was used for all analyses.
RESULTS

Profile of the patients and bacterial strains. A total of 74 children were enrolled in this study (Table 1). They included 39 boys and 35 girls, with median age of 1 year (IQR: 0.25-2). Sixty of the children (81.1%) were ≤ 2 years old. Seventy-four *H. influenzae* strains were isolated from these patients, including 37 (50.0%) BLNAR strains, 8 (10.8%) low-BLNAR strains, 2 (2.7%) BLPAR strains, and 2 (2.7%) BLPACR strains. The results of antimicrobial susceptibility testing are shown in Table 2. The serotypes of 74 *H. influenzae* isolates were all nontypeable.

Biofilm formation and invasion of BEAS-2B cells. The mean OD600 at 24 hours in the microtiter biofilm assay for the 74 *H. influenzae* isolates was 0.81 ± 0.77 (range: 0 – 3.5). Biofilm formation by the *H. influenzae* isolates was variable, but 70 (94.6%) out of 74 isolates showed the ability to form a biofilm even after 24 hours. The mean invasion rate of *H. influenzae* isolates for BEAS-2B cells was 0.29 ± 0.82. Forty-six (62.2%) of the 74 isolates showed the ability to invade BEAS-2B cells, and the highest invasion rate exceeded 5%.

Relationship between time to recovery and initial score or biofilm formation or invasion. Median of the time to recovery (when the severity score reached zero) was 9.5 (IQR: 7.3-19.0) days. Differences of the recovery time among patients with different initial
severity scores are shown in Figure 1. There was no significant association between the severity score and the recovery time ($P = 0.11$). In addition, there is no relationship between the time to recovery and the ability to form biofilms or invade cells (correlation coefficient was -0.06 and 0.01, respectively).

**Relationship between clinical outcome and biofilm formation or invasion of BEAS-2B cells.** Relapse and treatment failure were observed in 19 children (25.7%) and 6 children (8.1%), respectively, with both relapse and treatment failure occurring in 3 children (4.1%). The invasion rate of *H. influenzae* isolates for BEAS-2B cells was higher among the patients with relapse (0.57 ± 1.41%) than among those without relapse (0.19 ± 0.46%), but there was no significant difference of the invasion rate between relapse and non-relapse cases. There was also no significant difference of the invasion rates between patients with or without treatment failure. Moreover, there was no significant association between biofilm formation by *H. influenzae* and relapse or treatment failure (Figure 2).

**Factors influencing recovery.** Relationship between the relapse rate among patients and different severity scores at the initial examination was not significant ($P = 0.37$, Fisher’s exact test). Meanwhile, our regression analysis showed that the improvement of the severity score at 1 week after the initial examination was significantly associated with the time to
recovery ($P < 0.0001$). Only the improvement rate of the severity score was selected by the forward-backward procedure, and the association remained significant after adjustment for sex and age. The regression coefficient was $-0.26$ (95% CI: from -0.38 to -0.15), which indicates 10% improvement of the severity score at 1 week after the initial examination shortens the time to recovery of patients by 2.6 days.
DISCUSSION

AOM is a frequent complication of respiratory tract infection in children, and one of the main bacterial pathogens is *H. influenzae*. It was recently reported that AOM has become more difficult to treat with oral antibiotics when BLNAR *H. influenzae* is the causative pathogen (10). The global prevalence of BLNAR isolates of *H. influenzae* remains low, but these isolates have been emerging in some countries, particularly Japan (11). In the present study, 37 (50%) out of 74 *H. influenzae* strains showed intermediate resistance or resistance to AMP (MIC ≥ 2 μg/ml). However, there was no significant association between AMP resistance and the time to recovery or the relapse rate. We performed myringotomy and drainage of MEF in all of the patients on Day 1, and these procedures might have heavily influenced the improvement of AOM by antibiotic therapy.

A biofilm is a structured community of bacteria enveloped in a self-produced, extrapolymeric matrix that adheres to a surface, and biofilm production is a common cause of persistent and chronic bacterial infection (4). Recently, Torretta et al. investigated nasopharyngeal biofilm-producing pathogens in children with a history of mild/moderate recurrent AOM. They found that biofilm-producing pathogens were more frequently isolated from the nasopharynx in the recurrent AOM group than in the control group and *H. influenzae*...
was confirmed to be the main pathogen in the recurrent group (12). In addition, Bakaletz reported that biofilms contribute to both chronic otitis media and recurrent AOM (13). In the current study, nearly all of the *H. influenzae* isolates (94.6%) had the ability to form a biofilm. However, there was no significant association between biofilm formation and the clinical findings or outcome. AOM has a multifactorial etiology, and this may explain why we could not find a significant association between clinical findings or the outcome and the ability of causative bacteria to form biofilms.

Some reports have suggested that internalization of *H. influenzae* by epithelial cells has an important role in persistent and chronic infection by this microorganism (14, 15, 16). In the present study, 46 (62.2%) out of 74 isolates demonstrated the ability to invade BEAS-2B cells, indicating that many *H. influenzae* strains isolated from MEF of children with AOM can invade airway epithelial cells in vivo. Although there was no significant difference of the invasion rate between patients with or without relapse, the invasion rate of *H. influenzae* isolates for BEAS-2B cells was higher among the patients with relapse than among those without relapse. Presumably, when AOM is due to invasive *H. influenzae*, epithelial cells of the middle ear mucosa will be destroyed and damaged, so that AOM tends to relapse if the patient catches another respiratory tract infection.
This study showed that the improvement rate of the severity score at 1 week after the initial examination was significantly associated with the time to recovery. It is important for physicians and the parents of children with AOM to be able to predict the outcome of AOM at 1 week after the start of treatment. When there is little improvement of the score at 1 week, physicians should consider switching to another antibiotic.

In this study, we did not identify any significant association between clinical findings or the outcome and bacterial factors. Treatment failure and relapse of AOM in children are not only influenced by microbiological factors such as antibiotic resistance (11), viral co-infection (17, 18), biofilm formation (12, 13), and invasion of epithelial cells (19), but also by host factors including immaturity of the immune system (18), lack of breast-feeding (21), tubal dysfunction (22), recent antibiotic usage (23), and multiple episodes of AOM. In addition, environmental factors such as attending a day care center (2) and the presence of siblings (24) influence treatment failure and relapse of AOM. This complexity may explain why we could not identify a significant association with any of the bacterial factors. Further studies in a larger number of AOM patients are needed to more accurately assess risk factors for this disease.
ACKNOWLEDGMENTS

We thank Iku Kurokawa and Jun Masaki, who are clinical laboratory technologists at Tohoku Rosai Hospital, for their assistance in this study.

FUNDING

The authors have no support or funding to report. All work was funded by our department research budget.

COMPETING INTEREST

The authors have declared that no competing interests exist.
REFERENCES


β-glucan receptor in the nonopsonic entry of nontypeable *Haemophilus influenzae* into human monocytic and epithelial cells. J. Infect. Dis. **184**: 150-158.


Figure legends

Figure 1. Box and whisker plot of the time to recovery. The bold lines indicate the median, and the bottom and top of each box indicate the 25th and 75th percentiles, respectively. Differences of the time until recovery (when the severity score decreased to zero) between patients with different scores at the initial examination. There were no significant differences between them (P=0.46).

Figure 2. Dot plots of the mean OD600 (A) and the invasion rate (%) (B) in patients with or without relapse and dot plots of the mean OD600 (C) and the invasion rate (%) (D) in patients with or without treatment failure. The OD results are measures of the ability of the organisms to form biofilms. Although the invasion rate of H. influenzae isolates for BEAS-2B cells was higher among the patients with relapse (0.57 ± 1.41%) than among those without relapse (0.19 ± 0.46%), there was no significant difference. There was no significant association between biofilm formation and relapse or treatment failure.
Table 1. Characteristics of the patients and bacterial strains

<table>
<thead>
<tr>
<th></th>
<th>Relapse*</th>
<th>Treatment failure*</th>
<th>Neither relapse or treatment failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>19</td>
<td>6</td>
<td>52</td>
</tr>
<tr>
<td>Male : Female</td>
<td>10 : 9</td>
<td>3:3</td>
<td>27 : 25</td>
</tr>
<tr>
<td>Age, median (IQR)</td>
<td>1 (0.5-2)</td>
<td>1 (1-1)</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>Day care center attendance</td>
<td>9</td>
<td>3</td>
<td>24</td>
</tr>
</tbody>
</table>

*Haemophilus influenzae*

- BLNAR | 13 | 4 | 25
- low-BLNAR | 1 | 0 | 7
- BLNAS | 4 | 1 | 17
- BLPAR | 0 | 0 | 2
- BLPACR | 1 | 1 | 1

*There are three patients with both relapse and treatment failure*
Table 2. Susceptibility profile of *Haemophilus influenzae* isolates

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>range (μg/ml)</th>
<th>MIC90 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>max</td>
</tr>
<tr>
<td>ampicillin</td>
<td>≦0.12</td>
<td>≥16</td>
</tr>
<tr>
<td>amoxicillin-clavulanic acid</td>
<td>≦0.12</td>
<td>16</td>
</tr>
<tr>
<td>piperacillin</td>
<td>≦0.06</td>
<td>≥16</td>
</tr>
<tr>
<td>cefaclor</td>
<td>≦0.5</td>
<td>≥128</td>
</tr>
<tr>
<td>ceftriaxone</td>
<td>≦0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>cefditoren</td>
<td>≦0.03</td>
<td>0.5</td>
</tr>
<tr>
<td>meropenem</td>
<td>≦0.03</td>
<td>0.5</td>
</tr>
<tr>
<td>levofloxacin</td>
<td>≦0.03</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1. Box and whisker plot of the time to recovery. The bold lines indicate the median, and the bottom and top of each box indicate the 25th and 75th percentiles, respectively. Differences of the time until recovery (when the severity score decreased to zero) between patients with different scores at the initial examination. There were no significant differences between them ($P=0.46$).

Figure 2. Dot plots of the mean OD600 (A) and the invasion rate (%) (B) in patients with or without relapse and dot plots of the mean OD600 (C) and the invasion rate (%) (D) in patients with or without treatment failure. The OD results are measures of the ability of the organisms to form biofilms. Although the invasion rate of *H. influenzae* isolates for BEAS-2B cells was higher among the patients with relapse (0.57 ± 1.41%) than among those without relapse (0.19 ± 0.46%), there was no significant difference. There was no significant association between biofilm formation and relapse or treatment failure.