Improvement Rate of Acute Otitis Media Caused by *Haemophilus influenzae* at 1 Week Is Significantly Associated with Time to Recovery

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Acute otitis media (AOM) is the most frequent reason that children in the United States take antibiotics (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 the bacteria in a biofilm are more resistant to antibiotic therapy than are planktonic microorganisms, suggesting that biofilms might play an important role in the pathogenesis and chronicity of otitis media (3). In addition, an investigation of the mechanism of airway epithelium invasion by *H. influenzae* revealed that the bacteria are internalized by the adenoid cells of children (5). We also previously demonstrated that *H. influenzae* isolated from clinical samples can invade and destroy human bronchial epithelial cells (BEAS-2B cells) (6), 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 the 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 were (i) aged <6 years, (ii) were given a 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) had acute illness lasting ≤7 days, (iv) had no spontaneous perforation and no tympanostomy tubes, and (v) had follow-up until at least day 10 after the diagnosis of AOM by an otolaryngologist (3rd visit).

The MEF specimens from tympanocentesis were immediately collected with a sterile cotton swab (Seed swab no. 2; Eiken Chemical Co. Ltd., Tokyo, Japan). Each specimen was plated onto chocolate and sheep blood agar plates, which were incubated at 35°C under a 5% CO₂ atmosphere.
sphere for 18 to 24 h. The *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 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 was 0 at 7- to 10-day intervals. In addition, unscheduled visits were allowed at any time if the patient’s condition deteriorated.

Scores were assigned for temperature (0, <38°C; 1, 38.0 to 38.5°C; 2, 38.6 to 39°C; and 3, >39°C) and for irritability, ear tugging, and redness and bulging of the tympanic membrane (0, absent; 1, mild; 2, moderate; and 3, severe) (7).

The criteria for clinical failure and relapse were as follows. By definition at enrollment, the initial examination of the MEF always detected purulent, mucopurulent, or seropurulent fluid. The persistency of MEF for ≥2 weeks after the first visit was defined as a treatment failure. A relapse was defined as occurring when an ear that had responded previously developed new MEF at any time during the follow-up period. After examination and tympanocentesis at the first visit, each child was treated with either amoxicillin (60 mg/kg of body weight/day), amoxicillin-clavulanic acid (90/6.4 mg/kg/day), or cefditoren pivoxil (18 mg/kg/day). When the clinical findings for 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 MICs of ampicillin (AMP), amoxicillin-clavulanic acid, piperacillin, cepafolin, ceftriaxone, cefotidin, meropenem, and levofloxacin were determined by the broth dilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (8). *H. influenzae* ATCC 49247 was used as the reference strain. β-Lactamase production was assessed by the nitrocefin test. Among the β-lactamase-producing isolates, strains with MICs of ≥2 μg/ml for AMP were defined as β-lactamase-nonproducing AMP-resistant (BLNAR) *H. influenzae*, while those with MICs of 1 μg/ml were defined as low BLNAR and those with MICs of ≤0.5 μg/ml as β-lactamase-nonproducing 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 (MIC, ≥4/2 μg/ml) were classified as β-lactamase-producing amoxicillin-clavulanic acid-resistant (BLPACR) *H. influenzae*. The *H. influenzae* isolates were serotyped by slide agglutination with antisera purchased from Denka Seiken Co., Ltd. (Tokyo, Japan).

Microtiter biofilm assay. Biofilm formation by all *H. influenzae* isolates was assessed using 96-well microplates as described previously (4). The culture medium containing planktonic cells was stained with 1% crystal violet at room temperature. After the biofilm was rinsed three times with water, the dye bound to it was extracted with 230 μl of 95% ethanol for 15 min, and the amounts of dye extracted were quantified by measuring the optical density 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 here.

**Invasion assay with BEAS-2B cells.** Invasion assays using BEAS-2B cells were done with all isolates of *H. influenzae* from patients enrolled in this study as described previously (6). Bacterial suspensions of *H. influenzae* (about 6 × 10⁶ CFU/ml) were added at 10 μl/well to the cell monolayers (multiplicity of infection [MOI], 0.6) and incubated for 3 h at 37°C under a 5% CO₂ atmosphere, followed by 3 washes 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 the 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* PA01 and Escherichia coli RDEC-1 were used as the positive- and negative-control strains for the invasion assays. In the preliminary experiment, we confirmed that 1% Triton X-100 in PBS does not lyse *H. influenzae* isolates. The experiments were repeated at least 3 times with each strain. The bacterial invasion rate was calculated as (bacteria recovered from BEAS-2B cells [CFU/ml]÷inoculated bacteria [CFU/ml]) × 100 (%).

**Statistical analyses.** Statistical analyses were performed as follows. First, we examined differences in the times to recovery (when the severity score declined to 0) among patients with different severity scores at the initial examination by creating box-and-whisker plots and performing the Kruskal-Wallis test. We also examined differences in the relapse rates using Fisher’s exact test. Next, potential factors influencing the times to recovery were examined by multiple regression analyses, 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 in the severity score at 1 week after the initial examination. A forward-backward procedure was employed to construct a regression model. We confirmed the 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 analyses. R statistical environment software (version 2.14.2) was used for all analyses.

**RESULTS**

**Profiles 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 a median age of 1 year (interquartile range [IQR], 0.25 to 2 years). 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 h in the microtiter biofilm assay for the 74 *H. influenzae* isolates was 0.81 ± 0.77 (range, 0 to 3.5). Biofilm formation by the *H. influenzae* isolates was variable, but 70

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**TABLE 1 Characteristics of the patients and bacterial strains**

<table>
<thead>
<tr>
<th>Patient characteristic or bacterial strain</th>
<th>Relapse</th>
<th>Treatment failure</th>
<th>No relapse or treatment failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>9</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Male:female ratio</td>
<td>9:9</td>
<td>9:9</td>
<td>9:9</td>
</tr>
<tr>
<td>Age [median (IQR)] (yrs)</td>
<td>2 (1–1)</td>
<td>2 (1–1)</td>
<td>2 (1–1)</td>
</tr>
<tr>
<td>No. of patients attending day care centers</td>
<td>9</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

*a* There were three patients with both relapse and treatment failure.
(94.6%) out of 74 isolates showed the ability to form biofilms even after 24 h. 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.** The median time to recovery (when the severity score reached 0) was 9.5 days (IQR, 7.3 to 19.0 days). Differences in the recovery times among patients with different initial severity scores are shown in Fig. 1. There was no significant association between the severity score and the recovery time (*P* = 0.11). In addition, there was no relationship between the time to recovery among patients and the ability of the isolates to form biofilms or invade cells (correlation coefficients, −0.06 and 0.01, respectively).

**Relationship between clinical outcome and biofilm formation or invasion of BEAS-2B cells.** Relapses and treatment failures were observed in 19 (25.7%) and 6 (8.1%) children, 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 relapses (0.57 ± 1.41%) than among those without relapses (0.19 ± 0.46%), but there was no significant difference in the invasion rates between relapse and nonrelapse cases. There were also no significant differences in the invasion rates of isolates between patients with or those without treatment failure. Moreover, there was no significant association between biofilm formation by *H. influenzae* and relapse or treatment failure (Fig. 2).

**Factors influencing recovery.** The relationship between the relapse rates among patients and different severity scores at the initial examinations was not significant (*P* = 0.37, Fisher’s exact test). Meanwhile, our regression analysis showed that the improvement in 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% confidence interval [CI], −0.38 to −0.15), which indicates that a 10% improvement in the severity score at 1 week after the initial examination shortens the time to recovery of a patient by 2.6 days.

**DISCUSSION**

AOM is a frequent complication of respiratory tract infections 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 (9). The global prevalence of BLNAR isolates of *H. influenzae* remains low, but these isolates have been emerging in some countries, particularly Japan (10). In the present study, 37 (50%) out of 74 *H. influenzae* strains showed intermediate resistance or resistance to AMP (MICs ≥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 in the 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 infections (4). Recently, Torretta et al. investigated nasopharyngeal biofilm-producing pathogens in children with a history of recurrent mild/moderate 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 (11). In addition, Bakaletz reported that biofilms contribute to both chronic otitis media and recurrent AOM (12). In the current study, nearly all of the *H. influenzae* isolates (94.6%) had the ability to form biofilms. However, there was no significant association between biofilm formation and the clinical findings or the outcome. AOM has a multifactorial etiology, and this may explain why we did not find a significant association between the clinical findings or the outcome and the ability of causative bacteria to form biofilms.

Some reports have suggested that the internalization of *H. influenzae* by epithelial cells has an important role in persistent and chronic infections by this microorganism (13, 14, 15). 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 samples of children with AOM can invade the airway epithelial cells *in vivo*. Although there was no significant difference in the invasion rates between patients with or without relapse, the invasion rates of *H. influenzae* isolates for BEAS-2B cells were higher among the patients with relapse than among those without relapse. Presumably, when AOM is due to invasive *H. influenzae*, the epithelial cells of the middle ear mucosa will be

| Table 2 Susceptibility profile of *Haemophilus influenzae* isolates |
|------------------------|------------------|------------------|
| Antimicrobial agent     | Dose range (μg/ml) | MIC<sub>90</sub> (μg/ml) |
|                        | Minimum | Maximum |                      |
| Amoxicillin             | 0.12    | 16      | 8                    |
| Amoxicillin-clavulanic acid | 0.12    | 16      | 8                    |
| Piperacillin            | 0.06    | 16      | 0.25                 |
| Cefaclor               | 0.5     | 128     | 64                   |
| Ceftiraxone            | 0.03    | 0.25    | 0.25                 |
| Cefditoren             | 0.03    | 0.5     | 0.25                 |
| Meropenem              | 0.03    | 0.5     | 0.25                 |
| Levofloxacin           | 0.03    | 1       | 0.06                 |

**FIG 1** Box-and-whisker plot of times to recovery. The bold lines indicate medians, and the bottom and top of each box indicate the 25th and 75th percentiles, respectively. There was no significant difference in the times to recovery (when the severity scores decreased to 0) between patients with different scores at the initial examination (*P* = 0.46).
destroyed and damaged, so AOM tends to relapse if the patient acquires another respiratory tract infection.

This study showed that the improvement rate in 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 in the score at 1 week, physicians should consider switching to another antibiotic.

In this study, we did not identify any significant associations between the clinical findings or the outcomes and the bacterial factors. Treatment failures and relapses of AOM in children are influenced not only by microbiological factors, such as antibiotic resistance (10), viral coinfection (16, 17), biofilm formation (11, 12), and invasion of epithelial cells (18), but also by host factors, including immaturity of the immune system (17), lack of breastfeeding (19), tubal dysfunction (20), recent antibiotic usage (21), and multiple episodes of AOM. In addition, environmental factors such as attending a day care center (2) and the presence of siblings (22) 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 the risk factors for this disease.

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The authors declare no conflicts of interest.

REFERENCES


