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Antimicrobial Effect of Fluoroquinolones for the Eradication of Nontypeable *Haemophilus Influenzae* Isolates within Biofilms

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Biofilms can be defined as communities of microorganisms attached to a surface. Those bacterial biofilms cause serious problems, such as antibiotic resistance and medical device-related infections. Nontypeable *Haemophilus influenzae* (NTHi) is an important pathogen in respiratory infections, as it forms biofilms both in vitro and in vivo such as human middle ear. Recent reports indicate that otitis media, paranasal sinusitis and lower respiratory tract infections caused by *Haemophilus influenzae* have become more difficult to treat with oral antibiotic therapy. However, there has been no attention given to antibiotic eradication of NTHi biofilm. To investigate the antimicrobial effect of various antibiotics against NTHi biofilm formation, we conducted the following comparative study using both β-lactamase-negative ampicillin (AMP)-susceptible (BLNAS) and AMP-resistant (BLNAR) NTHi strains. In a microtiter biofilm assay, both levofloxacin and gatifloxacin, of the fluoroquinolone antibiotic group, significantly inhibited biofilm formation by BLNAS and BLNAR NTHi in a dose-dependent fashion compared to ampicillin of the penicillin antibiotic group, cefotaxime of the cephalosporin antibiotic group, and both erythromycin and clarithromycin of the macrolide antibiotic group. Furthermore, in flow cell chamber studies, confocal laser scanning microscopy counted survival bacteria in mature biofilm had been treated with gatifloxacin, ampicillin, cefotaxime and erythromycin. Only gatifloxacin completely killed the BLNAR NTHi isolates within biofilms without regard to the thickness of biofilm formation. The results of this study suggest that fluoroquinolones potentially have a role in therapy against diseases caused by both BLNAS and BLNAR NTHi isolates within biofilms. ———— *H. influenzae*; BLNAS; BLNAR; biofilm; fluoroquinolone; ceftriaxone; ampicillin; macrolide.

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Nontypeable *Haemophilus influenzae* (*H. influenzae*) is a gram-negative, pleomorphic bacterium that colonizes the human nasopharynx. NTHi can cause a variety of infections, including otitis media, sinusitis, conjunctivitis, bronchitis, and pneumonia (Murphy and Apicella 1987; Koyama et al. 2007). Recent reports state that β-lactamase-negative ampicillin (AMP)-resistant (BLNAR) strains have increased in some countries (Suzuki et al. 2003; Castanheira et al. 2006; Sunakawa and Farrell 2007), although their global prevalence remains low (Hoban and Felminghan 2002). Resistance in BLNAR strains results from mutations in the *ftsI* gene-encoding, penicillin-binding protein (*pbp*) 3, which mediates septal peptidoglycan synthesis (Hasegawa et al. 2004). Recent reports indicate that otitis media, paranasal sinusitis and lower respiratory tract infection caused by *H. influenzae* have become more difficult to treat with oral antibiotics therapy (Kaczmarek et al. 2004).

Biofilm is a structured community of bacterial cells enveloped in a self-produced, extrapolymeric matrix that is adherent to surfaces (Costerton et al. 1999; O'Toole et al. 2000). Bacterial biofilms are recognized as important causes of a variety of human infections, including infections of prosthetic devices, endocarditis, dental caries, pneumonia in cystic fibrosis, prostatitis and others (Singh et al. 2000; Donlan et al. 2001). It is well known that bacteria within biofilms are more resistant to antibiotic therapy than are planktonic organisms (Slinger et al. 2006). In order to develop strategies for the treatment of infections caused by bacteria in biofilms, including otitis media, it is important to elucidate the characteristics of bacterial pathogens within biofilms.

Recent reports state that NTHi forms biofilms both in vitro and in vivo, suggesting that biofilm formation in vivo might play an important role in the pathogenesis and chronicity of otitis media (Greiner et al. 2004; Jurcisek et al. 2005; Hall-Stoodley et al. 2006). Very little information is available concerning the effectiveness of various antimicrobials for the eradication of NTHi within biofilms. The aim of our study is to evaluate the antimicrobial effect of a variety of antibiotics on NTHi biofilms.

Materials and Methods

Bacterial strains and culture conditions

The H99 and H01 strains were clinical isolates obtained from different patients with respiratory tract infections. Before this experiment, microtiter biofilm assay was performed in each of approximately 20 BLNAS and 20 BLNAR strains obtained from different patients. The H99 and H01 strains were selected, because these strains had formed biofilms equally well. NTHi strains were reconstituted from frozen stocks and propagated on brain heart infusion (BHI) agar, or medium (Difco Laboratories, Detroit, MI, USA) supplemented with 10 μg/ml of hemin (Sigma Chemical Co., St. Louis, MO, USA) and 10 μg/ml of β-nicotinamide-adenine nucleotide (β-NAD) (Sigma) at 37°C and 5% CO_2 (Anderson et al. 1972).

Serotyping and PCR

H99 and H01 strains were serotyped by slide agglutination with antisera purchased from Difco Laboratories (Detroit, MI, USA). PCR was carried out for *H. influenzae* isolates by using mixed primers including the following (Wakunaga Pharmaceutical Co., Hiroshima): P6 primers to amplify the *p6* gene, which encodes the P6 membrane protein specific for *H. influenzae*; TEM-1 primers to amplify a part of the *β-lactamase* (*bla*) $_{\text{TEM-1}}$ gene; *pbp*3-1 primers to identify the same substitution as the low-BLNAR strains in the *fts I* gene; and *pbp* 3-2 primers to identify the same substitution as the BLNAR strains in the *fts I* gene (Hasegawa et al. 2004).

Antimicrobial susceptibility test

Minimum Inhibitory Concentrations (MICs) were determined using a broth microdilution method, according to guidelines from the Clinical and Laboratory Standards Institute (2007). Susceptibility of NTHi strains to the following 6 antibiotics was evaluated: ampicillin (AMP, Wako Pure Chemical Industries, Ltd., Osaka), cefotaxime (CTX, Wako), erythromycin (ERY, Dainippon Pharmaceutical Co., Osaka), clarithromycin (CLR, Abbott Japan Co., Tokyo), levofloxacin (LVX, Daiichi Sankyo Co., Ltd., Tokyo) and gatifloxacin (GAT, Kyorin Pharmaceutical Co., Tokyo). The antibiotics evaluated were those commonly approved for the treatment of bacterial infections from NTHi.

Microtiter biofilm assay

Biofilm formation in the H99 and H01 strains were assessed using a 96-well microplate, as previously described (Murphy and Kirkham 2002; Greiner et al. 2004). After being cultured for 48 hrs, the biofilms were exposed for 1 hr to differing concentrations (0.1 x MIC, 1 x MIC and 10 x MIC) of each antibiotics (AMP, CTX, ERY, CLR, LVX and GAT) solution every 12 hrs for 2 days (totally 4 doses). The evaluation of biofilm was performed immediately after the final exposure to the antibiotics solution. The culture medium containing planktonic cells was stained with 1% crystal violet at room temperature. After rinsing with water three times, the dye bound to the biofilm was extracted with 230 μl of 95% ethanol for 15 min. The extracted dye was quantified by measuring the absorbance at 600 nm (OD600) with a microplate reader. The strains were tested in quadruplicate for each experiment and the results were reported as three different experiments.

Biofilm growth in continuous flow cell chamber

Biofilms were formed in continuous flow cell chambers as described by Davies et al. (1998). The BLNAR H01 strain was grown in RPMI 1640 medium (Gibco BRI, Grand Island, NY, USA) supplemented with 1 μg/ml of protoporphyrin IX which is hemin without iron, 0.1 mg/ml of hypoxanthine, 0.1 mg/ml of uracil, 10 μg/ml of β-NAD, 0.8 mM of sodium pyruvate and 100 μM sialic acid, or supplemented BHI medium. The culture was diluted to an optical density (OD) of 0.15 at 600 nm and supplemented with RPMI 1640 medium. The cell suspensions of 1×10^8 colony-forming unit (cfu) /ml was used to inoculate a 37 × 5 × 5 mm flow cell chamber, filled with the prepared inoculum and incubated at 37°C for 1 hr to allow adherence to the glass coverslips (Seib et al. 2007). The chambers were then incubated for 48 hrs under 125 μl/min flow in supplemented fresh RPMI 1640 or BHI medium diluted 1:10 with sterile phosphate-buffered saline (PBS). After being cultured for 48 hrs, the biofilms were exposed for 1 hr to differing concentrations (0.1 x MIC, 1 x MIC and 10 x MIC) of each antibiotics (AMP, CTX, ERY and GAT) solution every 12 hrs for 2 days (totally 4 doses). After each exposure, the medium-containing antibiotic was replaced with fresh medium containing no antibiotic. Approximately 5 hrs after the final exposure to antibiotic solution, the biofilms were tested using a Dead/Live BacLight viability stain kit (Molecular Probes, Eugene, OR, USA), following the manufacturer's protocol, by counting the colonies.

Colony counting NTHi isolates within biofilms

To determine the antimicrobial effect of each antibiotics against NTHi isolates within biofilms, colony counting was performed after 48 hrs. To collect bacteria from the culture, the biofilms were removed from the flow chambers by flushing twice with 1 ml of sterile PBS at the end of each experiment. These washes were combined and aliquots (0.1 ml) of the suspension that had been subjected to serial 10-fold dilutions. These aliquots were sub-cultured onto supplemented BHI agars incubated at 37°C. The resultant colonies were counted to determine surviving colony-forming units (n = 3) at 48 hrs.

Quantification of biofilm structures

To evaluate the viability of biofilm structure after exposure to antibiotics, the biofilm formations were analysed by COMSTAT (BioCentrum-DTU, Lungby, Denmark) (Heydorn et al. 2000; Seib et al. 2007). COMSTAT is a program for quantification of three-dimensional biofilm structures. It analyzes stacks of images acquired with confocal laser scanning microscopy (CLSM). First, biofilms were stained using a Live/Dead BacLight bacterial viability stain kit (Molecular Probs, Eugene, OR, USA). This permits visualization of live and dead bacteria within the biofilms. Briefly, SYTO 9 and propidium iodide were mixed at a 1:1 ratio in sterile PBS solution. The staining solution was introduced into the chamber for 15 min at 37°C in the dark. Biofilm bacteria within the chamber were immediately visualized with CLSM (Nicon DIGITAL ECLIPSE C1, Nikon, Melville, NY, USA) using a modified stage. Three independent biofilm experiments were performed and at least four stacked images at 200 x magnification per experiment were obtained and average values were used for statistical analyses. Each of the four stacked images was selected from an apical surface area at random in the chamber and examined. The threshold value that best fit all image stacks of a trial was chosen and kept consistent for all stacks within the trial. Images were acquired at 2.0 μm intervals down through the biofilm Z-stack (μm). Therefore, the number of images in each stack varied according to the biomass and average thickness of the biofilm (Starner et al. 2006). Quantification of biofilm biomass and average thickness was calculated using a MatLab 5.3 (The MathWork, Inc., Natick, MA, USA), equipped with an Image Processing Toolbox and COMSTAT analysis. The image stacks of

"without" and "with" following exposure to each concentration of the antibiotic medium groups were averaged and compared.

Statistical tests and data analysis

All analyses of statistical significance were performed with one-tailed Student's *t*-tests using Microsoft Excel (Microsoft Corp., Redmond, WA, USA). *P* values less than were considered statistically significant. For quantification of biofilm mass, each datapoint used was the mean biomass of four randomly imaged areas from a single biofilm. Mean biomass values from different biofilm experiments were used for further statistical analyses (Starner et al. 2006).

Results

Characteristics of strains

The H99 and H01 strains used in this study are listed in Table 1. The H99 and H01 strain were both nontypeable as assayed by slide agglutination with antisera. The MIC of H99 against AMP was 0.06 mg/l and H99 did not have the mutation of *pbp* 3-1 and *pbp* 3-2 by PCR, whereas the MIC of H01 against AMP was 1.0 mg/l and H01 had the mutation of *pbp* 3-1 and *pbp* 3-2 by

Table 1. MICs of antibiotics and resistant gene in this study.

Strain	MIC (mg/L)						Resistant gene by PCR[g]		
	AMP[a]	CTX[b]	ERY[c]	CLR[d]	LVX[e]	GAT[f]	TEM-1	*pbp*3-1	*pbp*3-2
H-99	0.06	0.06	0.5	2.0	≦ 0.06	≦ 0.06	-	-	-
H-01	1.0	0.25	1.0	8.0	≦ 0.06	≦ 0.06	-	+	+

[a]AMP, ampicillin. [b]CTX, cefotaxime. [c]ERY, erythromycin. [d]CLR, clarithromycin. [e]LVX, levofloxacin. [f]GAT, Gatifloxacin. [g]TEM-1, type β-lactamase gene; TEM-1: -, None.
Penicillin binding protein genes; *pbp*3-1 and *pbp*3-2: +, altered; -, not altered.

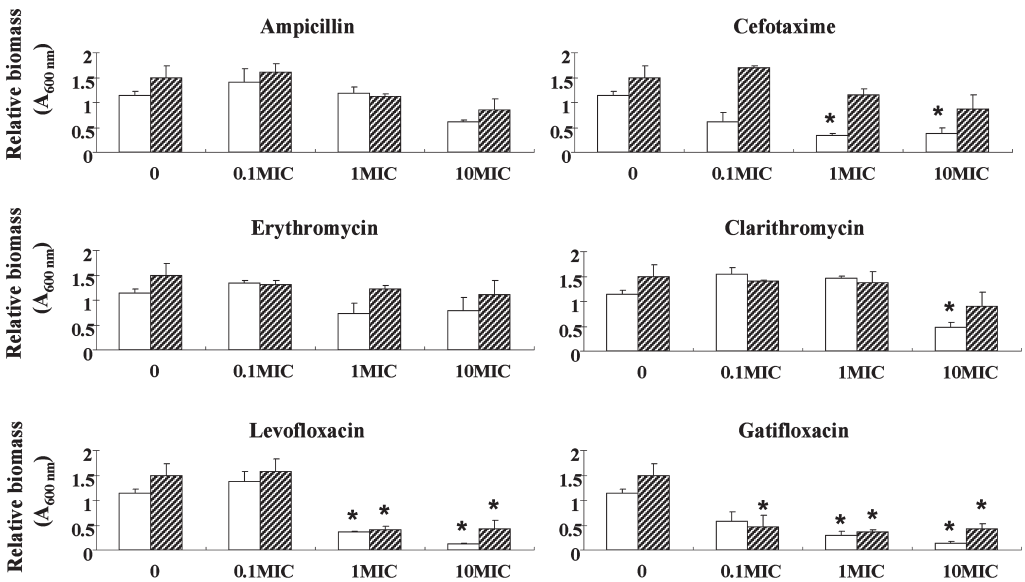

Fig. 1. Antimicrobial effect of 6 antibiotics against NTHi isolates within biofilms between BLNAS (H99, white bars) and BLNAR (H01, diagonally striped bars) strains by microtiter biofilm assay.
1 x and 10 x MIC of cefotaxime, and 10 x MIC of clarithromycin significantly inhibited only the BLNAS NTHi biofilms. By comparison, 1 x and 10 x MIC of levofloxacin and gatifloxacin inhibited both the BLNAS and BLNAR NTHi biofilms. Results are given as the average of 3 replicates with each performed in quadruplicate ± S.D.
$p < 0.001$ as compared with the same strain without exposure to antibiotics.

PCR. As a result, it was determined that H99 was a BLNAS and H01 was a BLNAR strain.

Antimicrobial effect of antibiotics against BLNAS and BLNAR NTHi biofilms by microtiter biofilm assay

The antimicrobial effect of 6 antibiotics against BLNAS and BLNAR NTHi biofilms were measured by microtiter biofilm assay (Fig. 1). As can be seen, 1 x and 10 x MIC of CTX, and 10 x MIC of CLR significantly inhibited only the BLNAS (H99) NTHi biofilm. By comparison, 1 x and 10 x MIC of LVX and GAT inhibited both the BLNAS (H99) and BLNAR (H01) NTHi biofilms (Fig. 1).

GAT completely kills BLNAR strain within biofilms

Using a flow-cell system, the antimicrobial effects of various antibiotics against the BLNAR (H01) strain within biofilms were evaluated after exposure to 0.1 x, 1 x and 10 x MIC of AMP, CTX, ERY and GAT (Fig. 2). AMP, ERY and CTX had no effect on viability at any concentration. However, the effect of GAT against the BLNAR (H01) strain within biofilms was dose-dependent and, under the concentration of 10 x MIC, killed completely. Qualitative evaluation using CLSM indicated that the total biomass of the biofilm exposed to each concentration of AMP and CTX were significantly higher than that of GAT (Fig. 3A) as assayed by COMSTAT software. Biofilms treated with GAT had a significantly lower ($p = 0.001$) total biomass than those treated with AMP and CTX. The total biomass of the biofilm after exposure to 0.1 x and 1,0 x MICs of ERY tended to be lower than that of GAT — but not significantly lower —, however, the total biomass of the 10 x MIC ERY treated biofilm was significantly higher ($p = 0.001$) than that of GAT. The average thickness of all the biofilms was similar whether or not the organisms were treated with antibiotics (Fig. 3B).

Discussion

NTHi is one of the most prevalent pathogens associated with respiratory tract infections and thus acquisition of antimicrobial resistance raises concern. The prevalence of BLNAR strains was reported to be 2.4% in the USA between 2002 and 2003 (Heilmann et al. 2005) and 9.3% in Spain between 1998 and 1999 (Marco et al. 2001). Their global prevalence of BLNAR strains remains relatively low. However, the prevalence of BLNAR in Japan has increased rapidly, from 5.8% in 2000 to 34.5% in 2004, even in *H. influ-*

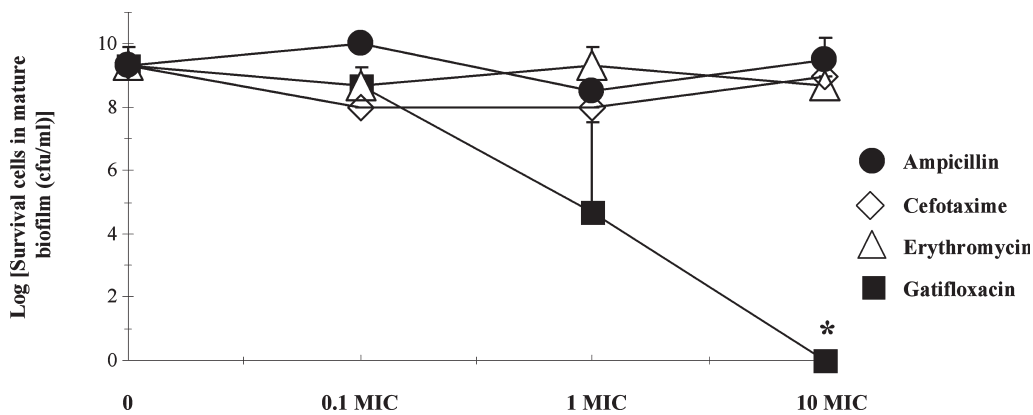

Fig. 2. Antimicrobial effect of 4 antibiotics of ampicillin (filled circle), cefotaxime (open diamonds), erythromycin (open triangles), or gatifloxacin (filled squares) against BLNAR NTHi isolate (H01) within biofilms in a continuous-flow cell chamber.
Ampicillin, erythromycin and cefotaxime had no effect on viability at any concentration, whereas the effect of gatifloxacin against the BLNAR strain within biofilms was dose-dependent and, under the concentration of 10 x MIC, killed completely. Results are given as average of 3 replicates ± S.D.
*$p < 0.001$ as compared with the strain without exposure to antibiotics.

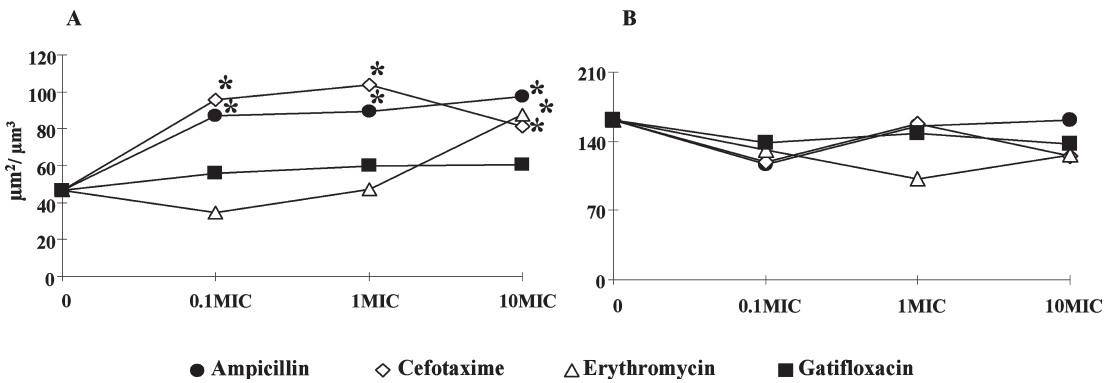

Fig. 3. Comparison of the biomass (A) and average thickness (B) of biofilms after exposure to 4 antibiotics of ampicillin (filled circle), cefotaxime (open diamonds), erythromycin (open triangles), or gatifloxacin (filled squares).
Biofilms exposed to gatifloxacin had a significantly lower total biomass than those to ampicillin and cefotaxime. The total biomass of the biofilms after exposure to 0.1 x and 1.0 x MICs of erythromycin tended to be lower than that of gatifloxacin, but the total biomass of the 10 x MIC erythromycin treated biofilm was significantly higher than that of gatifloxacin. The average thickness of all of the biofilms was similar whether or not the organisms were treated with antibiotics.
*$p < 0.001$ as compared with the strain exposed to without antibiotics.

enzae type b isolated from patients with meningitis (Hasegawa et al. 2004). The result has been an increasing number of cases of otitis media, which are difficult to treat, and an increasing incidence of treatment failures with oral antibiotics in young children (Starner et al. 2006). This is due in large part to antibiotic resistance, which points to a need for oral therapy to change along with surgical management of tympanostomy or tympanostomy tubes (Casey et al. 2004).

However, the pathogenesis of NTHi infection of airway cells involves both an intracellular life cycle as well as biofilm formation on the surface of the airway epithelia. Therefore, because NTHi invade human bronchial epithelial cells by macropinocytosis (Ketterer et al. 1999) and form biofilms both in vitro and in vivo (Greiner et al. 2004; Jurcisek et al. 2005; Hall-Stoodley et al. 2006), it is not clear whether drug resistance alone causes treatment difficulty and failure in otitis media among young children. To our knowledge, the antimicrobial effect of antibiotics against NTHi within biofilms remains unclear. Our data show that LVX and GAT have significant inhibitory effect against both BLNAS and BLNAR NTHi within biofilms compared to AMP, CTX, ERY and CLR. Our results agree with previous investigations that studied *P. aeruginosa* biofilm, as it has been shown that fluoroquinolones can penetrate exopolysaccharides (Kumon et al. 1994; Yassien et al. 1995). Since the use of fluoroquinolones in young children is limited in many countries because of adverse neurological side effects, the clinical antimicrobial effectiveness against BLNAS and BLNAR NTHi in biofilms has not been confirmed. AMP, CTX, ERY and CLR had no significant effect against either BLNAS or BLNAR NTHi biofilms, although it has been reported that macrolides have an inhibitory effect on *P. aeruginosa* biofilm formation and survival cells in biofilm (Favre-Bonté et al. 2003; Nalca et al. 2006).

There may be several limitations in the present study. First, only single BLNAS and BLNAR strains were investigated, although the biofilm formation among each of 20 BLNAS and 20 BLNAR strains were investigated before these studies and these two strains were selected. The second limitation is a lack of in vivo data.

In conclusion, our data demonstrate that fluoroquinolones could be effective therapeutic agents against biofilm diseases caused by both BLNAS and BLNAR NTHi. Further investigations, such as the chinchilla otitis media model or

clinical trial to determine the in vivo antimicrobial effect of fluoroquinolones on NTHi biofilms should be considered.

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References

Anderson, P., Peter G., Johnston, R.B., Jr., Wetterlow, L.H. & Smith, D.H. (1972) Immunization of humans with polyribophosphate, the capsular antigen of *Haemophilus influenzae*, type b. *J. Clin. Invest.,* **51**, 39-44.

Casey, J.R. & Pichichero, M.E. (2004) Changes in frequency and pathogens causing acute otitis media in 1995-2003. *Pediatr. Infect. Dis. J.,* **23**, 824-828.

Castanheira, M., Gales, A.C., Pignatari, A.C., Jones, R.N. & Sader, H.S. (2006) Changing antimicrobial susceptibility patterns among *Streptococcus pneumoniae* and *Haemophilus influenzae* from Brazil: Report from the SENTRY Antimicrobial Surveillance Program (1998-2004). *Microb. Drug Resist.,* **2**, 91-98.

Clinical and Laboratory Standards Institute (2007) Performance standards for antimicrobial susceptibility testing: M100-S17, 17th informational supplement. *CLSI*, Wayne, PA.

Costerton, J.W., Stewart, P.S. & Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science,* **284**, 1318-1322.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W. & Greenberg, E.P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science,* **280**, 295-298.

Donlan, R.M. (2001) Biofilm formation: a clinically relevant microbiological process. *Clin. Infect. Dis.,* **33**, 1387-1392.

Favre-Bonté, S., Köhler, T. & Van Delden, C. (2003) Biofilm formation by *Pseudomonas aeruginosa*: role of the C4-HSL cell-to-cell signal and inhibition by azithromycin. *J. Antimicrob. Chemother.,* **52**, 598-604.

Greiner, L.L., Watanabe, H., Phillips, N.J., Shao, J., Morgan, A., Zaleski, A., Gibson, B.W. & Apicella, M.A. (2004) Nontypeable *Haemophilus influenzae* strain 2019 produces a biofilm containing N-acetylneuraminic acid that may mimic sialylated O-linked glycans. *Infect. Immun.,* **72**, 4249-4260.

Hall-Stoodley, L., Hu, F.Z., Gieseke, A., Nistico, L., Nguyen, D., Hayes, J., Forbes, M., Greenberg, D.P., Dice, B., Burrows, A., Wackym, P.A., Stoodley, P., Post, J.C., Ehrlich, G.D. & Kerschner, J.E. (2006) Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA,* **296**, 202-211.

Hasegawa, K., Chiba, N., Kobayashi, R., Murayama, S.Y., Iwata, S., Sunakawa, K. & Ubukata, K. (2004) Rapidly increasing prevalence of beta-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. *Antimicrob. Agents Chemother.*, **48**, 1509-1514.

Hasegawa, K., Kobayashi, R., Takada, E., Ono, A., Chiba, N., Morozumi, M., Iwata, S., Sunakawa, K. & Ubukata, K.; Nationwide Surveillance for Bacterial Meningitis (2006) High prevalence of type b beta-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. *J. Antimicrob. Chemother.,* **57**, 1077-1082.

Heilmann, K.P., Rice, C.L., Miller, A.L., Miller, N.J., Beekmann, S.E., Pfaller, M.A., Richter, S.S. & Doern, G.V. (2005) Decreasing prevalence of beta-lactamase production among respiratory tract isolates of *Haemophilus influenzae* in the United States. *Antimicrob. Agents Chemother.,* **49**, 2561-2564.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B.K. & Molin, S. (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology,* **146**, 2395-2407.

Hoban, D. & Felmingham, D. (2002) The PROTEKT surveillance study: antimicrobial susceptibility of *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections. *J. Antimicrob. Chemother.,* **50**, 49-59.

Jurcisek, J., Greiner, L., Watanabe, H., Zaleski, A., Apicella, M.A. & Bakaletz, L.O. (2005) Role of sialic acid and complex carbohydrate biosynthesis in biofilm formation by nontypeable *Haemophilus influenzae* in the chinchilla middle ear. *Infect. Immun.,* **73**, 3210-3218.

Kaczmarek, F.S., Gootz, T.D., Dib-Hajj, F., Shang, W., Hallowell, S. & Cronan, M. (2004) Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* with unusually high resistance to ampicillin. *Antimicrob. Agents Chemother.,* **48**, 1630-1639.

Ketterer, M.R., Shao, J.Q., Hornick, D.B., Buscher, B., Bandi, V.K. & Apicella, M.A. (1999) Infection of primary human bronchial epithelial cells by *Haemophilus influenzae*: macropinocytosis as a mechanism of airway epithelial cell entry. *Infect. Immun.,* **67**, 4161-4170.

Koyama, J., Ahmed, K., Zhao, J., Saito, M., Onizuka, S., Oma, K., Watanabe, K., Watanabe, H. & Oishi, K. (2007) Strain-specific pulmonary defense achieved after repeated airway immunizations with non-typeable *Haemophilus influenzae* in a mouse model. *Tohoku J. Exp. Med.*, **211**, 63-74.

Kumon, H., Tomochika, K., Matunaga, T., Ogawa, M. & Ohmori, H. (1994) A sandwich cup method for the penetration assay of antimicrobial agents through Pseudomonas exopolysaccharides. *Microbiol. Immunol.,* **38**, 615-619.

Marco, F., Garcia-de-Lomas, J., Garcia-Rey, C., Bouza, E., Aguilar, L., Fernandez-Mazarrasa, C. & The Spanish Surveillance Group for Respiratory Pathogens (2001) Antimicrobial susceptibilities of 1,730 *Haemophilus influenzae* respiratory tract isolates in Spain in 1998-1999. *Antimicrob. Agents Chemother.*, **45**, 3226-3228.

Murphy, T.F. & Apicella, M.A. (1987) Nontypeable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. *Rev. Infect. Dis.,* **9**, 1-15.

Murphy, T.F. & Kirkham, C. (2002) Biofilm formation by nontypeable *Haemophilus influenzae*: strain variability, outer membrane antigen expression and role of pili. *BMC Microbiol.,* **2**, 7.

Nalca, Y., Jäsch, L., Bredenbruch, F., Geffers, R., Buer, J. & Hässler, S. (2006) Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach. *Antimicrob. Agents Chemother.,* **50**,

1680-1688.

O'Toole, G., Kaplan, H.B. & Kolter, R. (2000) Biofilm formation as microbial development. *Annu. Rev. Microbiol.,* **54**, 49-79.

Seib, K.L., Wu, H.J., Srikhanta, Y.N., Edwards, J.L., Falsetta, M.L., Hamilton, A.J., Maguire, T.L., Grimmond, S.M., Apicella, M.A., McEwan, A.G. & Jennings, M.P. (2007) Characterization of the OxyR regulon of *Neisseria gonorrhoeae*. *Mol. Microbiol.,* **63**, 54-68.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J. & Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature,* **407**, 762-764.

Slinger, R., Chan, F., Ferris, W., Yeung, S.W., St. Denis, M., Gaboury, I. & Aaron, S.D. (2006) Multiple combination antibiotic susceptibility testing of nontypeable *Haemophilus influenzae* biofilms. *Diagn. Microbiol. Infect. Dis.,* **56**, 247-253.

Starner, T.D., Zhang, N., Kim, G., Apicella, M.A. & McCray, P.B., Jr. (2006) *Haemophilus influenzae* forms biofilms on airway epithelia: implications in cystic fibrosis. *Am. J. Respir. Crit. Care. Med.,* **174**, 213-220.

Sunakawa, K. & Farrell, D.J. (2007) Mechanisms, molecular and sero-epidemiology of antimicrobial resistance in bacterial respiratory pathogens isolated from Japanese children. *Ann. Clin. Microbiol. Antimicrob.,* **13**, 6-7.

Suzuki, K., Nishimura, T. & Baba, S. (2003) Current status of bacterial resistance in the otolaryngology field: results from the Second Nationwide Survey in Japan. *J. Infect. Chemother.,* **9**, 46-52.

Yassien, M., Khardori, N., Ahmedy, A. & Toama, M. (1995) Modulation of biofilms of *Pseudomonas aeruginosa* by quinolones. *Antimicrob. Agents Chemother.,* **39**, 2262-2268.