Clonal spread of β-lactamase-producing amoxicillin–clavulanate-resistant (BLPACR) strains of non-typeable Haemophilus influenzae among young children attending a day care in Japan

2010/04/28

Clonal Spread of β-lactamase-producing amoxicillin-clavulanate-resistant (BLPACR) Strains of Non-typeable *Haemophilus influenzae* among Young Children Attending A Day Care in Japan

Makoto Ito, MD, PhD[1)]*, Muneki Hotomi, MD, PhD[2)], Yumiko Maruyama, MD, PhD[1) 3)], Miyako Hatano, MD, PhD[1)], Hisashi Sugimoto, MD, Tomokazu Yoshizaki, MD, PhD[1)],

and

Noboru Yamanaka, MD, PhD[2)]

[1)] Department of Otolaryngology-Head and Neck Surgery, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

[2)] Department of Otolaryngology-Head and Neck Surgery, Wakayama Prefectural Medical School, Wakayama, Japan

[3)] Department of Otolaryngology, Kurobe Civic Hospital, Kurobe, Japan

Text pages: 9 Figure: 1 Tables: 3

Abbreviated title: Clonal Spread of BLPACR of Non-typeable *Haemophilus influenzae* in Day Care

Running Head: BLPACR of NTHi in Day Care

Address first author and proofs are to be sent to:

Makoto Ito, MD, PhD; Associate Professor

Department of Otolaryngology-Head and Neck Surgery, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

Tel: 72-265-2413; Fax: 72-234-4265

E-mail: makoto@med.kanazawa-u.ac.jp

Word count: 2458

ABSTRACT

***Objective*:** Resistant strains of non-typeable *Haemophilus influenzae* (NTHi) are one of the principal causes of recurrent acute otitis media (otitis prone), rhinosinusitis, and pneumonia in young children. β-lactamase-nonproducing ampicillin-resistant (BLNAR) strains are particularly common in Japan, and β-lactamase-producing amoxicillin-clavulanate resistant (BLPACR) strains are now emerging. We investigated the nasopharyngeal carriage status of these resistant strains among children attending a same day care center during a 10-year period.

***Methods*:** From 1999 to 2008, we obtained nasopharyngeal swab specimens from young children attending a same day care center and examined the incidence of resistant strains of NTHi. Antimicrobial resistance of NTHi was identified based on PCR analysis of mutation of the penicillin binding protein (PBP) genes. Pulsed-field gel electrophoresis (PFGE) was performed to examine the clonal relationship of each resistant strain.

***Results*:** The prevalence of resistant strains of NTHi among the children attending this day care has significantly increased during the past 10 years and most of this day care children recently have resistant strains with PBP gene mutations in their nasopharynx. Genetically BLPACR (gBLPACR) strains have rapidly increased since 2007 and PFGE analysis demonstrated that all gBLPACR were clonally identical. This is the first report of apparent clonal dissemination of gBLPACR strains of NTHi occurring in a certain environment such as day care.

***Conclusions*:** The rapidly increasing prevalence of resistant strains, in particular gBLPACR, in this day care center may predict a high incidence of these resistant bacteria from clinical isolates in the near future and potential serious medical problems worldwide.

Keywords: non-typeable *Haemophilus influenzae* (NTIs); β-lactamase-producing amoxicillin-clavulanate-resistant (BLPACR); β-lactamase-nonproducing ampicillin-resistant (BLNAR); pulsed-field gel electrophoresis (PFGE); day care

List of abbreviations: AOM; acute otitis media, NTHi: non-typeable *Haemophilus influenzae*, gBLPACR; genetically β-lactamase-producing amoxicillin-clavulanate-resistant, gBLNAR; genetically β-lactamase-nonproducing ampicillin-resistant, gBLPAR: genetically β-lactamase-producing ampicillin-resistant, gBLNAS; genetically β-lactamase-nonproducing ampicillin-susceptible, Pc^r; penicillin resistance, *S.pneumoniae* (SP); *Streptococcus pneumoniae*, PCR; polymerase chain reaction, PBP; penicillin binding protein, RTIs; respiratory tract infections, PFGE; pulsed-field gel electrophoresis

INTRODUCTION

Non-typeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae* (*S. pneumoniae*) are the principal causes of acute otitis media (AOM), pneumonia and meningitis in young children. Recently, rapid increases in β-lactamase-nonproducing ampicillin-resistant (BLNAR) strains of NTHi and a penicillin resistant (Pc^r) strain of *S. pneumoniae* from respiratory tract specimens have been reported in Japan [1,2]. BLNAR strains of NTHi and Pc^r *S. pneumoniae* are the principal causes of recurrent AOM (otitis prone) among young children, especially children attending day care.

Over the past three decades, a rapid increase in Pc^r *S. pneumoniae* has been reported in most areas of the world [3-5]. The high prevalence of Pc^r *S. pneumoniae* has resulted in the limited use of penicillin for the empirical treatment of infectious diseases [6]. These penicillin-resistant bacteria are becoming less susceptible to other commonly prescribed oral anti-microbial drugs, including the extended spectrum cephalosporins [7,8]. A similar situation is now emerging among NTHi isolates from patients with respiratory tract infections (RTIs) [9]. In Japan, the BLNAR strains were not identified before 1984 then increased 23.1% to 37.8% from 1996 to 1999. BLNAR strains are particularly common in Japan and France. BLNAR strains evolved significantly during the past decade in some countries while oral cephalosporin antimicrobial agents were being commonly used for the treatment of RTIs, including AOM and sinusitis. The increase in the percentage of BLNAR strains has led to serious problems in the treatment of infectious disease in Japan. Furthermore, another group of resistant strains with combined mechanisms of altered penicillin binding proteins (PBPs) and TEM β-lactamase classified as β-lactamase-producing amoxicillin-clavulanate-resistant (BLPACR) strains are now emerging [10,11]. BLPACR strains have another resistant mechanism of β-lactamase producing in addition to the BLNAR resistance with PBP gene mutations. BLPACR are highly resistant to amoxicillin/clavulanate and other commonly prescribed oral anti-microbial drugs compared to BLNAR. The high prevalence of BLPACR represents potentially more serious clinical problems than BLNAR.

Children attending day care centers have more frequent episodes of otitis prone than those cared for at home [12-14]. Several lines of studies have shown that the incidence of nasopharyngeal carriage of resistant strains of *S. pneumoniae* and NTHi is high in children attending day care centers, and attendance at a day care center is a risk factor for otitis prone [15,16]. Previously, we described a strong relationship between exposure to other children in day care and nasopharyngeal carriage of Pc^r *S. pneumoniae* among young children in Japan [1]. In addition to *S. pneumoniae*, the prevalence of nasopharyngeal carriage of other respiratory pathogens, such as NTHi and *Moraxella catarrhalis*, in children attending day care is also very high in Japan[1]. In contrast, the carriage rates of these pathogens from children who were healthy and cared for at home were much lower than those from day care children [1].

Recently, Ubukata et al. defined antimicrobial resistances of NTHi genetically based on the mutation status of the PBP genes [10]. BLPACR strains of NTHi were recently isolated and, at least for now, generally continue to be isolated at very low frequencies (0.04 to 2.5%) worldwide.

The resistance patterns of these RTIs pathogens differ and may change dramatically over time. In the present study, to reveal the bacteriological change in the nasopharyngeal flora of healthy children, the carriage status of antibiotic resistant strains of NTHi were examined at the same day care center over a 10-year period.

MATERIALS AND METHODS

From 1999 to 2008, we obtained nasopharyngeal swab specimens once a year from children between 5 months and 3 years of age attending the same day care center during January to March in Kanazawa City, Japan. Each child was sampled only one time in each year. None of the participants in these studies showed any symptoms of RTIs or AOM on the day of the survey. Written informed consent was obtained from the parents of each child. If a parent refused to consent, the child was excluded from the study.

The parents completed a self-reporting questionnaire at the time of consent. The questionnaire included questions about previous hospitalization and the number of episodes of AOM, rhinosinusitis and pneumonia. The number of participants was 34 in 1999, 42 in 2000, 36 in 2005, 35 in 2007 and 31 in 2008. Nasopharyngeal swab specimens for culture were obtained by trained investigators using aluminum shaft ear, nose and throat swabs inserted as far into the nose as possible, parallel to the roof of the mouth. Antimicrobial resistances of NTHi were identified based on polymerase chain reaction (PCR) analysis of mutation of the PBP genes as described by Ubukata et al. [10]. Four sets of primers were obtained from Wakunaga Pharmaceutical Co. (Hiroshima, Japan): P6 primers to amplify the P6 gene which encodes the P6 membrane protein specific for NTHi; TEM-1 primers to amplify a part of the $bla_{\text{TEM-1}}$ gene; PBP3-S primers to identify an Asn 526→Lys amino acid substitution in the *ftsI* gene; and PBP3-BLN primers to identify an ASN526→Lys and Ser385→Thr amino-acid substitution in the *ftsI* gene. On the basis of the PCR-based genotyping, the NTHi strains were classified into four genotypes: genetically β-lactamase-nonproducing ampicillin-susceptible strains (gBLNAS), without amino acid substitutions in the *ftsI* gene and β-lactamase gene; genetically BLNAR strains (gBLNAR), with an amino acid substitution in the *ftsI* gene but without the β-lactamase gene; genetically β-lactamase-producing ampicillin-resistant strains (gBLPAR), with the β-lactamase gene but without an amino acid substitution in the *ftsI* gene; and genetically BLPACR strains (gBLPACR), with β-lactamase gene and an amino acid substitution in the *ftsI* gene. In this study, we have designated PCR-based genotypes as gBLNAS, gBLNAR, gBLPAR and gBLPACR to distinguish them from the phenotypes, which are written without the introductory “g.”

Pulsed-field gel electrophoresis (PFGE) was performed with all NTHi isolates obtained in 2008 to examine the clonal relationship of each resistant strain. Association between variables was assayed using the chi-squared test and Fisher Exact Test, using the StatView computer software (Abacus Concepts, Inc., Baltimore, Maryland, USA).

RESULTS

A total of 178 nasopharyngeal swab specimens from young children in the day care center were examined in this study. The mean age of the children was 20.4 months (1999), 25.1 months (2000), 22.9 months (2005), 24.1 months (2007), and 25.1 months (2008). Most of the children entered this day care in April and we obtained nasopharyngeal swab specimens during January to March. These children have been in this day care at least nine months.

Multiple pathogenic bacteria were cultured from the nasopharynx. The chief bacteria found in children attending day care were *S.pneumoniae* (148 strains, 83.1%), NTHi (155 strains, 87.1%) and MC (138 strains, 77.5%). Nasopharyngeal carriage of these specific respiratory pathogens in each year is listed in Table 1. In 1999, 26 strains (76.5%) of NTHi were isolated from the nasopharynx of 34 children attending the day care center. The carrier rates of NTHi were 39/42 (92.9%) isolates in 2000, 34/35 (97.1%) isolates in 2007 and 29/31 (93.5%) in 2008. Recently, almost all young children attending this day care center were carriers of NTHi. The difference of carriage rates of NTHi was not statistically different in each year.

The percentages of the resistant isolates for NTHi in each year are listed in Table 2. In 1999, gBLNAR were identified in 6/26 (23.1%) NTHi isolates. The other 20/26 (76.9%) strains of NTHi were gBLNAS. None of the children showed AOM or RTIs symptoms on the day of the medical examination. In 2000, antibiotic resistance in NTHi isolates was 11/39 (28.3%) and resistant rate is almost same as 1999. In 2005, the carrier rate of gBLNAR had significantly increased to 21/27 (77.8%) NTHi isolates in the same day care center ($p<0.05$). In 2007, gBLNAR were identified in 6/34 (17.6%) of isolates. gBLPACR isolates of NTHi were initially identified in 10/34 (29.4%) isolates in 2007. The total carrier rate of antibiotic resistance in NTHi was 16/34 (47.1%) isolates. In 2008, the carrier rate of resistant strains (gBLNAR+gBLPACR) was 28/31 (90.3%) children and the carrier rate of antibiotic resistance in NTHi was 28/29 (96.6%). The

incidence of gBLPACR significantly increased to 24/29 (82.8%) isolates ($p<0.01$) compared with that before 2005. In summary, the prevalence of gBLNAR among the children attending day care significantly increased from 1999-2000 to 2005. Furthermore, gBLPACR strains of NTHi have been rapidly increasing since 2007. As a result, the total carrier rate of all resistant strains of NTHi (gBLNAR and gBLPACR) in the children attending this day care center significantly increased during the past decade. Most of the children in this day care recently have resistant strains of NTHi, which had acquired PBP gene mutations, in their nasopharynx.

PCR-based genotypes and MICs distributions. The MICs of gBLPACR, gBLNAR and gBLNAS for eight antimivrobial agents against are listed in Table 3. The MIC50s and MIC90s of amoxicillin/clavulanate and other β-lactams for resistant strains of NTHi were higher than those for gBLNAS isolates (Table 3).

Genetic distribution of NTHi strains by PFGE. PFGE analysis classified the NTHi strains into five PFGE types. Although the PFGE patterns of gBLNAR and gBLNAS (total five strains) were classified into four different patterns, all of the gBLPACR (23 strains) were clonally identical and were different from the other strains of NTHi (Figure 1). Our PFGE results demonstrate the first evidence that the apparent clonal dissemination of gBLPACR of NTHi occurred in a certain environment such as day care.

DISCUSSION

In this study, we focused on resistant strains of NTHi isolated from the nasopharynx of young children attending day care and investigated clonal dissemination of gBLPACR. NTHi and *S. pneumoniae* are recognized as part of the normal nasopharyngeal flora in healthy children but remain a major cause of bacterial infections of the respiratory tract, such as pneumonia, rhinosinusitis, and AOM [1,17]. Hotomi et al demonstrated that the MIC50s of amoxicillin/clavulanate and cephalosporin for gBLNAR isolates were 4 to 64 times higher than

those for gBLNAS isolates[18]. PBP gene mutations of NTHi, identified by PCR, were related to antibiotic resistance. Intractable infections due to resistant strains of these pathogens have become a significant clinical problem worldwide. Single or occasional episodes of AOM are relatively easy to treat, but recurrent episodes of AOM (otitis prone) would represent an onerous burden for both child and caregiver. The high prevalence of BLNAR and Pc^r *S. pneumoniae* in Japan is a serious problem and is thought to be the principal cause of otitis prone seen among young children. BLNAR isolates of NTHi were first reported in 1980 [19] and generally continue to be isolated at low frequencies in Western Europe and the USA [20-23]. However, recent surveillance studies have reported higher proportions of BLNAR isolates [10,11,17,24,25]. There have been reports from many countries such as Spain, France, Poland, India, Korea and Japan, in which 9.3%, 18.6%, 12.8%, 14.4%, 29.3% and 13.9% to 65.1%, respectively, of NTHi isolates were BLNAR strains [2,11,24-29]. In Japan, this strain appeared in 1997 and increased rapidly among isolates from patients with RTIs, AOM and rhinosinusitis. The mechanism of resistance in the BLNAR strains involves decreased affinities of PBPs for β-lactam antibiotics[29]. In the present study, we have reported a high prevalence of resistant strains of NTHi among young children attending a single day care in Japan. The carrier rates of gBLNAR in the NTHi isolates increased from 23.1-28.2% in 1999-2000 to 77.8% in 2005. The carrier rate of resistant strains (gBLNAR and gBLPACR) in the NTHi isolates was quite high (96.6%) in 2008. Furthermore, NTHi were isolated from about 95% of children attending this day care center recently and these carrier rates were much higher than those from clinical specimens [25,28-31]. Thus the carrier rate of resistant strains (gBLNAR and gBLPACR) of NTHi in children attending this day care center has been rapidly increasing during the past decade. Because day care attendance is a risk factor for upper RTIs and otitis prone, both the high prevalence of resistant strains in the isolates and the very high isolate rates of NTHi from healthy young children potentially pose significant clinical problems.

The prevalence of resistance strains of NTHi varies in different countries but gBLPACR

strains of NTHi are isolated at very low frequencies around the world [11,31], except in Korea where its frequency is relatively high (8.3%) [27]. In Japan, β-lactamase-producing strains were identified in 2.5% of isolates and β-lactamase-producing strains with mutations in ftsl (gBLPACR) were identified in 0.3% of isolates [25]. The most important finding in the present study was that gBLPACR of NTHi were initially identified in 29.4% of subjects in 2007 and then this rate markedly increased to 82.8% in 2008. The present study is the first report to show that gBLPACR strains of NTHi are capable of spreading in a certain environment such as a day care center. Because gBLPACR of NTHi are resistant to amoxicillin/clavulanate and other commonly prescribed oral anti-microbial drugs, the high prevalence of gBLPACR will cause significant problems in the clinical setting of treating community-acquired infections, including pneumonia, AOM, and rhinosinusitis.

Another important finding of the present study is that all gBLPACR isolates were clonally identical by PFGE analysis and were different from the other strains of NTHi. Our PFGE analysis revealed clonal spread of only one gBLPACR isolate with altered PBPs in this day care center. This represents the first evidence of the apparent clonal epidemic of gBLPACR of NTHi occurred. The contributions of clonality to the isolation of gBLPACR were not determined in previous studies. The present study demonstrated that gBLPACR of NTHi are capable of broad dissemination worldwide, similar to the gBLNAR. The clinical significance of gBLPACR of NTHi is as yet unknown. However, the MICs of amoxicillin-clavulanate for all gBLPACR in the present study were high (8 μg/ml). Furthermore, gBLPACR isolates are generally highly ampicillin resistant and show elevated levels of resistance to expanded and broad-spectrum cefalosporins compared to non-gBLPACR. The high prevalence of gBLPACR from the clinical isolates may cause more serious problems than gBLNAR for the treatment of patients with otitis prone, intractable RTIs and rhinosinusitis. The rapidly increasing prevalence of resistant strains of NTHi in this day care center may predict a high incidence of these resistant bacteria being isolated from clinical samples in the

near future and possible serious medical problems worldwide.

CONCLUSIONS

Most of the children attending this day care center recently had resistant strains of NTHi with PBP gene mutations in their nasopharynx. gBLPACR strains have rapidly increased since 2007. PFGE analysis demonstrated that all gBLPACR strains in 2008 were clonally identical. This represents the first report in the would about the apparent clonal dissemination of gBLPACR of NTHi occurred like an epidemic. Because BLPACR strains are generally multi-drug resistant, BLPACR strains are now emerging and clonal spread of BLPACR observed in this study might be an alarm of increasing more serious bacterial infectious diseases with NTHi in the future.

ACKNOWLEGMENTS

We wish to thank Dr. Higashi, T (Department of Environmental and Molecular Bio-Informatics, Kanazawa University Graduate School of Medical Science) for her valuable support with the statistical analysis; and the participating children, their parents and teachers for allowing us to conduct the study. This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (#19591961, #19591987, #18591882, #18791228, #19791226) and the Ministry of Health, Labor and Welfare (#H21-clinical research-general-007).

Conflict of interest

We have no conflict of interest with any other people or organizations.

REFERENCES

[1] Ito M, Ito K, Yoshizaki T, Nishimura T, Miwa T, Furukawa M. Nasopharyngeal

penicillin-resistant Streptococcus pneumoniae strains among young children in Japan. *Otol Neurotol* 2002;23:349-352.

[2] Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K, et al. Rapidly increasing prevalence of beta-lactamase-nonproducing, ampicillin-resistant Haemophilus influenzae type b in patients with meningitis. *Antimicrob Agents Chemother* 2004;48:1509-1514.

[3] Chiba N, Kobayashi R, Hasegawa K, Morozumi M, Nakayama E, Tajima T, et al. Antibiotic susceptibility according to genotype of penicillin-binding protein and macrolide resistance genes, and serotype of Streptococcus pneumoniae isolates from community-acquired pneumonia in children. *J Antimicrob Chemother* 2005;56:756-760.

[4] Richter SS, Heilmann KP, Coffman SL, Huynh HK, Brueggemann AB, Pfaller MA, et al. The molecular epidemiology of penicillin-resistant Streptococcus pneumoniae in the United States, 1994-2000. *Clin Infect Dis* 2002;34:330-339.

[5] Lu CY, Lee PI, Hsueh PR, Chang SC, Chiu TF, Lin HC, et al. Penicillin-nonsusceptible Streptococcus pneumoniae infections in children. *J Microbiol Immunol Infect* 1999;32:179-186.

[6] Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, Ubukata K, et al. Evaluation of PCR primers to screen for Streptococcus pneumoniae isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. *J Antimicrob Chemother* 2001;48:915-918.

[7] Nagai K, Davies TA, Jacobs MR, Appelbaum PC. Effects of amino acid alterations in penicillin-binding proteins (PBPs) 1a, 2b, and 2x on PBP affinities of penicillin, ampicillin, amoxicillin, cefditoren, cefuroxime, cefprozil, and cefaclor in 18 clinical isolates of penicillin-susceptible, -intermediate, and -resistant pneumococci. *Antimicrob Agents Chemother* 2002;46:1273-1280.

[8] Jacobs MR, Felmingham D, Appelbaum PC, Gruneberg RN. The Alexander Project 1998-2000: susceptibility of pathogens isolated from community-acquired respiratory tract infection to commonly used antimicrobial agents. *J Antimicrob Chemother* 2003;52:229-246.

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

[10] Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, et al. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant Haemophilus influenzae. *Antimicrob Agents Chemother* 2001;45:1693-1699.

[11] Dabernat H, Delmas C, Seguy M, Pelissier R, Faucon G, Bennamani S, et al. Diversity of beta-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of Haemophilus influenzae. *Antimicrob Agents Chemother* 2002;46:2208-2218.

[12] Wald ER, Guerra N, Byers C. Frequency and severity of infections in day care: three-year follow-up. *J Pediatr* 1991;118:509-514.

[13] Wald ER, Dashefsky B, Byers C, Guerra N, Taylor F. Frequency and severity of infections in day care. *J Pediatr* 1988;112:540-546.

[14] Collet JP, Burtin P, Kramer MS, Floret D, Bossard N, Ducruet T. Type of day-care setting and risk of repeated infections. *Pediatrics* 1994;94:997-999.

[15] Arnold KE, Leggiadro RJ, Breiman RF, Lipman HB, Schwartz B, Appleton MA, et al. Risk factors for carriage of drug-resistant Streptococcus pneumoniae among

children in Memphis, Tennessee. *J Pediatr* 1996;128:757-764.
[16] Pons JL, Mandement MN, Martin E, Lemort C, Nouvellon M, Mallet E, et al. Clonal and temporal patterns of nasopharyngeal penicillin-susceptible and penicillin-resistant Streptococcus pneumoniae stains in children attending a day care center. *J Clin Microbiol* 1996;34:3218-3222.
[17] Hasegawa K, Kobayashi R, Takada E, Ono A, Chiba N, Morozumi M, et al. 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* 2006;57:1077-1082.
[18] Hotomi M, Fujihara K, Billal DS, Suzuki K, Nishimura T, Baba S, et al. Genetic characteristics and clonal dissemination of beta-lactamase-negative ampicillin-resistant Haemophilus influenzae strains isolated from the upper respiratory tract of patients in Japan. *Antimicrob Agents Chemother* 2007;51:3969-3976.
[19] Markowitz SM. Isolation of an ampicillin-resistant, non-beta-lactamase-producing strain of Haemophilus influenzae. *Antimicrob Agents Chemother* 1980;17:80-83.
[20] Doern GV, Brueggemann AB, Pierce G, Holley HP, Jr., Rauch A. Antibiotic resistance among clinical isolates of Haemophilus influenzae in the United States in 1994 and 1995 and detection of beta-lactamase-positive strains resistant to amoxicillin-clavulanate: results of a national multicenter surveillance study. *Antimicrob Agents Chemother* 1997;41:292-297.
[21] Karlowsky JA, Critchley IA, Blosser-Middleton RS, Karginova EA, Jones ME, Thornsberry C, et al. Antimicrobial surveillance of Haemophilus influenzae in the United States during 2000-2001 leads to detection of clonal dissemination of a beta-lactamase-negative and ampicillin-resistant strain. *J Clin Microbiol* 2002;40:1063-1066.
[22] Felmingham D, Washington J. Trends in the antimicrobial susceptibility of bacterial respiratory tract pathogens--findings of the Alexander Project 1992-1996. *J Chemother* 1999;11 Suppl 1:5-21.
[23] Doern GV. Antimicrobial resistance among lower respiratory tract isolates of Haemophilus influenzae: results of a 1992-93 western Europe and USA collaborative surveillance study. The Alexander Project Collaborative Group. *J Antimicrob Chemother* 1996;38 Suppl A:59-69.
[24] Marco F, Garcia-de-Lomas J, Garcia-Rey C, Bouza E, Aguilar L, Fernandez-Mazarrasa C. Antimicrobial susceptibilities of 1,730 Haemophilus influenzae respiratory tract isolates in Spain in 1998-1999. *Antimicrob Agents Chemother* 2001;45:3226-3228.
[25] Hotomi M, Sakai KF, Billal DS, Shimada J, Suzumoto M, Yamanaka N. Antimicrobial resistance in Haemophilus influenzae isolated from the nasopharynx among Japanese children with acute otitis media. *Acta Otolaryngol* 2006;126:130-137.
[26] Skoczynska A, Kadlubowski M, Wasko I, Fiett J, Hryniewicz W. Resistance patterns of selected respiratory tract pathogens in Poland. *Clin Microbiol Infect* 2007;13:377-383.
[27] Kim IS, Ki CS, Kim S, Oh WS, Peck KR, Song JH, et al. Diversity of ampicillin resistance genes and antimicrobial susceptibility patterns in Haemophilus influenzae strains isolated in Korea. *Antimicrob Agents Chemother* 2007;51:453-460.
[28] Kubota T, Higa F, Kusano N, Nakasone I, Haranage S, Tateyama M, et al. Genetic analyses of beta-lactamase negative ampicillin-resistant strains of Haemophilus

influenzae isolated in Okinawa, Japan. *Jpn J Infect Dis* 2006;59:36-41.
[29] Sakai A, Hotomi M, Billal DS, Yamauchi K, Shimada J, Tamura S, et al. Evaluation of mutations in penicillin binding protein-3 gene of non-typeable Haemophilus influenzae isolated from the nasopharynx of children with acute otitis media. *Acta Otolaryngol* 2005;125:180-183.
[30] Yamanaka N, Hotomi M, Billal DS. Clinical bacteriology and immunology in acute otitis media in children. *J Infect Chemother* 2008;14:180-187.
[31] Hasegawa K, Yamamoto K, Chiba N, Kobayashi R, Nagai K, Jacobs MR, et al. Diversity of ampicillin-resistance genes in Haemophilus influenzae in Japan and the United States. *Microb Drug Resist* 2003;9:39-46.

FIGURE LEGEMDS

Table 1: Nasopharyngeal carriage of specific respiratory pathogens in children attending a day care. Almost all young children attending this day care center were carriers of SP and/or NTHi.

Abbreviations: NTHi: non-typeable *Haemophilus influenzae*, SP; *Streptococcus pneumoniae*, MC; *Moraxella catarrhalis*

Table 2: Nasopharyngeal carriage of NTHi in children attending a day care. Changes in resistance among NTHi strains, by year, as identified by PCR. The carrier rates of gBLPACR dramatically increased after 2007.

Abbreviations: NTHi: non-typeable *Haemophilus influenzae*, gBLPACR; genetically β-lactamase-producing amoxicillin-clavulanate-resistant, gBLNAR; genetically β-lactamase-nonproducing ampicillin-resistant, gBLNAS; genetically β-lactamase-nonproducing ampicillin-susceptible

Table 3: MIC distributions for eight antimicrobial agents against 24 isolates of gBLPACR (upper), 3 isolates of gBLNAR (middle) and 2 isolates of gBLNAS (lower) in 2008.

Figure 1: PFGE analysis of NTHi isolates in 2008. All of the gBLPACR were clonally identical and were different from the other strains of NTHi.

Table 1. Nasopharyngeal carriage of specific respiratory pathogens in children attending day care.

Year	No. of children	No. (%) of SP	No. (%) of NTHi	No. (%) of MC	No. (%) of non- SP, NTHi
1999	34	33 (97.1)	26 (76.5)	17 (50.0)	0 (0)
2000	42	28 (66.7)	39 (92.9)	37 (88.1)	1 (2.4)
2005	36	32 (88.9)	27 (75.0)	36 (100)	3 (8.3)
2007	35	32 (91.4)	34 (97.1)	30 (85.7)	0 (0)
2008	31	23 (74.2)	29 (93.5)	18 (58.1)	1 (3.2)
total	178	148 (83.1)	155 (87.1)	138 (77.5)	5 (2.8)

Table 2. Nasopharyngeal carriage of NTHi in children attending day care.

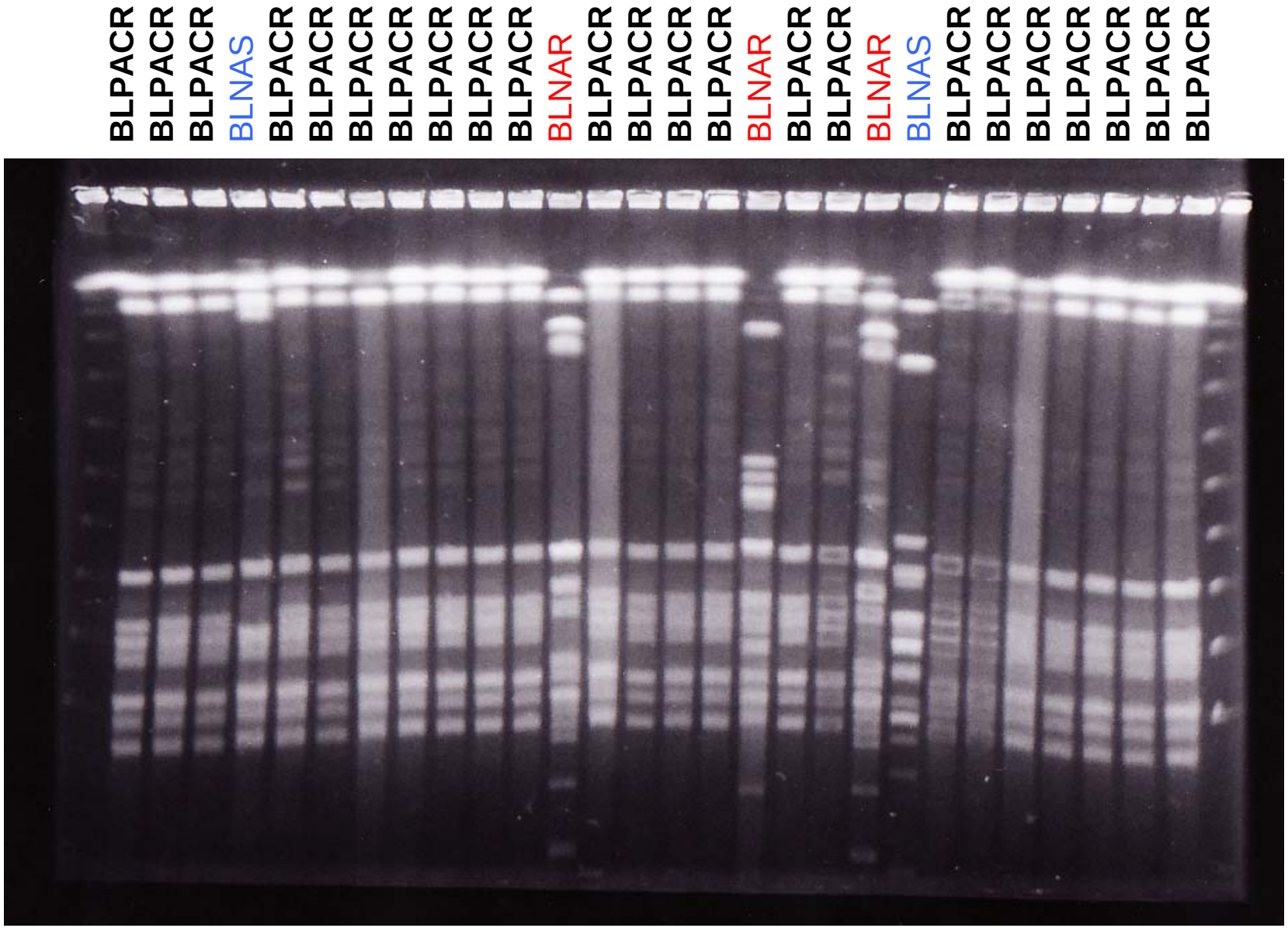
Changes in resistance among NTHi strains, by year, as identified by PCR. The carrier rate of gBLPACRwere dramatically increased since 2007.

No. (%) of NTHi isolates	1999	2000	2005	2007	2008
gBLPACR	0	0	0	10 (29.4)	24 (82.8)
gBLNAR	6 (23.1)	11 (28.2)	21 (77.8)	6 (17.6)	3 (10.3)
gBLNAS	20 (76.9)	28 (71.8)	6 (22.2)	18 (52.9)	2 (6.9)
Total NTHi	26 (100)	39 (100)	27 (100)	34 (100)	29 (100)

Table 3
MIC distributions for eight antimivrobial agents against 24 isolates of gBLPACR (upper), 3 isolates of gBLNAR (middle) and 2 isolates of gBLNAS (lower) in 2008.

	PCR-based genotype	No. of isolates with MIC (ug/ml)											MIC (ug/ml)		
		≦0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	Range	50%	90%
GRNX	gBLPACR	21	3										≦0.008-0.015	≦0.008	0.015
	gBLNAR	3											≦0.008	≦0.008	≦0.008
	gBLNAS	2											≦0.008	≦0.008	≦0.008
LVFX	gBLPACR		24										0.015	0.015	0.015
	gBLNAR		3										0.015	0.015	0.015
	gBLNAS	1	1										≦0.008-0.015	≦0.008	0.015
GFLX	gBLPACR		23	1									0.015-0.03	0.015	0.015
	gBLNAR	3											≦0.008	≦0.008	≦0.008
	gBLNAS	2											≦0.008	≦0.008	≦0.008
MFLX	gBLPACR		24										0.015	0.015	0.015
	gBLNAR		3										0.015	0.015	0.015
	gBLNAS		2										0.015	0.015	0.015
CDTR-PI	gBLPACR				24								0.06	0.06	0.06
	gBLNAR			2			1						0.03-0.25	0.03	0.25
	gBLNAS	1	1										≦0.008-0.015	≦0.008	0.015
AMPC /CVA	gBLPACR										1	23	4-8	8	8
	gBLNAR							1	1			1	0.5-8	1	8
	gBLNAS						1	1					0.25-0.5	0.25	0.5
AZM	gBLPACR					1		6	17				0.125-1	1	1
	gBLNAR							2		1			0.5->2	0.5	2
	gBLNAS						1	1					0.25-0.5	0.25	0.5
ABPC	gBLPACR									24(>2)			>2	>2	>2
	gBLNAR							2		1 (>2)			0.5->2	0.5	>2
	gBLNAS							2					0.5	0.5	0.5

Figure.1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