DISCUSSION

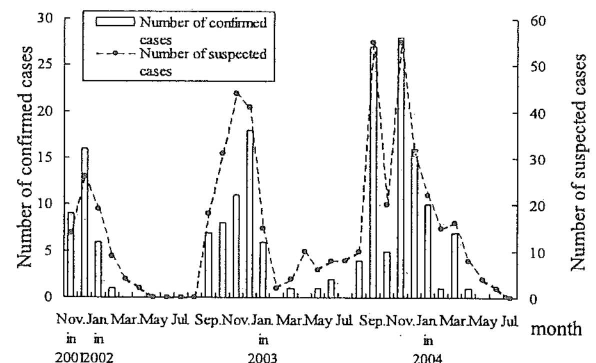
The present investigation of the patterns of circulation of RSV infections in a community over three seasons by genotyping of the second hypervariable region of G protein (21, 22, 28) demonstrated that multiple genotypes cocirculate each year. In the present study RSV infections started in early winter, and the rates declined in the spring. The predominant subgroup changed from subgroup A to subgroup B over the three epidemic seasons, in line with the findings of earlier reports (4, 8, 15). In our study, we monitored patients over three seasons; however, the 2001-2002 season began in November 2001. The peak month for RSV infection was December 2001 in Niigata City, as was the case in a national survey of RSV infection (20), and we considered that our analysis may have been developed or implemented partially in the 2001-2002 season.

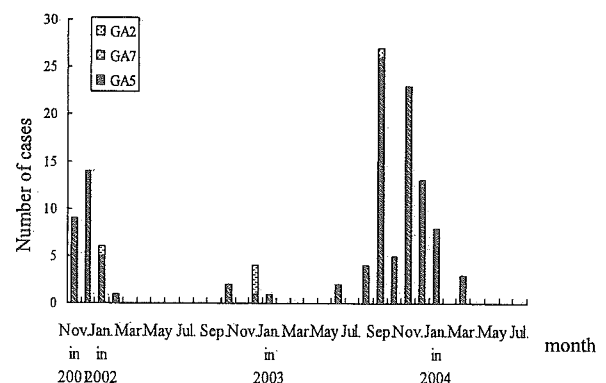
Our phylogenetic analysis revealed that genotype GA5 of subgroup A was predominant in the 2001-2002 and 2003-2004 seasons, while a new genotype of subgroup B, which featured a 60-nucleotide insertion in the second variable region of G protein (BA viruses), was predominant in the 2002-2003 season. Our observations indicate that multiple genotypes cocirculate in a single epidemic and that the genotypes in each epidemic may differ, as described previously (22, 28). Genotypes of both subgroups A and B showed temporal clustering by year of detection, which supported previous findings (6). Strains detected at the end of the previous season tended to be predominant in the next season, which might be associated with antigenic evasion from host immunity.

Viruses of genotypes similar to our BA viruses were also reported in Sapporo, Japan, in 2002 (GenBank accession number AB117522). The BA virus reported by Trento et al. (27) had an exact duplication of 60 nucleotides in the C-terminal one-third of the G-protein gene and illustrated a new type of drastic change introduced in G protein during the natural propagation of RSV. Our strains of the new genotype and strains from Sapporo were demonstrated to have 1 amino acid substitution in the insert region compared to the sequence of the BA strain. It is noted that the specific strains circulated in two countries, one in South America and another in Northeast Asia, after approximately 3 years with only a minor modification of the amino acid, which could support the robustness of the virus. The C terminus of the G-protein molecule has been shown to be immunologically relevant. Therefore, it is suggested that the 60-nucleotide insertion in the C-terminal one-third of the G-protein gene and the amino acid replacement compared with the amino acid in prototype BA strains change its antigenic structure, which confers an evolutionary advantage that allows reinfection of individuals previously exposed to the ancestor virus. However, as an emerging strain, our strain of the new genotype of subgroup B was not associated with new epidemiological or clinical features compared with those of the other clades during the three seasons that we studied. Further studies are required to determine the effect of the insertion on the immune response to RSV and susceptibility to infection and disease.

It has been reported that the severity of RSV infection may vary with the specific virus genotype (16); however, in the present study, no differences in the epidemiological or clinical manifestations, such as age or an illness that required hospitalization, were detected among these genotypes. Thus, we need continued observations to determine whether the greater severity of illness is associated with specific genotypes.

The variability of RSV strains may contribute to the cause of repeated infections, and children infected with subgroup A strains appear to be more likely to be reinfected than those





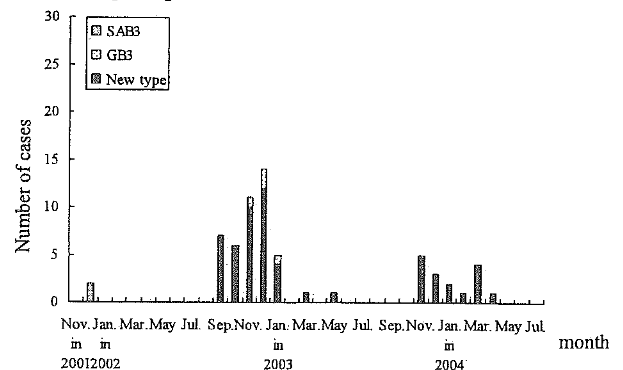


FIG. 2. Monthly distribution of 499 suspected and 185 confirmed cases of RSV infection (A), 122 cases of subgroup A RSV infection (B), and 63 cases of subgroup B RSV infection (C) from November 2001 to July 2004. Each subgroup is classified as genotype GA2, GA5, GA7, GB3, or SAB3 or the new genotype of subgroup B with a 60-nucleotide insertion.

infected with subgroup B strains (18). Only 8 (4.3%) of our 177 RSV patients became reinfected over the study period. With such a small number of patients, it is impossible to discuss the relationship between reinfection and genetic diversity, even with the new genotype of subgroup B strains. Furthermore, small numbers of reinfections may have been detected in our study because the patients visited other medical care facilities or the patients may have had mild symptoms during the second infections.

In conclusion, our molecular analysis of RSV in Niigata, Japan, confirmed that plural genotypes cocirculate each year and that the predominant genotype may shift with the season. A new genotype of subgroup B with a 60-nucleotide insertion, named BA-like virus, was found to be a predominant genotype, but it was not associated with new epidemiological or clinical features compared with those of the other genotypes that were present during the three seasons that we studied. Finally, our results provide support for genotype designation by RT-PCR methods as an effective tool for characterization of RSV circulation patterns in communities.

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The possible west-east spread of influenza across Europe during the influenza seasons of the previous few years was mapped in 2004 using data from the European Influenza Surveillance Scheme (EISS) database [1]. A method used in Japan has now been applied to European data for the 2004-5 season, and the results echo the pattern arising from the previous EISS analysis.

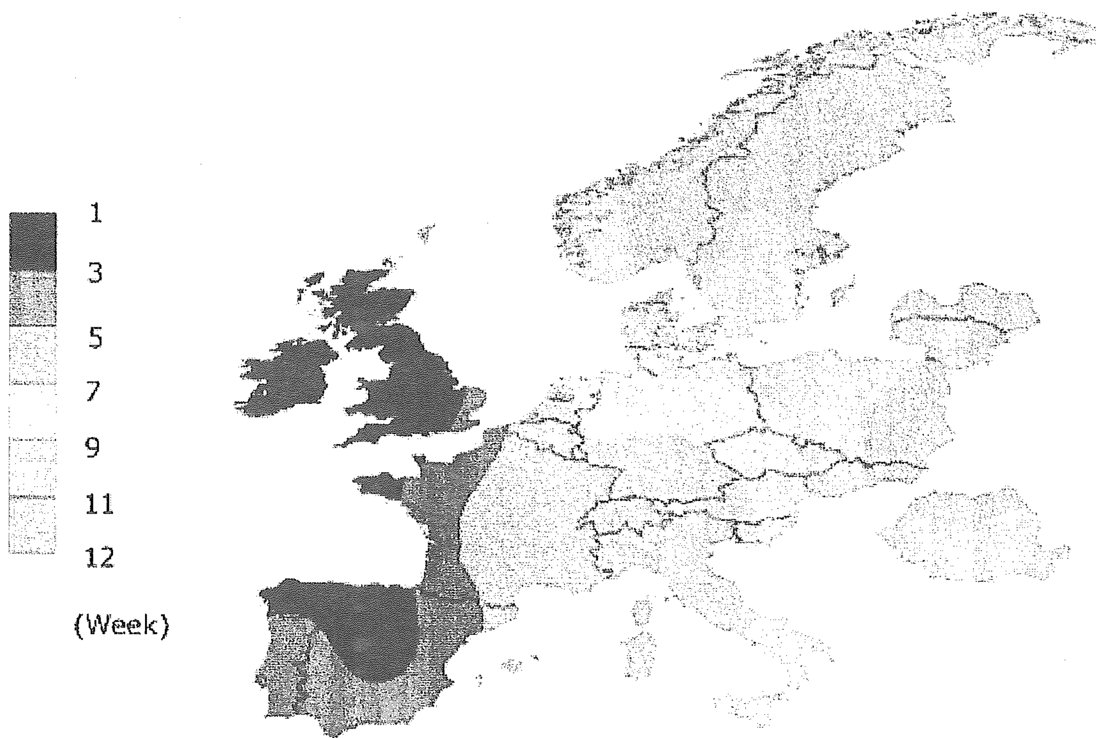
To analyse the movement and speed of influenza epidemic diffusion in Japan in the last few years, a geographic information system (GIS) and the Kriging method were used. Kriging is an interpolation method of spatial prediction to estimate unknown point values by using known point values. The weights reflect the distances between locations for which a value is being predicted and the locations with measured values. It is considered the best linear unbiased estimator if it reflects the best minimum mean square error, and can minimise estimation error variance. The method has previously been used to illustrate and clarify spatiotemporal relationships in epidemiological research, e.g., for rotavirus in the United States and influenza-like illness in France and Germany [2,3,4]. Kriging maps have illustrated nationwide epidemic spread in concentric circles from western-central Japan to eastern Japan [5].

The Kriging method was used to show the peak activity week of influenza in the 23 European countries that reported to EISS during the 2004-05 season. The earliest peak activity was observed in Ireland and the United Kingdom (including Scotland) in week 1 in 2005, and the last was in Norway in week 12. The time difference when countries measured peak activity comparing the earliest country (or countries) with the rest of the countries was calculated, and the values were entered under each country in attributed tables in a computer package ArcGIS 8.

These peak difference values and central locations of the nations were used to generate contours by the ordinary Kriging method after adjusting semivariograms in Geostatistical Analyst, extension software to ArcGIS 8.

The Kriging map clearly demonstrated seeming west-east as well as south-north spread of influenza across Europe in the season [Figure]. This mapping analysis also visually supported the result from the previous analysis by peak week of clinical activity versus the longitude of each country 1. A separate analysis for Germany [6], using 2004-05 national data also indicated the south-north/east patterns observed in the EISS European analysis.

Figure



The isobars on the contour maps represent interpolated time of peak activity distributed spatially at 2-week intervals. Europe countries included in this spatial analysis were Austria, Belgium, Czech Republic, Denmark, France, Germany, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, the Netherlands, Norway, Poland, Portugal, Romania, Slovak Republic, Slovenia, Spain, Switzerland, Sweden, and the United Kingdom (including Scotland).

The contour map generated by the GIS software matched the empirical values well in central Europe where countries are often smaller, and where there was the highest density of points, whereas standard error became relatively higher (and so the map probably became less accurate) where the locations were sparse (e.g. only two points in Iberian Peninsula), or where the measured values had local outliers (e.g. 'outlying' peak in Spain in week 2 compared with Portugal (week 5) and France in week 6).

Compared to this original data analysis carried out by EISS, which looked at the spread of influenza activity using graphs, the GIS method provides a better visualisation of the influenza activity by presenting the data in a single map showing both the west-east and south-north spread of influenza activity. This technique will therefore be used to further analyse the European data, for example, by increasing the number of locations (e.g., integrating regional data for France and England) and study years.

There are of course social factors (e.g., traffic, or population density), meteorological conditions (e.g., temperature, or winds), or host susceptibility and virus antigenic conditions that could potentially be incorporated into the further analysis. The results demonstrate that GIS, using Kriging contour maps, may be an effective surveillance tool to investigate the dynamics of an influenza epidemic in real time. Furthermore, in the case of pandemic influenza, this kind of analysis could be useful for focusing control measures, such as vaccines, antiviral agents, or even human resources, to maximise an effective public health response.

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Prevalence of Human Metapneumovirus and Influenza Virus Infections Among Japanese Children During Two Successive Winters

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Background: Human metapneumoviruses (hMPVs) are recognized as a leading cause of respiratory infections in young infants in many countries. The objective of this study was to identify links between hMPV and influenza virus infections among children with influenza-like illness.

Method: This study was conducted in 2 influenza seasons (2002–2003 and 2003–2004) at 2 pediatric outpatient clinics in Niigata city, Japan. Nasopharyngeal swabs or aspirates were collected from influenza-like illness patients, and hMPV and influenza were detected by reverse transcription-polymerase chain reaction (RT-PCR). A nucleotide sequence of 352 nucleotides segment of the *F* gene was performed.

Results: A total of 765 influenza viruses and 84 hMPV were identified from 1498 nasopharyngeal swabs or aspirates by virus isolation and RT-PCR, respectively. hMPV-positive rates in patients with influenza-like illness were 5.7 and 5.2% in the 2002–2003 and 2003–2004 seasons, respectively. Epidemic curves of influenza and hMPV patients showed similar patterns with peaks in February in 2 influenza seasons. hMPV infections occurred frequently in infants and school children. Approximately 46% of hMPV patients were coinfected with influenza A viruses, but those coinfected cases were not clinically distinct from the others. No coinfection with influenza B viruses was found. Phylogenetic analysis of the hMPV fusion gene sequences revealed that 2 distinct hMPV cocirculated and that completely identical strains in subgroup A were observed over 2 years.

Conclusions: HMPV plays an important pathogenic role in patients with influenza-like illness in winter seasons, often in coinfections with influenza A viruses.

Key Words: metapneumovirus, influenza, surveillance, coinfection, epidemiology

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Human metapneumoviruses (hMPVs) were first isolated from children with respiratory tract illness (RTI) in the Netherlands,[1] and are now recognized as a leading cause of RTI in young infants in many countries.[2–4] The proportion of cases positive for hMPV varies from 1.5 to 10% with the age group, underlying medical condition and study setting.[5] The seasonal distribution is mainly during winter to spring,[6,7] and affected patients are usually younger than 3 years old.[8]

Because hMPV is genetically closer to respiratory syncytial virus (RSV) than to other human viruses, the clinical symptoms and seasonal distribution of hMPV are comparable with those of RSV.[9,10] Influenza viruses are also important winter viruses as well as RSV with similar clinical manifestations. Although hMPV coinfections occur with RSV and influenza viruses, or severe acute respiratory syndrome (SARS),[11,12] little is known about its role as a copathogen.

The objective of the present study was to assess the prevalence of hMPV infection and of coinfections with influenza viruses in children with influenza-like illness during 2 successive winters in Japan.

MATERIALS AND METHODS

Study Design. This study was conducted during 2 influenza seasons, from December 2002 to March 2003 and from January to April 2004, at 2 pediatric outpatient clinics in Niigata city, Japan, the capital of Niigata Prefecture, with a total population of ~500,000. The 2 clinics are located in the central part of the city separated by a distance of 2.4 km. There are ~2000 outpatient visits per months in each clinic, and 70% of these reside within a 10-km radius.

Influenza-like illness cases were defined on the basis of a sudden fever, cough and throat pain. Informed consent was obtained from parents or patients. The parents were given a diary card to record temperatures 3 times daily and any symptoms occurring up to 5 days after the therapy started. Single use of antipyretics was allowed when a patient had a fever of >38.5°C, and a request was made that this be recorded in the patient's dairy card for mailing to the clinic. When a temperature fell after antipyretic use, the patient was eliminated from the analysis of treatment efficacy with antiviral drugs, such as amantadine or oseltamivir. This study was approved by the medical faculty ethics committee of the Niigata University Graduate School of Medical and Dental Sciences.

Nasopharyngeal swabs or aspirates were collected from influenza-like illness patients. Diagnosis of influenza A or B

patient was made with rapid antigen test kits (Directigen Flu A+B; Becton Dickinson Japan, Tokyo, Japan) at first visits, and treatment with antiviral drugs such as amantadine or oseltamivir was recommended. Samples were placed in virus transport media and transferred to the Department of Infectious Control and International Medicine, Niigata University Graduate School of Medical and Dental Sciences, within 5 days of sampling and kept frozen at −80°C until further examination.

Detection of Influenza Viruses and hMPV. For influenza virus isolation, supernatants of nasopharyngeal swabs from patients were inoculated into Madin-Darby canine kidney cells. Subtypes were determined by hemagglutination inhibition tests with type-specific antisera.[13]

Detection of the hMPV genome was performed by reverse transcription-polymerase chain amplification (RT-PCR). Briefly viral RNA was extracted from nasopharyngeal aspirate specimens and reverse transcription reactions were performed for complementary DNA synthesis as described previously.[13] Heminested PCR was performed to detect the *F* gene of hMPV.[14] Genetic sequencing was conducted with fluorescent dye-labeled terminators on an ABI 310 Sequencer model (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Nucleotide sequences of 352 nucleotide segments of the F protein were aligned and submitted to distance-based criteria analysis with Clustal W 1.7 (DDBJ) for phylogenetic tree analysis.

Statistical Analyses. Statistical analysis to compare mean values was performed with Scheffé's test. Comparison of proportions was accomplished with a 2×m table. Statistical significance was defined as $P < 0.05$.

RESULTS

Influenza-Like Illness Cases. A total of 1116 specimens from 1044 individuals with influenza-like illness presenting at the 2 clinics in Niigata City were enrolled in this study in the 2002–2003 season, and 382 specimens were obtained from 367 individuals at one of the 2 clinics in the 2003–2004 season (Table 1; Fig. 1). Patients older than 20 years of age were excluded from the study. The median age of the influenza-like illness patients was 6.45 ± 4.43 years in the 2002–2003 season and 5.02 ± 3.41 in the 2003–2004 season. The male-female ratio overall was 1.2:1.

Influenza Cases. Influenza A viruses were isolated from 543 (48.7%) of 1116 specimens and influenza B viruses from 46 (4.1%) in the 2002–2003 season, and from 172 (45.0%) and 4 (1.0%) of 382, respectively, in 2003–2004 (Table 1; Fig. 1).

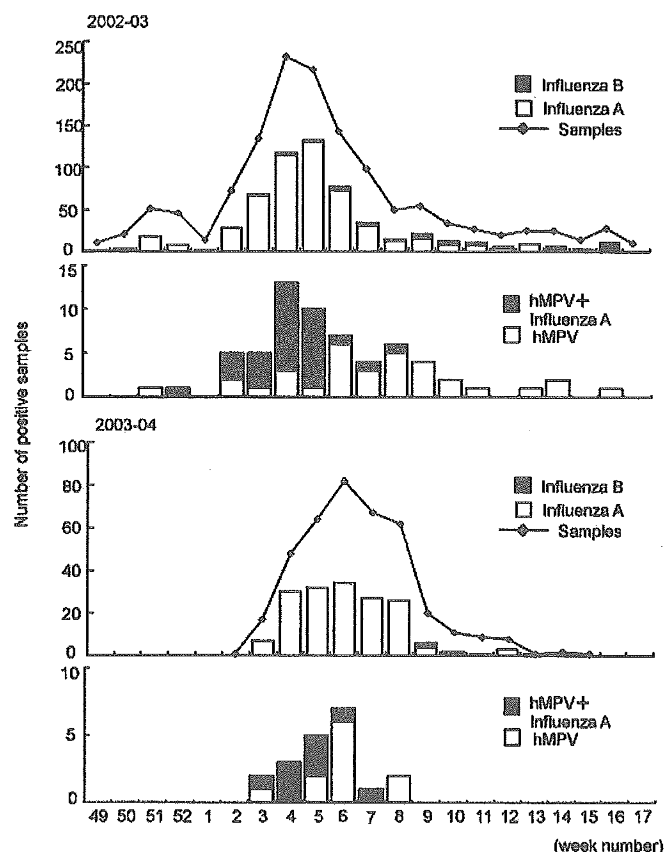

FIGURE 1. Numbers of children with influenza-like illness, positive for the influenza A virus, influenza B virus, hMPV, and hMPV/influenza A coinfection in 2002–2003 and 2003–2004 influenza epidemic seasons by week.

TABLE 1. Detection of Influenza A, Influenza B, hMPV and hMPV/Influenza Coinfections in the 2002–2003 and 2003–2004 Winter Seasons

Virus	2002–2003* (1116 Samples)		2003–2004† (382 Samples)	
	Positive	Age of Patient (yr)	Positive	Age of patient (yr)
Influenza A	543 (48.7)‡	7.15 ± 4.38§	172 (45.0)	6.04 ± 3.82
Influenza B	46 (4.1)	7.41 ± 4.26	4 (1.0)	5.62 ± 0.90
hMPV	64 (5.7)	6.13 ± 5.45	20 (5.2)	5.26 ± 3.06
hMPV only	34	5.53 ± 4.47‖	11	5.18 ± 3.52‖
hMPV + influenza A	30	6.91 ± 4.08‖	9	5.43 ± 4.02‖
hMPV + influenza B	0		0	

*Influenza season in 2002–2003 was from December 2002 to March 2003.
†Influenza season in 2003–2004 was from January 2004 to April 2004.
‡Numbers in parentheses, percent.
§Median ± SD.
‖Not statistically significant.

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Influenza A H3N2 was the predominant serotype, and influenza A H1N1 was not isolated in either of the winters. The median age of patients with an influenza A infection was 7.15 ± 4.38 years in the 2002–2003 season and 6.04 ± 3.82 in the 2003–2004 season. With influenza B the ages were 7.41 ± 4.26 and 5.62 ± 0.90, respectively. Five patients hospitalized with influenza A in the 2002–2003 season were clinically diagnosed as having croup in 2 cases and pneumonia in 3 cases.

hMPV Cases. In the 2002–2003 season, 64 (5.7%) of 1116 specimens were positive for hMPV by RT-PCR, the median age of affected patients being 6.13 ± 5.45 years (Table 1; Fig. 1). Of the 64, 30 (46.9%) were coinfected with influenza A viruses (mean age, 6.91 ± 4.08 years). In 2003–2004 season, 20 (5.2%) of 382 specimens were positive for hMPV, the median age being 5.26 ± 3.06 years, with 9 (45.0%) coinfected with influenza A viruses (mean age 5.43 ± 4.02 years). Coinfection with hMPV and influenza B was not observed in either season. The male-female ratio overall was 1.5:1.

We followed 220 influenza A patients and 8 of 39 coinfected patients for 5 days after the onset of illness (Fig. 2). Both groups were treated with oseltamivir. The median body temperature on the first day was 38.7 ± 0.9°C in the influenza A patients and 38.9 ± 0.7°C in the coinfected patients. Alleviation of fever after treatment was apparent on the second day, and values were 37.0°C or below on the third day. Other clinical manifestations such as coughing, rhinorrhea and throat pain did not differ between the groups.

Distribution of hMPV. Weekly distributions of patients with clinically diagnosed influenza-like illness, influenza virus and hMPV infections were similar in the 2 influenza epidemic seasons (Fig. 1). The peaks of the infections also coincided. Coinfection with hMPV and influenza viruses occurred frequently from weeks 2 to 5 of 2003 and from weeks 3 to 5 of 2004.

hMPV Phylogeny. Almost one-half (43 of 84) of the hMPV specimens were selected for sequence analysis (30 of 64 specimens in 2002–2003 and 13 of 20 specimens in 2003–2004). Phylogenetic analysis of the hMPV *F* gene sequences revealed that 2 distinct hMPV to have circulated during the study period. Of the 43 strains identified, 40 were classified as group A and only 3 as group B viruses. Group A strains were isolated in both seasons, but group B strains only in the 2002–2003 season. Nucleotide identities between group A and B strains were 84.9–87.1%, and those among group A were 93.6 to 96.1%. Strains with 100% nucleotide homology were detected in group A in both seasons: 11 strains in 2002–2003; and 6 in 2003–2004.

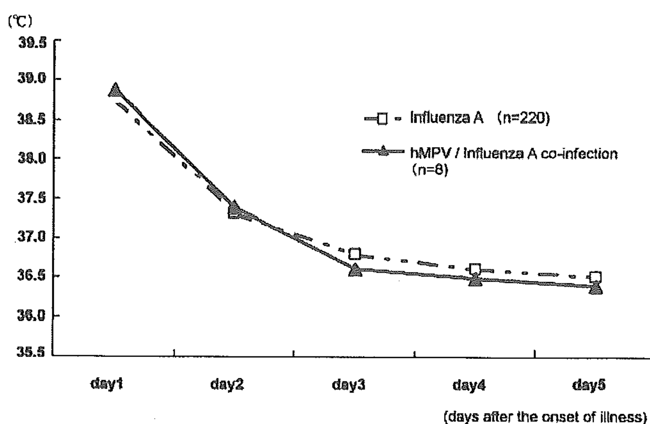

FIGURE 2. Mean daily temperatures for each day of 8 children coinfected with influenza A viruses and human metapneumovirus (hMPV) and 220 controls infected with influenza A viruses, all treated with oseltamivir.

DISCUSSION

Our present findings are clearly in line with the medical literature. hMPV infections are responsible for at least 5–7% of RTIs in hospitalized children and for at least 3% of cases who visit a general practitioner for RTIs.[5] Coinfections have been reported to occur at a rate of 4–70% in cases with hMPV and RSV[15]and 1–3% in cases with RSV and various other viruses.[16,17] In the present study of a general community, hMPV-positive rates in patients with influenza -like illness were 5.7% in 2002–2003 and 5.2% in 2003–2004. In United Kingdom and Dutch studies, hMPV was detected less frequently in influenza-like illness patients than in others with acute respiratory infections.[7,18] The data suggest that hMPV is as important as pathogen as RSV, especially in winter seasons.

The predominant influenza virus was have found to be influenza A H3N2, accounting for ~46% coinfections with hMPV. Most previous publications referred to detection of hMPV excluding patients who tested positive for influenza viruses and RSV, presumably leading to an underestimation of the incidence of hMPV infections. We could not find any coinfection cases with influenza B in the present study, but this might have been a result of the low number of cases. Thus we need further investigations to define coinfections, because the cases of hMPV and influenza were identified by different diagnostic techniques (RT-PCR and virus isolation, respectively) in this study.

Symptoms and genetic characteristics of hMPV and RSV infections are frequently compared, particularly with regard to clinical manifestations.[1,9,19] hMPV can cause severe respiratory disease in children younger than age 5 years,[9] and coinfection with RSV is a risk factor for severe bronchiolitis.[11,20] However, we did not experience any hospitalized patients with hMPV alone or with coinfection in the 2 seasons. Our observations are line with the reported wide spectrum of clinical symptoms associated with hMPV infection in patients of all ages, ranging from mild upper RTI to severe disease requiring hospitalization.[1,9,21]

hMPV may exist in coinfections with influenza viruses, RSV and SARS,[11,12,22,23] but little is known about its role as a copathogen. Animal studies indicate that the disease caused by the SARS coronavirus is not enhanced by subsequent inoculation with hMPV.[24,25] Our present preliminary study indicated the effectiveness of anti-influenza virus drugs against coinfection with influenza A viruses in 8 patients, showing that their body temperatures decreased within 24 hours after oseltamivir treatment, as in control cases of individual influenza A patients. These observations lead us to

conclude that hMPV is not an important copathogen, but further study to elucidate this point is warranted.

Two distinct genotypes of hMPV were detected during the study period. The group A strain was predominant in 2 seasons as in other reports,[4,26] and the group B was found only in the 2002–2003 season. Different hMPV subgroups produce clinical syndromes of varying severity. Although group B patients appear to have more severe symptoms than group A patients in hospitalized cases,[27] we had no hospitalized patients in either group.

In conclusion, we here provide evidence that hMPV is one of the important viruses causing influenza-like illness in children in the winter and that coinfection with hMPV and influenza viruses occurs frequently. Additional studies should now be undertaken to characterize the role of hMPV in coinfection with other viruses and its severity.

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ORIGINAL ARTICLE

Antimicrobial resistance in Haemophilus influenzae isolated from the nasopharynx among Japanese children with acute otitis media

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Abstract

Conclusion. High prevalence of penicillin-binding protein (PBP) gene mutated (PGM) strains of *H. influenzae* should be taken into account when treating otitis media in children. *Objective.* To evaluate prevalence of β-lactamase nonproducing ampicillin-resistant (BLNAR) strains of *Haemophilus influenzae* with mutations in *ftsI* gene encoding penicillin-binding protein 3 (PBP3) among children with otitis media. *Methods.* A total of 644 nasopharyngeal isolates of *H. influenzae* was collected from pediatric acute otitis media (AOM) patients with or without otitis media with effusions (OME) at the clinics of Otolaryngology-Head and Neck Surgery, Wakayama Medical University Hospital and six affiliated hospitals in Wakayama prefecture between January 1999 and December 2003. Minimal inhibitory concentrations (MICs) of ampicillin (AMP), cefditoren (CDN), cefdinir (CFD), cefaclor (CCL), cefpodoxime (CPD), and cefcapene (CFPN) were determined by the microbroth dilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS). Types of mutations in PBP3 gene (*ftsI*) were evaluated by a polymerase chain reaction (PCR)-based genotyping method. β-Lactamase gene (*bla*) was also identified by PCR. *Results.* β-Lactamase-producing (BLP) strains with the *bla* gene were identified in 16 (2.5%) of isolates. PGM strains were identified in 279 (43.3%) isolates. There were 242 (37.6%) PGM1-nonBLP strains with mutations in variable mutated locus of *ftsI*, 35 (5.4%) PGM2-nonBLP strains with mutations in highly mutated locus of *ftsI*, 2 (0.3%) BLP-PGM strains with mutations in *ftsI* and producing β-lactamase. BLP-nonPGM strains producing β-lactamase without mutations in *ftsI* were identified in 14 (2.2%) isolates. MICs of PGM1-nonBLP strains to AMP were 0.5–2.0 μg/ml. The MIC_{90} of CDN to the PGM1-nonBLP strains was lowest (0.06 μg/ml). Proportions of PGM1-nonBLP strains rapidly increased during 1999 to 2002 and then decreased in 2003. In contrast, PGM2-nonBLP strains increased in 2003.

Keywords: *β-Lactamase non-producing ampicillin-resistant H. influenzae (BLNAR), acute otitis media, PCR, nasopharynx*

Introduction

Haemophilus influenzae (H. influenzae) is a leading cause of bacterial acute otitis media (AOM) among children [1,2]. The pathogen initially colonizes the nasopharynx and subsequently infects the middle ear cavity via the Eustachian tube [3,4]. An increase in the presence of *H. influenzae* and parallel decreases in the commensal flora in the nasopharynx were a substantial risk for development of and clinical course of AOM during childhood [5]. Antimicrobial-resistant pathogens, especially penicillin-resistant *Streptococcus pneumoniae* (PRSP), have become the major causes of intractable otitis media [5]. Antimicrobial resistance in *H. influenzae* has also evolved significantly during the last 20 years, while ampicillin (AMP) has long been considered the drug of first choice for the treatment of infection due to *H. influenzae* [6].

Two well-known mechanisms of resistance to β-lactams in *H. influenzae* have been reported. One is the production of either TEM-1 or ROB-1 type β-lactamase [7,8]. The other is designated as β-lactamase nonproducing ampicillin-resistant (BLNAR) and involves a decreasing affinity of penicillin-binding proteins (PBPs) to β-lactams caused by conformational changes with genetic

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mutations [9–12]. Recent studies revealed that increasing resistance to β-lactams in BLNAR strains is closely related to mutations in *ftsI* gene encoding PBP3, which mediates septum peptidoglycan formation [13,14]. The resistant strains have increased rapidly in Japan [15]. The alarming increases of BLNAR in Japan suggest that attention should be paid to surveillance of antimicrobial resistance in this pathogen. However, current bioassays have difficulties in distinguishing such resistant isolates, because the strains usually show relatively low levels of resistance to β-lactams [16]. The variability in the magnitude of minimal inhibitory concentrations (MICs) to ABPC among BLNAR strains also complicates the definition of the strains. The National Committee for Clinical Laboratory Standards (NCCLS) defined the strain with no detection of β-lactamase and ABPC MICs of $\geq$4 μg/ml as BLNAR *H. influenzae* [17]. Strains for which the MIC to AMP is 2 μg/ml are defined as intermediate or indeterminate. In Japan, on the other hand, the strains with MICs to AMP between 1 and 2 μg/ml are usually defined as BLNAR [8]. Attempts to distinguish the BLNAR strains focus on the more distributable classifications of the strain according to the genetic characteristics such as mutations in *ftsI* encoding PBP3 that mediate an increasing resistance to β-lactams [16,18].

In this study, we investigated the prevalence of BLNAR strains and polymerase chain reaction (PCR)-based genotypes of mutations in PBP3 gene (*ftsI*) among *H. influenzae* isolated from the nasopharyx of children with AOM.

Materials and methods

H. influenzae strains

A total of 644 *H. influenzae* nasopharyngeal isolates was collected from pediatric AOM patients with or without otitis media with effusions (OME) at the clinics of Otolaryngology-Head and Neck Surgery, Wakayama Medical University Hospital and six affiliated hospitals in Wakayama prefecture between January 1999 and December 2003. Nasopharyngeal cultures were obtained with a rayon-tipped flexible swab. Informed consent was obtained from each patient's parents or guardians prior to the collection of samples according to the requirements of the institutional review board.

H. influenzae strains were identified and confirmed by colony morphology, Gram's staining, growth in chocolate but not in blood agar plates, catalase test, and X and V factor requirement. Production of β-lactamase was examined by nitrocefinase disc (Nippon Becton Dickinson Company Ltd, Tokyo, Japan). The *p6* gene encoding outer membrane protein P6, which is highly conserved among *H. influenzae*, was also amplified by PCR to confirm the isolates as *H. influenzae*. The isolates were stored in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA) containing 10% glycerol at −80°C before the study. Multiple isolates cultured within 3 months from the same patient were considered duplicate isolates, and only the first isolate was included in this study.

Antimicrobial susceptibility test

Antimicrobial susceptibilities were determined with the MIC by microbroth dilution method according to the recommendations of the NCCLS [17]. Antibiotics employed in this study were ampicillin (AMP), cefditoren (CDN), cefdinir (CFD), cefaclor (CCL), cefpodoxime (CPD), and cefcapene (CFPN).

PCR-based genotyping of H. influenzae

The oligonucleotide primers to amplify PBP3 gene (*ftsI*) including both a variable mutated locus (PBP3-S) and a highly mutated locus (PBP3-BLN), and β-lactamase gene (*bla*) used in this study were as follows. For PBP3-S: forward 5′-GATACTACGT CCTTTAAATTAAG-3′, reverse 5′-GCAGTAAA TGCCACATACTTA-3′; product size 551 bp, for PBP3-BLN: forward 5′-TTCAAGTAACCGTGG TGTGAC-3′, reverse 5′-GCAGTAAATGCCACA-TATTTC-3′; product size 465 bp, for *bla*: forward 5′-TAAGAGAATTATGCAGTGCTGCC-3′, reverse 5′-TCCATAGTTGCCTGACTCCCC-3′; product size 458 bp [16]. To confirm the isolated pathogen as *H. influenzae*, primers for *p6*, forward 5′-ACGATGCTGCAGGCAATGGT-3′; reverse 5′-TCCATAGTTGCCTGACTCCCC-3′; product size 198 bp were used [16]. Briefly, a single colony of *H. influenzae* on chocolate agar plates was lysed in 30 μl of lysis solution (1 M Tris, pH 8.9, 4.5 v/v nonident P-40, 4.5 v/v Tween 20, 10 mg/ml Proteinase K) for 10 min at 60°C and for 5 min at 94°C in the programmable thermal cycler (Gene Amp PCR System 9700, Perkin Elmer, Norwalk, CT, USA). The reaction mixtures consisted of 2 μl of bacterial lysate, 0.8 μl of 10 mM of dNTP mixture, 0.5 U of *Taq* DNA polymerase, 2.5 μl of 10 x PCR buffer, 0.5 μl of 25 mM $MgCl_2$, 5.0 μl Q-solution (Qiagen Gmbh, Germany), and 60 ng of the appropriate sets of primer mixtures. The reaction mixture was subjected to amplification in the programmable thermal cycler that consisted of denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for

30 s, and extension at 72°C for 30 s and further extension at 72°C for 10 min. Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis. Because of the mutations in *ftsI*, the amplification of PBP3-S locus was successful in non-mutated genes of susceptible strains, but failed in strains with mutated genes. On the other hand, primers for PBP3-BLN were designed to amplify the locus that shows similar mutations among nonsusceptible strains.

Results

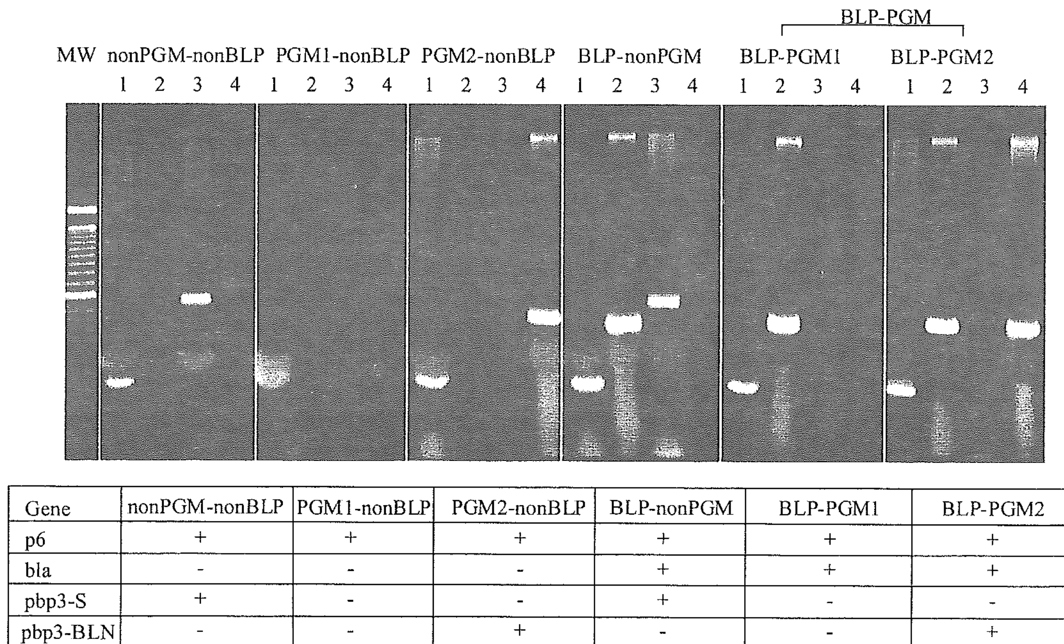
PCR-based genotyping of H. influenzae isolates

Based on PCR-based genotyping *H. influenzae* isolates were segregated into the three major groups: the strain without mutations in PBP3 gene (*ftsI*) and β-lactamase gene (nonPGM-nonBLP), the PBP3 gene mutated strain, and the β-lactamase-producing strain (BLP). PBP3 gene mutated (PGM) strains were further divided into two subgroups: PGM1-nonBLP strain with mutations in a variable mutated locus of *ftsI* (PBP3-S) and PGM2-nonBLP strain with mutations in a highly mutated locus (PBP3-BLN) without producing β-lactamase. β-Lactamase-producing (BLP) strains were also divided into two subgroups based on the presence or absence of mutations in *ftsI*: BLP-PGM strain with mutations in PBP3 gene (*ftsI*) and BLP-nonPGM strain without mutations in *ftsI* (Figure 1).

Among 644 *H. influenzae* isolates, mutations in *ftsI* were identified in 279 (43.3%) isolates, of which 242 (37.6%) were PGM1-nonBLP strains, 35 (5.4%) were PGM2-nonBLP strains, and 2 (0.3%) were BLP-PGM strains. Fourteen (2.2%) isolates with a detectable β-lactamase gene lacked mutations in *ftsI* (BLP-nonPGM). All strains having β-lactamase gene produced TEM-1 type β-lactamase. The remaining 351 isolates (54.5%) had no mutation in *ftsI* and did not produce β-lactamase (nonPGM-nonBLP).

Relationship between mutations in ftsI, β-lactamase production and susceptibility to AMP

Table I shows MIC range, MIC_{50} value, MIC_{90} value to AMP and the PCR-based genotypes of *H. influenzae* isolates. The isolates were classified based on susceptibility to AMP according to the NCCLS criteria. In all, 534 (82.9%) isolates were susceptible (MIC $\leq$1 μg/ml), 79 (12.3%) isolates were intermediately resistant (MIC = 2 μg/ml), and 31 (4.8%) isolates were resistant (MIC $\geq$4 μg/ml) to AMP. Among the 534 susceptible isolates, 349 (65.4%) isolates were nonPGM-nonBLP strains, 175 (32.8%) isolates were PGM1-nonBLP strains, and 10 (1.9%) isolates were PGM2-nonBLP strains. Among the 79 intermediately resistant strains, 2 (2.5%) isolates were nonPGM-nonBLP, 64 (81.0%) isolates were PGM1-nonBLP, and 13 (16.5%) isolates were PGM2-nonBLP. Among the 31 resistant



Gene	nonPGM-nonBLP	PGM1-nonBLP	PGM2-nonBLP	BLP-nonPGM	BLP-PGM1	BLP-PGM2
p6	+	+	+	+	+	+
bla	-	-	-	+	+	+
pbp3-S	+	-	-	+	-	-
pbp3-BLN	-	-	+	-	-	+

Figure 1. PCR-based genotypes of *H. influenzae*. nonPGM-nonBLP, β-lactamase non-producing ampicillin-sensitive strain (without PBP gene mutations and production of β-lactamase); PGM1-nonBLP, strain with mutations in variable mutated locus of *ftsI* and no production of β-lactamase; PGM2-nonBLP, strain with mutations in highly mutated locus of *ftsI* and no production of β-lactamase; BLP-PGM, strain with mutations in *ftsI* and productions of β-lactamase. Lane 1, p6; lane 2, bla; lane 3, pbp3-S; lane 4, pbp3-BLN.

Table I. Relationship between *pbp* gene and susceptibility to ampicillin ($n = 644$).

Antimicrobial agent	PCR-based genotype		Number of isolated strains and their MIC (µg/ml)											MIC (µg/ml)		
			≤0.03	0.06	0.125	0.25	0.5	1	2	4	>4	16	>16	MIC_{50}	MIC_{90}	Range
Ampicillin	β-Lactamase-nonproducing	NonPGM-nonBLP		1	1	179	151	17	2					0.25	0.5	0.06–2
		PGM1-nonBLP				44	46	85	64	3				1	2	0.25–4
		PGM2-nonBLP				2	1	7	13	11	1			2	4	0.25–>4
	β-Lactamase-producing	BLP-nonPGM								2	12			0.5	2	4–>4
		BLP-PGM									2			>4	>4	>4
	Total		0	1	1	225	198	109	79	16	15	0	0			

nonPGM-nonBLP, β-lactamase nonproducing ampicillin-sensitive strain (without PBP gene mutations and production of β-lactamase); PGM1-nonBLP, strain with mutations in variable mutated locus of *ftsI* and no production of β-lactamase; PGM2-nonBLP, strain with mutations in highly mutated locus of *ftsI* and no production of β-lactamase; BLP-PGM, strain with mutations in *ftsI* and production of β-lactamase.

strains, 3 (9.7%) isolates were PGM1-nonBLP, 12 (38.7%) isolates were PGM2-nonBLP, 2 (6.5%) isolates were BLP-PGM, and 14 (45.1%) isolates were BLP-nonPGM.

Relationship between mutation in ftsI, β-lactamase production and susceptibility to oral cephalosporins

The MIC range, MIC_{50} value, MIC_{90} value for oral cephalosporins and the PCR-based genotypes of *H. influenzae* isolates are shown in Table II. Although some PGM strains were not highly or intermediately resistant to AMP, the strains showed relatively decreased susceptibilities to oral cephalosporins. Both PGM1-nonBLP and PGM2-nonBLP strains showed decreased susceptibilities to CFD (PGM1-nonBLP: MIC_{50} 1 µg/ml, MIC_{90} 4 µg/ml; PGM2-nonBLP: MIC_{50} 2 µg/ml, MIC_{90} 8 µg/ml), CCL (PGM1-nonBLP: MIC_{50} 16 µg/ml, MIC_{90} >16 µg/ml; PGM2-nonBLP: MIC_{50} >16 µg/ml, MIC_{90} > 16 µg/ml), CDN (PGM1-nonBLP: MIC_{50} ≤0.03 µg/ml, MIC_{90} 0.06 µg/ml; PGM2-nonBLP: MIC_{50} 0.125 µg/ml, MIC_{90} 0.25 µg/ml), CFPN (PGM1-nonBLP: MIC_{50} 0.06, MIC_{90} 0.25 µg/ml; PGM2-nonBLP: MIC_{50} 0.5 µg/ml, MIC_{90} 2 µg/ml) and CPD (PGM1-nonBLP: MIC_{50}, 0.25 µg/ml, MIC_{90} 0.5 µg/ml; PGM2-nonBLP: MIC_{50} 2 µg/ml, MIC_{90} >4 µg/ml).

Year to year prevalence of antimicrobial-resistant H. influenzae

The prevalence of PGM1-nonBLP strains of *H. influenzae* steadily increased between 1999 and 2002 from 28.5% to 49.3%, then decreased to 23.9% in 2003. The prevalence of PGM2-nonBLP strains was 2.3–8.9% during 1999–2002 and then increased to 19.7% in 2003. On the other hand, nonPGM-nonBLP were highly identified at 62.9% in 1999 and decreased to 40.5% during 1999–2002. The prevalence of this strain increased again to 54.9% in 2003. There is no obvious change in the prevalence of β-lactamase-producing strains (BLP-PGM) (Figure 2).

Discussion

The problems of antimicrobial resistance in *H. influenzae* were further complicated by the description of β-lactamase non-producing ampicillin-resistant (BLNAR) strains in the 1980s [19–23]. In the USA, β-lactamase-producing *H. influenzae* strains are predominantly resistant to AMP. The BLNAR strains were identified in < 2% of strains until the 1990s. The prevalence of BLNAR (MIC ≥4 µg/ml) strains increased up to 2.5–10.1% in 1994–1995

Table II. Relationship between PCR-based genotypes and MICs to cephalosporins ($n = 644$).

Antimicrobial agent	PCR-based genotypes		Number of isolated strains and their MIC (μg/ml)												MIC (μg/ml)		
			≤0.03	0.06	0.125	0.25	0.5	1	2	4	>4	8	16	>16	MIC_{50}	MIC_{90}	Range
CDN	β-Lactamase-nonproducing	NonPGM-nonBLP	344	3	1	2	1								≤0.03	≤0.03	≤0.03–0.5
		PGM1-nonBLP	216	13	3	7	3								≤0.03	0.06	≤0.03–0.5
		PGM2-nonBLP	13	3	9	9	1								0.125	0.25	≤0.03–0.5
	β-Lactamase-producing	BLP-nonPGM	11	3											≤0.03	0.06	≤0.03–0.06
		BLP-PGM	2												≤0.03	≤0.03	≤0.03
	Total		586	22	13	18	5	0	0	0	0	0	0	0			
CPD	β-Lactamase-nonproducing	NonPGM-nonBLP	6	201	111	22	7	3		1					0.06	0.125	≤0.03–4
		PGM1-nonBLP	1	46	54	74	53	7	2	4	1				0.25	0.5	≤0.03–>4
		PGM2-nonBLP		2	3		6	3	11	6	4				2	>4	0.06–>4
	β-Lactamase-producing	BLP-nonPGM	2	3	3	6									0.125	0.25	≤0.03–0.25
		BLP-PGM				1	1								0.25	0.5	0.25–0.5
	Total		9	252	171	103	67	13	13	11	5	0	0	0			
CCL	β-Lactamase-nonproducing	NonPGM-nonBLP				1	1	13	199	100		25	9	3	2	8	0.25–>16
		PGM1-nonBLP						2	36	29		47	75	53	16	>16	1.0–>16
		PGM2-nonBLP								2		6	9	18	>16	>16	4–>16
	β-Lactamase-producing	BLP-nonPGM					1		2	2		5	3	1	8	>16	0.5–>16
		BLP-PGM												2	>16	>16	>16
	Total		0	0	0	1	2	15	237	133	0	83	96	77			
CFD	β-Lactamase-nonproducing	NonPGM-nonBLP			13	239	85	10	3	1					0.25	0.5	0.125–4
		PGM1-nonBLP			1	34	59	64	57	17		8	2		1	4	0.125–16
		PGM2-nonBLP				2	1	6	12	6		5	3		2	8	0.25–16
	β-Lactamase-producing	BLP-nonPGM			1	4	2	6		1					0.5	2	0.125–16
		BLP-PGM							1	1					2	4	2.0–4
	Total		0	0	15	279	147	86	73	26	0	13	5	0			
CFPN	β-Lactamase-nonproducing	NonPGM-nonBLP	323	12	7	5	3		1						≤0.03	≤0.03	≤0.03–2
		PGM1-nonBLP	79	70	53	30	6	2	2	2					0.06	0.25	≤0.03–2
		PGM2-nonBLP	4	1	3	5	5	9	6						0.5	2	≤0.03–4
	β-Lactamase-producing	BLP-nonPGM	9	5											≤0.03	0.06	≤0.030.06
		BLP-PGM			2										0.125	0.125	0.125
	Total		415	88	65	40	14	11	9	2	0	0	0	0			

CDN, cefditoren; CPD, cefdinir; CCL, cefaclor; CFD, cefpodoxime; CFPN, cefcapene; nonPGM-nonBLP, β-lactamase nonproducing ampicillin-sensitive strain (without PBP gene mutations and production of β-lactamase); PGM1-nonBLP, strain with mutations in variable mutated locus of *ftsI* and no production of β-lactamase; PGM2-nonBLP, strain with mutations in highly mutated locus of *ftsI* and no production of β-lactamase; BLP-PGM, strain with mutations in *ftsI* and production of β-lactamase.

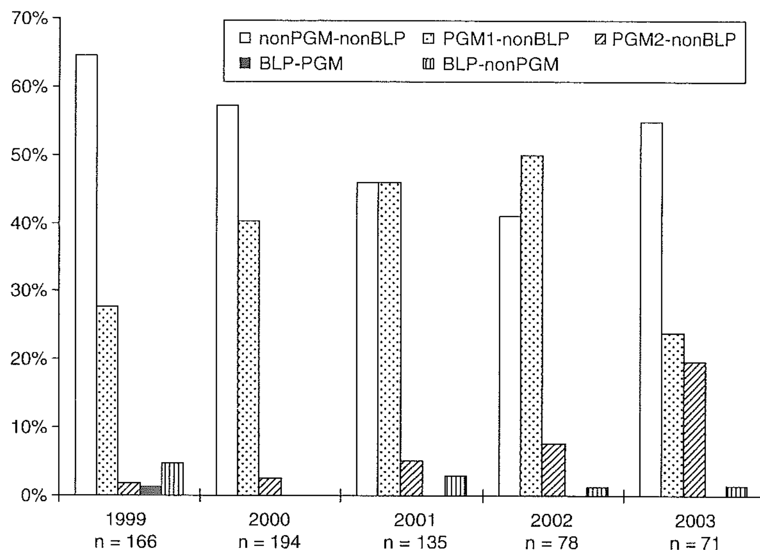


Figure 2. Year to year distributions of *pbp* gene mutated strains ($n = 644$). nonPGM-nonBLP, β-lactamase non-producing ampicillin-sensitive strain (without PBP gene mutations and productions of β-lactamase); PGM1-nonBLP, strain with mutations in variable mutated locus of *ftsI* and no production of β-lactamase; PGM2-nonBLP, strain with mutations in highly mutated locus of *ftsI* and no production of β-lactamase; BLP-PGM, strain with mutations in *ftsI* and production of β-lactamase.

[14]. Although BLNAR strains were identified only in 2.1% in 1988 and 5.0% in 1991, the prevalence of the strains (MIC to AMP > 1.0 μg/ml) increased up to 37.8% of isolates during 1996–1997 [15]. The countrywide surveillance of *H. influenzae* isolated from the upper respiratory tract during 1998–1999 in Japan showed that BLNAR (MIC to AMP 1.0 μg/ml) were identified in 23.1% of isolates and β-lactamase-producing strains were identified only in 6.0% [24]. The most recent surveillance among meningitis patients showed that BLNAR (similar genotype to PGM2-nonBLP) were identified at 13.9% and Low-BLNAR (similar genotype to PGM1-nonBLP) were identified at 30.6% by PCR-based genotypes [25]. In contrast to this surveillance focused on type b strains among the invasive disease, we focused on the prevalence of BLNAR in nontypeable strains among the nasopharyngeal isolates from pediatric patients with AOM and applied similar PCR-based genotypes in this study. The PGM strains were identified in 43.9% of all isolates. Among PGM strains, 1.2% of PGM1-nonBLP strains and 34.3% of PGM2-nonBLP strains were resistant to AMP according to the criteria recommended by the NCCLS. The strains showed decreased susceptibilities to CCL and CFD rather than to AMP, although they were still susceptible to recently developed cephalosporins such as CDN, CFPN and CPD. The higher affinities of cephalosporins for PBP3 rather than those of penicillins cause the decreased activities of cephalosporins against the PGM strains. The recent precise study of genetic and molecular characteristics of BLNAR strains by Karczmarek et al. showed the close relationship, but not alone, between reductions in penicillin binding and mutations in PBP3 [26]. The PCR-based genotyping method applied in this study shows their potential antimicrobial resistance and produces rapid information about resistance for clinicians. However, there were still some discrepancies between PCR-based genotypes and susceptibilities to β-lactams. Of 534 isolates susceptible to ABPC, 185 were identified with mutations in *ftsI* (PGM1-nonBLP or PGM2-nonBLP). These strains might have different mutations from those in typical BLANR strains which will not reduce affinities to β-lactams. It is necessary to assess sequences of these strains to reveal any relationships between PBP3 gene mutations and susceptibility to β-lactams.

According to the year to year distributions, the PGM1-nonBLP strains rapidly increased in Japan from 1999 to 2002 and then decreased in 2003. In contrast to the decrease of PGM1-nonBLP strains, PGM2-nonBLP strains increased in 2003. In 1999, we changed the antimicrobial treatment policies for outpatients. Amoxicillin has been used as the drug of first choice since 1999. The prevalence of antimicrobial resistance varies from country to country depending on different consumption patterns of antibiotics. Ubukata et al. also suggested the apparent relationship between clinical use of oral cepharosporins and increase in the numbers of BLNAR [18]. Frequent use of oral cephalosporins in Japan, in contrast to the USA, would likely lead to the higher prevalence of PGM strains. Our treatment policies might influence the year to year distribution of PGM strains. Moreover, in contrast to the apparent rapid spread of certain subtypes of type b

BLNAR strains among Japanese meningitis patients, the clonal dissemination of BLNAR strains among nontypable *H. influenzae* frequently isolated from the nasopharynx still remains unclear. Further surveillance of genetic and molecular characteristics is required to evaluate the spread of BLANR among non-invasive diseases in Japan.

Finally, the clinical importance of BLNAR strains has not been well documented yet. Brook *et al.* reported a relationship between antimicrobial-resistant *H. influenzae* and clinical outcomes of AOM [27] they found a higher recovery rate of antimicrobial-resistant *H. influenzae* from the nasopharynx of children who had otitis media that recurred after amoxicillin therapy than those with AOM [27]. Physicians should pay attention to the alarming increasing of the BLNAR strains among *H. influenzae* isolates.

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ORIGINAL ARTICLE

Evaluation of mutations in penicillin binding protein-3 gene of non-typeable *Haemophilus influenzae* isolated from the nasopharynx of children with acute otitis media

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Abstract

Conclusion. Younger children tend to harbor more resistant strains because they are exposed to these pathogens more often through contacts with siblings or attendance at day-care centers and are frequently treated with antibiotics. The high prevalence of BLNAR strains should be taken into account in the treatment of AOM in young children. *Objective.* Non-β-lactamase-producing ampicillin-resistant (BLNAR) strains with mutations in penicillin-binding protein (PBP) genes of *Haemophilus influenzae* have been prevalent recently among younger children. *Material and methods.* We investigated mutations in the *ftsI* gene encoding PBP-3 of *H. influenzae* isolated from the nasopharynx of children with acute otitis media (AOM) using polymerase chain reaction (PCR). *Results.* Strains containing the *bla* gene (β-lactamase-producing ampicillin-resistant) were identified in 4.7% of cases. Strains with mutations in the *ftsI* gene (BLNAR) were identified in 23.3% of cases. Strains without mutations in the *ftsI* gene and that did not contain the *bla* gene (non-β-lactamase-producing ampicillin-susceptible) were identified in 70.7% of cases. Strains with both expression of the *bla* gene and mutations in the *ftsI* gene (β-lactamase-producing amoxicillin–clavulanate-resistant) were identified in 1.3% of cases. The MICs of ampicillin against the strains evaluated in this study were 0.5–2.0 μg/ml. Cefditoren-pivoxil had the lowest MIC_{90} against the strains (0.06 μg/ml). Strains with mutations in the *ftsI* gene (BLNAR) were broadly identified among young children.

Keywords: *Acute otitis media, nasopharynx, non-β-lactamase-producing ampicillin-resistant Haemophilus influenzae, polymerase chain reaction*

Introduction

Acute otitis media (AOM) represents an enormous national health problem because of its vast incidence, the socioeconomic cost of its medical management and, especially, because of the rapid emergence of antibiotic resistance in pathogens. Non-typeable *Haemophilus influenzae* is one of the important causative pathogens for AOM. Because this pathogen has been susceptible to antibiotics in the past, the clinical outcomes of AOM resulting from antimicrobial treatment have been favorable. However, there has recently been an alarming increase in antimicrobial resistance in *H. influenzae* [1]. The most antimicrobial-resistant *H. influenzae* strains have been used to produce β-lactamase in the US and Europe. In contrast, non-β-lactamase-producing ampicillin-resistant (BLNAR) strains are gradually becoming more prevalent among children in Japan [2,3]. The mechanism of resistance against antibiotics is based on decreased affinity of β-lactams for penicillin-binding proteins (PBPs) as a result of genetic mutations in PBP genes [4,5]. Recent advances in molecular technologies have enabled investigators to evaluate the genetic mutations responsible for antibiotic resistance [6,7]. Polymerase chain reaction (PCR) has proved to be extremely helpful in understanding antibiotic resistance.

In this study, we investigated mutations in the *ftsI* gene encoding PBP-3 using PCR and evaluated the antimicrobial susceptibilities of *H. influenzae* isolated from the nasopharynx of children with AOM.

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Material and methods

H. influenzae strains

A total of 150 strains of *H. influenzae* isolated from the nasopharynx of 106 children (61 boys, 45 girls; age range 0–8 years; mean ± SD 3.0 ± 1.8 years) with AOM were evaluated. The children had been healthy for > 2 months prior to the study and had received no antibiotics for > 1 month before diagnosis. Diagnostic criteria for AOM included recent onset of symptoms of fever, crying and otalgia, changes in the tympanic membrane in the form of decreased landmarks, and bulging contours and erythema of the tympanic membrane as observed under a pneumatic otoscope. Nasopharyngeal cultures were obtained with a small rayon-tipped flexible swab at the first medical examination. *H. influenzae* strains were identified by means of colony morphology on chocolate agar plates, Gram staining, catalase and oxidase production and requirement for V and X factors. Production of β-lactamase was identified by means of an acidometric method.

Antimicrobial susceptibility test

The MICs of antibiotics were determined using the usual microbroth dilution method. The antibiotics employed in the study were ampicillin (ABPC), cefditoren-pivoxil (CDTR), cefdinir (CFDN), cefaclor (CCL) and cefpodoxime proxetil (CPDX). The MICs of the strains were provided by Meiji Seika Pharmaceuticals (Tokyo, Japan).

PCR analysis for H. influenzae

Three sets of oligonucleotide primers (Wakunaga Pharmaceutical Co. Ltd, Osaka, Japan) were used [8]. Primer sets for the P6 gene encoding outer membrane protein P6 were used to confirm the isolate as *H. influenzae*. Primer sets for the *bla* gene encoding a part of TEM-I-type β-lactamase were used to assess the production of β-lactamase. Primer sets for the *ftsI* gene encoding the transpeptidase domain of PBP3 were used to evaluate mutations in the PBP3 gene. A single colony of *H. influenzae* on a chocolate agar plate (Nippon Becton Dickinson, Tokyo, Japan) was lysed in 30 μl of lysis solution (1 M Tris, pH 8.9, 4.5% nonident P-40, 4.5% Tween 20, 10 mg/ml proteinase K) for 20 min at 60°C and 5 min at 94°C in a programmable thermal cycler (Gene Amp PCR System 9700; Perkin Elmer, Norwalk, CT). Two-μl portions of the bacterial lysate were added to 2 tubes, each containing 30 μl of reaction mixture. The reaction mixtures consisted of 3 μl of 10 × PCR buffer, 3 μl of dNTP mixture at a concentration of 2 mM, 1.2 U of Tth DNA polymerase (TOYOBO Biochemicals, Osaka, Japan) and 3 pmoles of both sense and reverse primers. The mixture was subjected to 30 cycles of amplification in the programmable thermal cycler. Each cycle consisted of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C. Amplified DNA fragments were analyzed using 3% agarose gel electrophoresis.

Results

Detection of mutations in the ftsI gene and expression of the bla gene using PCR

All *H. influenzae* strains possessed the P6 gene. Strains containing the *bla* gene [β-lactamase-producing ampicillin-resistant (BLPAR)] were identified in 4.7% of cases. Strains with mutations in the *ftsI* gene (BLNAR) were identified in 23.3% of cases. Strains without mutations in the *ftsI* gene and that did not contain the *bla* gene [non-β-lactamase-producing ampicillin-susceptible (BLNAS)] were identified in 70.7% of cases. Strains with both expression of the *bla* gene and mutations in the *ftsI* gene [β-lactamase-producing amoxicillin–clavulanate-resistant (BLPACR)] were identified in 1.3% of cases.

Relationship between PCR analysis and MICs of antibiotics

Strains with mutations in the *ftsI* gene (BLNAR) showed relative resistance to ABPC (MIC 0.5–2.0 μg/ml). β-lactamase-producing strains (BLPAR) were highly resistant to ABPC (MIC > 4 μg/ml). The susceptibilities of other β-lactams are shown in Figure 1. The MIC_{90} values of antibiotics against the strains with *ftsI* gene mutations (BLNAR) were 0.06 μg/ml for CDTR, 0.5 μg/ml for CPDX, 2 μg/ml for CFDN and > 16 μg/ml for CCL (Figure 2).

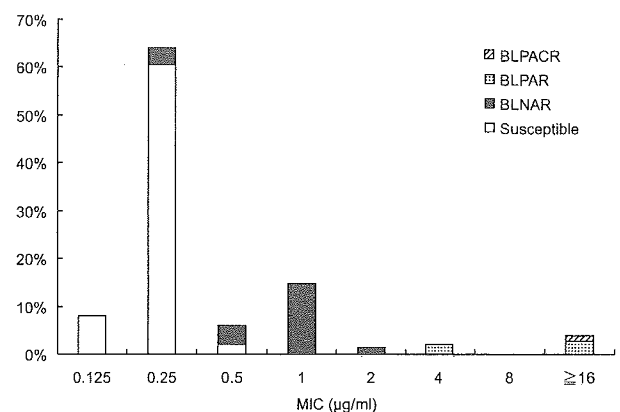

Figure 1. MICs of ampicillin against *H. influenzae* strains with mutations in the *ftsI* gene ($n = 150$).