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Rapid Detection of Varicella–Zoster Virus Infection by a Loop-Mediated Isothermal Amplification Method

Souki Okamoto,[1] Tetsushi Yoshikawa,[2]* Masaru Ihira,[3] Kayoko Suzuki,[4] Kaoru Shimokata,[1] Yukihiro Nishiyama,[5] and Yoshizo Asano[1]

[1]*Department of Medicine, Division of Respiratory Medicine, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan*
[2]*Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Aichi, Japan*
[3]*Department of Medical Information Technology, Fujita Health University College, Toyoake, Aichi, Japan*
[4]*Department of Dermatology, Central Hospital of Tokai Medical Institute, Tokai, Aichi, Japan*
[5]*Department of Virology, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan*

The reliability of varicella–zoster virus (VZV) loop-mediated isothermal amplification (LAMP) was evaluated for rapid diagnosis of viral infection. VZV-specific primers only amplified VZV DNA; no LAMP products were observed in reactions performed with other viral DNA templates. The specificity of this method was confirmed by two independent determinations, agarose gel electrophoresis and a turbidity assay. The sensitivity of VZV LAMP, determined by agarose gel electrophoresis, were 500 copies/tube. Detection using the turbidity assay, however, gave a sensitivity of 1,000 copies/tube. After these initial validation studies, reliability of VZV LAMP was evaluated for the detection of viral DNA in clinical specimens. Thirty-two swab samples collected from patients with vesicular skin eruptions were tested for VZV DNA. VZV was confirmed in sample numbers 10–32 by VZV real-time PCR, a previously established technique. VZV LAMP products were detected using turbidity from samples 13 to 32 (sensitivity; 87.0%, specificity; 100%, positive predictive value; 100%, negative predictive value; 75%). Although low levels of VZV DNA could be detected in the three samples exhibiting divergent results (samples numbers 10–12), no VZV LAMP product was detected in these samples, indicating a higher detection limit for this assay. Requirement of a DNA extraction step in the VZV LAMP method was examined in next experiment. The turbidity assay detected a VZV LAMP product in all of the 20 positive swab samples (samples numbers 13–32), regardless of DNA extraction. ***J. Med. Virol. 74:677–682, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: VZV; LAMP; rapid diagnosis; direct detection

INTRODUCTION

Varicella–zoster virus (VZV), the causative agent of chicken pox and herpes zoster, often results in central nervous system manifestations and pneumonia in both immunocompetent and immunocompromised individuals. Immunocompromised patients may also suffer severe clinical manifestations characterized by hemorrhagic, progressive, and disseminated infection with a potentially fatal outcome. The diagnosis of visceral herpes zoster in patients lacking cutaneous herpes zoster eruptions is difficult in stem cell transplant recipients [Stemmer et al., 1993; Rogers et al., 1995; David et al., 1998; de Jong et al., 2001]. Meanwhile, herpes simplex virus (HSV), another member of the Alphaherpesvirinae subfamily, causes a wide variety of clinical manifestations including pathologies of the skin, lips, oral cavity, eyes, genital tract, and central nervous system. This virus also results in generalized and disseminated

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*Correspondence to: Tetsushi Yoshikawa, MD, Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan 4701192. E-mail: tetsushi@fujita-hu.ac.jp

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infection in immunocompromised patients, including transplant recipients, patients with congenital or acquired immunodeficiency, and neonates. Due to these similarities in symptoms, differential diagnosis between VZV infection and HSV infection can be difficult in immunocompromised patients [Rubben et al., 1997]. Several additional infectious agents also induce similar clinical features. While a family of antiviral drugs is effective against both VZV and HSV infections, the antiviral activities of these drugs differ significantly between the two viruses. As early administration of these drugs can improve dramatically a patient's prognosis, rapid and correct diagnosis of viral infections is crucial for patient management.

Viral isolation and serological assays are standard methods used for the diagnosis of VZV infection. Both viral isolation and serological testing, however, require substantial time and skill to obtain final results. Additional laboratory diagnostic techniques to diagnose VZV infections include the direct detection of viral antigens in infected cells collected from the vesicular lesions of patients [Rawlinson et al., 1989; Schirm et al., 1989; Perez et al., 1994; Coffin and Hodinka, 1995; McCarter and Ratkiewicz, 1998]; the sensitivity of this method is problematic. Detection of VZV DNA by polymerase chain reaction (PCR) increases the sensitivity of viral detection from that seen with either antigen detection or cell culture methods [Dlugosch et al., 1991; Beards et al., 1998; Sauerbrei et al., 1999]. While quantitative analysis of viral DNA by real-time PCR is a valuable tool, suggested for the bedside monitoring of VZV infections [Espy et al., 2000; Dworkin et al., 2002; Ishizaki et al., 2003; van Doornum et al., 2003; Weidmann et al., 2003; Asano et al., 2004; Schmutzhard et al., 2004], this methodology has not yet become common procedure in hospital laboratories, due to the necessity of specific expensive equipment (a thermal cycler).

Recently, Notomi et al. [2000] reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), which amplifies DNA with high specificity, efficiency, and speed. The most significant advantage of LAMP is its ability to amplify specific sequences of DNA under isothermal conditions between 63 and 65°C. Thus, LAMP requires only simple, cost-effective equipment amenable to use in hospital laboratories. LAMP also exhibits high specificity and high amplification efficiency. As LAMP uses four primers recognizing six distinct sequences on target DNA, its specificity is extremely high. This method also exhibits extremely high amplification efficiency, due in part to its isothermal nature; there is no time lost due to changes in temperature. In addition, the reaction can be conducted at the optimal temperature for polymerase enzyme function. The inhibition reactions that often occur at later stages of PCR-based amplification are also reduced. Thus, this method may be a valuable tool for the rapid diagnosis of infectious diseases [Enosawa et al., 2003; Iwamoto et al., 2003; Kuboki et al., 2003; Ihira et al., 2004; Parida et al., 2004; Yoshikawa et al., 2004] in both commercial and hospital laboratories. In this study, we sought to establish a LAMP-based VZV DNA amplification technique and examine its reliability for the detection of viral DNA from clinical specimens.

MATERIALS AND METHODS

Study Design

VZV (Oka-vaccine strain) DNA was used as a positive control to determine the appropriate conditions for VZV LAMP and to establish a baseline sensitivity and specificity. DNA from HSV-1 (KOS), HSV-2 (186), varicella–zoster virus (Oka-vaccine strain), human cytomegalovirus (AD-169), human herpesvirus 6 B (Z29), and human herpesvirus 7 (RK) were used to determine the specificity of VZV LAMP. A plasmid containing the VZV target sequence was used to determine assay sensitivity.

To determine the reliability of VZV LAMP for the detection of viral DNA in clinical specimens, 32 swab samples collected from patients with vesicular skin eruptions were examined. Swabs, collected from patients at the outpatient clinic of Fujita Health University Hospital and Central Hospital of Tokai Medical Institute, were placed into 1 ml of sterilized water. Detection of VZV DNA was attempted in samples by LAMP with or without DNA extraction. The results of VZV LAMP were compared with results of VZV real-time PCR to assess the reliability of LAMP as a rapid diagnostic tool detecting VZV infection.

DNA Extraction

For initial validation studies establishing VZV LAMP as a diagnostic method, viral DNA was extracted from HSV-1 (KOS)-, HSV-2 (186)-, varicella–zoster virus (Oka-vaccine strain)-, human cytomegalovirus (AD-169)-, HHV-6 B (Z29)-, and HHV-7 (RK)-infected cells using a QIAamp Blood Kit (QIAGEN, Chatsworth, CA). This DNA extraction kit was also used to extract DNA from 200 μl swab samples collected from the patients. After extraction, DNA was eluted in 100 μl of elution buffer and stored at −20°C.

VZV LAMP

LAMP reactions were conducted as described by Notomi et al. [2000] and Nagamine et al. [2002]. LAMP requires a set of four specially designed primers (B3, F3, BIP, FIP) recognizing a total of six distinct target DNA sequences (B1–B3, F1–F3). Primers for VZV LAMP were designed against VZV gene 62 using Primer Explorer V software (FUJITSU, Tokyo, Japan). The location and sequence of each primer in the target DNA sequence are shown in Figure 1. BIP for VZV gene 62 (VZBIP) consisted of the B1 direct sequence (20 nucleotides; nt) and the B2 complementary sequence (20 nt) for the VZV Oka-vaccine strain. Primer FIP for VZV gene 62 (VZFIP) consisted of the F1 complementary sequence (20 nt) and the F2 direct sequence (18 nt). Primer B3 (VZB3) and F3 (VZF3) for VZV gene 62 are

A Location and name of each target sequence as a primer in VZV ORF62 gene

Nucleotide position

1391 ATGATCAGAAGCCTCACATCCTCCGGGTCTGGGATCTGCCGCATCCAGGC
F3 F2

1441 GCACCTCCGTCGCAGCGCCTCCACTCCGCTGGGTGGACCAAACCGTCGGT
LPF (loop primer F) F1

1491 CTCCTCCGCCCGGACGCCGAGCGGCGATTTCCGCCAAGGCGCCGGGATCA
B1

1541 AAGCTTAGCGCAGGGCGCCAGGCCGTGGGAAACAATGGGTCGTCGACCAG
LPB (loop primer B) B2

1591 ACGGGCGATGGTTTCGGGGGTACAGTACGCCTTGCGAGCCTGGTCCGACG
B3

B Sequence of each primer

Name of primers	Sequence
VZBIP	5'- GGCGCCGGGATCAAAGCTTA GGTCGACGACCCATTGTTTC - 3' (B1-B2c)
VZFIP	5'- GACGGTTTGGTCCACCCAGC TCTGGGATCTGCCGCATC - 3' (F1c-F2)
VZB3	5'- CGTACTGTACCCCCGAAAC - 3'
VZF3	5'- TCAGAAGCCTCACATCCTCC - 3'
VZLPB	5'- CGCAGGGCGCCAGGCCGTGG -3'
VZLPF	5'- AGTGGAGGCGCTGCGACGGA -3'

Fig. 1. The locations and names of the target sequences used as primers for VZV LAMP within the VZV ORF62 gene (A). The name and sequence of each primer for VZV LAMP is shown (B). B2c, sequence complementary to B2; F1c, sequence complementary to F1.

complimentary to sequences outside of the F2-B2 regions. As additional loop primers increase the amplification efficiency, loop primers for gene 62 (VZLPB and VZLPF) were also synthesized. VZLPB was composed of the LPB sequence, while VZLPF is made up of the LPF complementary sequence. LAMP reactions were performed with a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). A reaction mixture (25 μl) containing 1.6 μM of the FIP and BIP primers, 0.8 μM of each outer primer (F3 primer and B3 primer), 0.8 μM of each loop primer (LPF primer and LPB primer), 2× reaction mix (12.5 μl), *Bst* DNA polymerase (1 μl), and 5 μl sample. Reaction mixtures were incubated at 63°C for 30 min. A TERAMECS LA200 (Teramecs, Kyoto, Japan) was then used to measure turbidity after 30 min of LAMP [Mori et al., 2002]. The cutoff value of turbidity used to distinguish negative from positive samples was fixed at 0.1, higher than the mean plus 3 SD of the turbidity of five negative samples. After turbidity measurement, LAMP products were subjected to electrophoresis on a 1.5% agarose gel. Gels were visualized under UV light after ethidium bromide staining. Great care was taken to avoid contamination between samples; different rooms were used for DNA extraction, LAMP set up, and gel analysis. In addition, pipette tips with filters for aerosol protection were used to minimize contamination.

Real-Time PCR for VZV

Real-time PCR was used to measure the quantity of VZV DNA in each sample. The sequences of primers and probes used have been described by Kimura et al. [2000]. PCR reactions were performed using the TaqMan PCR Kit (PE Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Standard curve for measuring VZV DNA was constructed using the C_T values obtained from a serially diluted plasmid containing the target sequence, pVZTA. The C_T value from each sample was plotted on a standard curve, allowing copy numbers to be automatically calculated using Sequence Detector v1.6 software (PE Applied Biosystems). Each sample was tested in duplicate; the copy number of each sample is represented as the mean of the two values.

Cloning of VZV DNA

To determine the sensitivity of VZV LAMP, a plasmid containing the target VZV DNA sequence was constructed. Upstream (VZVU; 5'-GGGCAACGGATCGTAGATG) and downstream (VZVD; 5'-AGGCTCGCAAGGCGTACT) primers spanning the sequences between the F3 and B3 primers were synthesized. VZV DNA (Oka-vaccine strain) was amplified by conventional PCR using these two primers. The PCR product was cloned into the pGEM-T plasmid using the pGEM-T Vector

System II (Promega, Madison, WI), according to the manufacturer's instructions. Standard dilutions of the constructed plasmid (pGEMVZ) were used to evaluate the lower detection limit of LAMP. To create the plasmid (pVZTA) used to make a standard curve for VZV real-time PCR, viral DNA was amplified from the Oka-vaccine strain using the real-time PCR primers. Subsequently, the PCR product was cloned into pGEM-T using the pGEM-T Vector System (Promega), according to the manufacturer's instruction.

RESULTS

During assay development, we evaluated the specificity of VZV-specific primers. VZV LAMP was performed on DNA extracted from HSV-1 (KOS)-, HSV-2 (186)-, VZV (Oka)-, HCMV (AD-169)-, HHV-6 B (Z29)-, and HHV-7 (RK)-infected cells. As LAMP products contained several inverted-repeat structures, positive samples demonstrate multiple bands of different sizes upon agarose gel electrophoresis. VZV-specific LAMP primers amplified only the VZV DNA; no LAMP products were detected in reactions performed with other viral DNAs (Fig. 2). The specificity of the primers was also tested by turbidity assay. Elevation of turbidity was observed only in VZV DNA-containing samples; no elevation of turbidity could be observed in samples containing other viral DNAs (Fig. 2).

The sensitivity of VZV-specific LAMP was also determined (Fig. 3). Serial dilutions of the pGEMVZ plasmid were used to determine the detection limit of VZV LAMP. The sensitivity of VZV LAMP determined by agarose gel electrophoresis and turbidity assay were 500 copies/tube and 1,000 copies/tube, respectively (Fig. 3).

After these initial validation studies, the reliability of VZV LAMP was determined as a rapid method for viral DNA detection from clinical specimens. Thirty-two swab samples were collected from patients with vesicular skin eruptions (Table I). VZV DNA was detected in sample numbers 10–32 by VZV real-time PCR. VZV LAMP products were detected by turbidity assay in samples 13–32. Although low levels of VZV DNA were detected in samples 10–12 by real-time PCR, no VZV LAMP product could be observed in these samples (sensitivity; 87.0%, specificity; 100%, positive predictive value; 100%, negative predictive value; 75%). As both the rapidity and simplicity of a diagnostic method are important for both commercial and hospital laboratory use, the requirement for DNA extraction in VZV LAMP was examined in the next experiment. As the results of the turbidity assay for samples with and without DNA extraction were the same (Table I), DNA extraction does not appear to be necessary for LAMP diagnostic testing.

To determine if culture medium contained an inhibitor of the LAMP reaction, detection of VZV LAMP

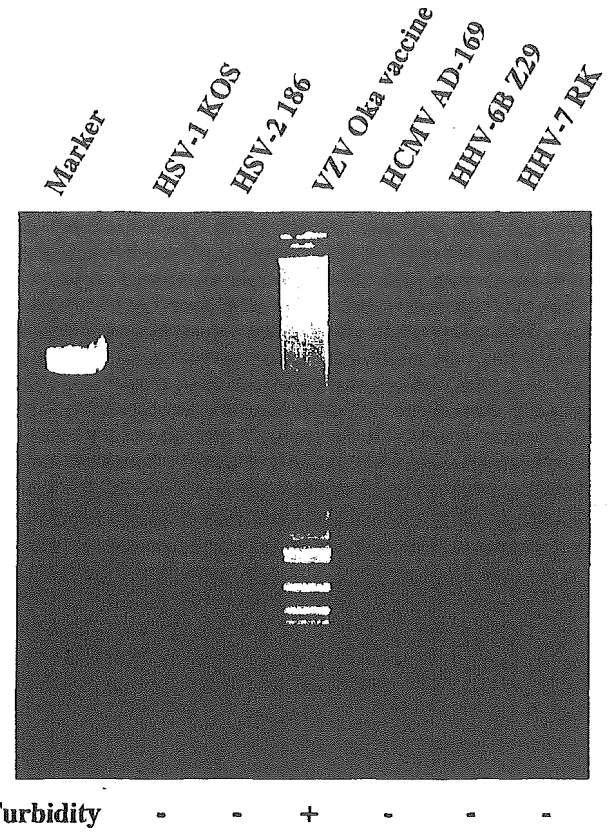

Fig. 2. DNA extracted from herpes simplex virus (HSV)-1 (KOS)-, HSV-2 (186)-, varicella–zoster virus (Oka-vaccine strain)-, human cytomegalovirus (AD-169)-, human herpesvirus 6 B (Z29)-, and human herpesvirus 7 (RK)-infected cells was amplified by VZV LAMP to determine the specificity of this method. The specificity of the method was confirmed by agarose gel electrophoresis and turbidity assay. Marker, 123-base-pair DNA ladder marker.

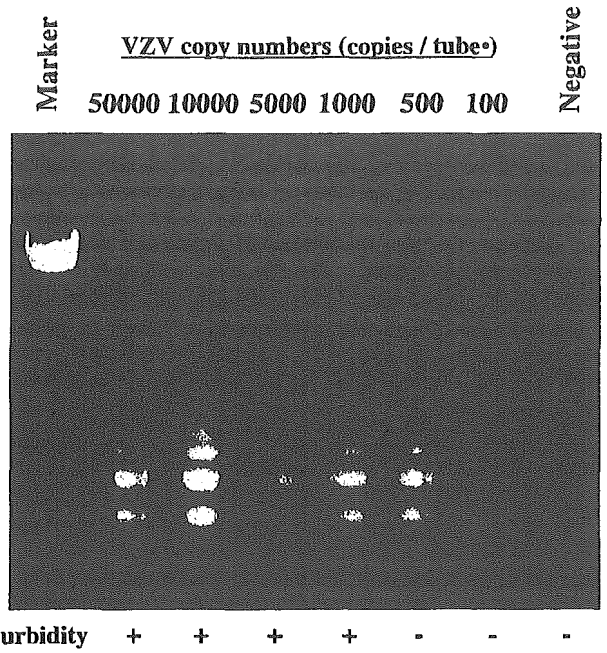

Fig. 3. Serial dilutions of the pGEMVZ plasmid DNA were amplified by VZV LAMP to determine the method's detection limit. Two different detection methods (agarose gel electrophoresis and turbidity assay) were used to determine the lowest detection limit. Marker, 123-base-pair DNA ladder marker.

TABLE I. Comparison Between VZV LAMP and the Previously Established Real-Time PCR

No. of samples	Results of VZV LAMP: DNA extraction for preparing the samples: Yes	Results of VZV LAMP: DNA extraction for preparing the samples: No	Copy numbers determined by real-time PCR (copies/tube)
1	−	−	0
2	−	−	0
3	−	−	0
4	−	−	0
5	−	−	0
6	−	−	0
7	−	−	0
8	−	−	0
9	−	−	0
10	−	−	19
11	−	−	36
12	−	−	76
13	+	+	137
14	+	+	6089
15	+	+	126375
16	+	+	1205567
17	+	+	1569136
18	+	+	1750154
19	+	+	2189819
20	+	+	3161031
21	+	+	3280522
22	+	+	3402132
23	+	+	3579352
24	+	+	3775060
25	+	+	3828846
26	+	+	4337990
27	+	+	4960072
28	+	+	6744228
29	+	+	7252607
30	+	+	7375980
31	+	+	8043041
32	+	+	9815983

Detection of VZV LAMP products were attempted from samples with and without DNA extraction. Only the DNA extracted samples were used to carry out real-time PCR.

products was attempted from either sterilized water or culture medium containing plasmid DNA including the target sequence. Although VZV LAMP products could be detected in sterilized water samples, no LAMP products were detected in culture medium samples (data not shown).

DISCUSSION

To establish a new diagnostic procedure, detailed evaluation of the specificity and the sensitivity is crucial. VZV-specific LAMP specifically amplified VZV DNA; no cross-reactivity was observed with other human herpesviruses, including another member of the Alphaherpesvirinae family, HSV (Fig. 2). This specificity was confirmed by two independent detection methods, agarose gel electrophoresis and turbidity assay. The detection limits for VZV LAMP were 500 copies/tube and 1,000 copies/tube for agarose gel electrophoresis and turbidity assay, respectively. Although the turbidity assay is less sensitive than agarose gel electrophoresis [Yoshikawa et al., 2004], the ease and rapidity of the turbidity assay makes it more appropriate for bedside monitoring. Furthermore, as tubes were not opened at any time during amplification and detection of amplified DNA, this system also minimizes potential contamination. Turbidity increases gradually during the LAMP reaction due to the increasing number of amplified product DNA [Mori et al., 2002]. Therefore, increase in LAMP reaction time may result in the improvement of assay sensitivity. Quantitative analysis of LAMP products, which appears to be an effective tool for monitoring disease activity, is therefore possible using the turbidity assay. Initial validation studies demonstrated that VZV LAMP has both a high specificity and sensitivity for the amplification of viral DNA. Moreover, all amplification steps are completed using an LA-200, a cheaper piece of equipment than that used for real-time PCR. As the reaction requires only 30 min to perform, VZV LAMP has major advantages as a rapid diagnostic procedure for hospital laboratories.

The reliability of VZV LAMP was evaluated for the detection of viral DNA from clinical specimens. As the turbidity assay appears to be the most appropriate evaluation for clinical laboratory use, this assay was used for clinical sample analysis. Most of the positive samples contained high copy numbers of viral DNA. LAMP products were detected in 20 (87%) of the 23 positive samples determined to be positive by real-time PCR. While three false negative samples containing low levels of viral DNA, likely below the detection limits of VZV LAMP, were found, no false positive samples were observed in this analysis, likely due to the high specificity and low contamination of LAMP. Thus, high sensitivity and specificity of VZV LAMP in the analysis of clinical specimens collected from skin lesions were demonstrated. Typically, large quantities of viral DNA [Kimura et al., 2000], clearly higher than the detection limit of VZV LAMP, can be detected from skin eruptions in varicella and zoster patients by real-time PCR. Moreover, ocular specimens collected from patients with posterior uveitis or acute retinal necrosis also demonstrated high copies of viral DNA, although sample numbers were limited [Dworkin et al., 2002; Asano et al., 2004]. Thus, the sensitivity of VZV LAMP is likely sufficient to detect viral DNA from clinical specimens. The efficacy of VZV LAMP in detecting viral DNA from ocular samples is now under investigation.

When swabs were placed in sterilized water, VZV LAMP product could be detected directly from samples without DNA extraction. To our knowledge, this is the first report demonstrating direct amplification of VZV DNA from swab samples of skin eruptions without DNA extraction. As DNA extraction requires approximately 30 min, omission of this DNA extraction step could save great deal of time and labor in preparation of the samples for LAMP reaction. This is a major advantage for rapid diagnosis in hospital laboratories. Direct amplification from swab samples, in combination with the ease of the turbidity assay for detection of LAMP products, would enable completion of the entire amplification within 30 min. Therefore, we emphasize that

swab should be placed into sterilized water for viral DNA amplification by LAMP. The rapid diagnosis of VZV infection in central nervous system is also important. As HSV LAMP products were detected directly (without DNA extraction) in cerebrospinal fluids collected from patients with neonatal herpes simplex virus infection (unpublished data), VZV LAMP products might be detected in cerebrospinal fluids containing sufficient amounts of viral DNA without DNA extraction. Further investigations should be carried out to elucidate the reliability of the method for rapid diagnosis of central nervous infections caused by VZV. Moreover, direct detection of VZV DNA in swab samples also suggests that skin eruptions may contain large quantities of naked viral DNA, as well as complete virions, another important point from a virological standpoint.

In summary, a novel VZV DNA detection system was developed employing the nucleoside amplification method, LAMP. This method is highly specific and sensitive. The advantages of VZV LAMP for use in hospital laboratories are its requirements for minimal simple and cost-effective equipment and a short completion time (30 min).

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High prevalence of type b β-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced

Keiko Hasegawa[1], Reiko Kobayashi[1], Emi Takada[1], Akiko Ono[1], Naoko Chiba[1], Miyuki Morozumi[1], Satoshi Iwata[2], Keisuke Sunakawa[3] and Kimiko Ubukata[1]*
on behalf of the Working Group of the Nationwide Surveillance for Bacterial Meningitis

[1]*Laboratory of Infectious Agents Surveillance, Kitasato Institute for Life Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo, Japan;* [2]*National Hospital Organization Tokyo Medical Center, Tokyo, Japan;* [3]*Department of Infectious Disease, Kitasato University School of Medicine, Kanagawa, Japan*

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***Objectives*:** To study yearly changes in resistance and to identify *ftsI* mutations in β-lactamase-non-producing ampicillin-resistant (BLNAR) and TEM-1 β-lactamase-producing amoxicillin/clavulanic acid-resistant (BLPACR) isolates of *Haemophilus influenzae* from patients with meningitis.

***Methods*:** Between January 2000 and December 2004, we received 621 isolates of *H. influenzae* from 285 member institutions of the Nationwide Surveillance Study Group for Bacterial Meningitis. All isolates were analysed by PCR to identify resistance genes and tested for susceptibility to β-lactams. The *ftsI* gene was sequenced in all BLNAR and BLPACR isolates.

***Results*:** All but four isolates were of serotype b. The isolates could be divided into six classes, namely β-lactamase-non-producing ampicillin-susceptible (25.0%), TEM-1 β-lactamase-producing ampicillin-resistant (11.0%), β-lactamase-non-producing low-level ampicillin-resistant with N526K or R517H substitution in the *ftsI* gene (30.4%), BLNAR with an S385T substitution together with either N526K or R517H substitution in *ftsI* (22.2%), BLPACR-I with either a N526K or R517H substitution in *ftsI* (9.5%) and BLPACR-II with an S385T substitution together with either a N526K or R517H substitution in *ftsI* (1.9%). The prevalence of BLNAR has increased rapidly, from 5.8% in 2000 to 34.5% in 2004. All BLNAR and BLPACR-II strains were classified into nine subgroups on the basis of substitution patterns in the *ftsI* gene. The MICs of cephalosporin antibiotics for *H. influenzae* transformants into which the *ftsI* genes from BLNAR strains of each of the nine subgroups were introduced increased to varying degrees depending on the mutations.

***Conclusions*:** The results suggest that introduction of *H. influenzae* type b (Hib) vaccination into infants and children is necessary for the prevention of severe Hib infections in Japan.

Keywords: *H. influenzae*, BLNAR, PCR

Introduction

The *Haemophilus influenzae* type b (Hib) vaccine has not been introduced in Japan, and in this country *H. influenzae* remains a major cause of meningitis in children aged ≥3 months.[1] Surveillance indicates that the incidence of Hib meningitis in Japan is ~10/100 000 in children under 5 years. This is in marked contrast to the low rates in countries where the Hib vaccine has been introduced.[2]

In the past 6 years, β-lactam-resistant strains of *Streptococcus pneumoniae* and Hib isolates from patients with meningitis have been rapidly increasing in parallel with their increase in patients with respiratory tract infections (RTIs) in Japan.[1,3–5] Of these pathogens, β-lactamase-non-producing ampicillin-resistant (BLNAR)

*Corresponding author. Tel: +81-3-5791-6385; Fax: +81-3-5791-6386; E-mail: ubukatak@lisci.kitasato-u.ac.jp

Table 1. MIC distributions and resistance genes identified by PCR in *H. influenzae* strains isolated in Japan (n = 621)

Antimicrobial agent and resistance class	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)
Ampicillin			
BLNAS	0.25	0.5	0.031–0.5
BLPAR (TEM-1)	8	16	2–64
low-BLNAR	1	2	0.5–2
BLNAR	2	4	1–16
BLPACR-I	16	32	2–64
BLPACR-II	32	64	16–64
Cefotaxime			
BLNAS	0.016	0.031	0.004–0.063
BLPAR (TEM-1)	0.016	0.031	0.008–0.063
low-BLNAR	0.063	0.125	0.031–0.25
BLNAR	0.5	1	0.125–2
BLPACR-I	0.063	0.125	0.016–0.25
BLPACR-II	0.5	1	0.125–1
Ceftriaxone			
BLNAS	0.004	0.008	0.002–0.016
BLPAR (TEM-1)	0.004	0.008	0.002–0.008
low-BLNAR	0.016	0.031	0.004–0.125
BLNAR	0.125	0.25	0.031–0.5
BLPACR-I	0.016	0.031	0.004–0.125
BLPACR-II	0.125	0.25	0.031–0.25
Meropenem			
BLNAS	0.031	0.063	0.008–0.125
BLPAR (TEM-1)	0.063	0.063	0.031–0.063
low-BLNAR	0.125	0.25	0.031–0.5
BLNAR	0.125	0.25	0.031–0.5
BLPACR-I	0.125	0.25	0.031–0.5
BLPACR-II	0.125	0.25	0.063–0.25

BLNAS, β-lactamase-non-producing ampicillin-susceptible *H. influenzae*; BLPAR, β-lactamase-producing ampicillin-resistant *H. influenzae*; low-BLNAR, β-lactamase-non-producing low-level ampicillin-resistant *H. influenzae*; BLNAR, β-lactamase-non-producing ampicillin-resistant *H. influenzae*; BLPACR, β-lactamase-producing amoxicillin/clavulanic acid-resistant *H. influenzae*.

Hib isolates have exponentially increased from 5.8% in 2000 to 34.5% in 2004. Although *H. influenzae* strains showing decreased affinities for β-lactams were first described in the USA in the 1980s,[6,7] these strains are rare in the USA and also in the EU.[8,9] The ampicillin resistance mechanism in BLNAR strains results from mutations in the *ftsI* gene encoding penicillin-binding protein 3 (PBP3), which mediates septal peptidoglycan synthesis in the cell wall. We have identified three mutations associated with decreased susceptibility to β-lactams.[10] BLNAR strains have the amino acid substitutions N526K or R517H and S385T, while strains with the amino acid substitution N526K or R517H alone, and which have slightly lower ampicillin MICs than BLNAR strains, are designated low-BLNAR (Table 1).

In this paper, we describe the results obtained by the Nationwide Surveillance Study Group for Bacterial Meningitis (NSBM) Working Group for Hib isolates in meningitis, with regard to yearly changes in genotypic resistance and verification of the correlation between antibiotic susceptibility and diversity of the *ftsI* gene.

Materials and methods

Strains

A total of 621 *H. influenzae* isolates were collected from clinical laboratories at 285 Japanese medical institutions participating in the NSBM Working Group between January 2000 and December 2004. These institutions were recruited on the basis of their proximity to major metropolitan areas. PCR was immediately performed on all isolates as described below to determine the *ftsI* mutations, after which production of β-lactamase was confirmed by a nitrocefin test (Showa Chemical Inc., Tokyo, Japan). Isolates were all stored at −80°C for subsequent testing.

The samples were collected after obtaining informed consent from family members in the case of infants and children.

PCR

PCR was carried out using six sets of primers reported previously:[8,11] the six targets included the 16S rRNA gene (for identification of *H. influenzae*), part of the TEM β-lactamase gene,[12] part of the ROB β-lactamase gene,[13] an N526K amino acid substitution in the *ftsI* gene, an S385T amino acid substitution in the *ftsI* gene and a portion of the Hib-specific *capB* locus.[14] PCR cycling conditions were 35 cycles at 94°C for 15 s, at 53°C for 15 s and at 72°C for 15 s.

Isolates suspected of having an R517H substitution on the basis of their susceptibility to ampicillin and cefotaxime were subjected to direct sequencing to detect this substitution because useful primers could not be designed.[8]

Antibiotic susceptibility

Isolates were tested for susceptibility to ampicillin (Meiji Seika Kaisha, Tokyo, Japan), cefotaxime (Aventis Pharma Ltd, Tokyo, Japan), ceftriaxone (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) and meropenem (Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) using an agar dilution method.[15]

Sequencing

The 1.0 kb DNA fragment of the *ftsI* gene encoding the PBP3 transpeptidase domain was amplified from the chromosomal DNA of all BLNAR and TEM-1 β-lactamase-producing amoxicillin/clavulanic acid-resistant (BLPACR) strains identified by the PCR method described above and was sequenced by the method reported previously.[10]

Transformation

An open reading frame that corresponded to the *ftsI* gene was amplified and transformed into the Rd strain (ATCC 51907) as described previously[10] in a cuvette with a 0.1 cm electrode gap using a Micro-Pulser™ electroporation apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The conditions for electroporation were 1.8 kV/cm with time constants of 5.8–5.9 ms. Colonies grown on selective agar plates containing cefotaxime at 0.031 and 0.063 mg/L were selected at random, and the MICs of the β-lactam antibiotics for the colonies were determined. The *ftsI* gene was also sequenced for several transformants to confirm gene transfer.

Results

Age distribution of cases

Among isolates from 621 cases of meningitis, 617 (99.4%) were type b and 4 were non-typeable. The prevalence of *H. influenzae*

Table 2. Changes by year in resistant strains identified by PCR in *H. influenzae* isolated from meningitis patients

Resistance class	Year					Total
	2000	2001	2002	2003	2004	
BLNAS	24 (34.8)	44 (31.4)	35 (21.1)	26 (20.0)	26 (22.4)	155 (25.0)
BLPAR	14 (20.3)	19 (13.6)	18 (10.8)	8 (6.2)	9 (7.8)	68 (11.0)
Low-BLNAR	24 (34.8)	41 (29.3)	52 (31.3)	40 (30.8)	32 (27.6)	189 (30.4)
BLNAR	4 (5.8)	19 (13.6)	35 (21.1)	40 (30.8)	40 (34.5)	138 (22.2)
BLPACR-I	3 (4.3)	15 (10.7)	22 (13.3)	12 (9.2)	7 (6.0)	59 (9.5)
BLPACR-II	0 (0.0)	2 (1.4)	4 (2.4)	4 (3.1)	2 (1.7)	12 (1.9)
Total	69	140	166	130	116	621 (100.0)

meningitis peaked in patients who were 1 year of age (n = 167, 26.9%), and was next highest in patients aged between 7 and 11 months (n = 120, 19.3%), and then in patients who were 1–6 months aged (n = 106, 17.1%). The total number of isolates from patients aged between 1 and 11 months (n = 226, 36.4%) was higher than that in patients who were 1 year of age. Hib meningitis gradually became less prevalent after the age of 2 years and was rare by the age of 5 years. β-Lactam-resistant strains including BLNAR strains were isolated at the same rate in all age groups.

Susceptibility of H. influenzae to intravenous agents

Table 1 shows the correlation between resistance genes and susceptibility to the four β-lactams used for empirical treatment of meningitis. The isolates could be grouped into the following six classes: β-lactamase-non-producing ampicillin-susceptible (BLNAS) (n = 155; 25.0%); TEM-1 β-lactamase-producing ampicillin-resistant (BLPAR) (n = 68; 11.0%); low-BLNAR (n = 189; 30.4%), BLNAR (n = 138; 22.2%); BLPACR-I with either a N526K or R517H substitution in the *ftsI* gene (n = 59; 9.5%) and BLPACR-II with an S385T substitution together with either a N526K or R517H substitution in the *ftsI* gene (n = 12; 1.9%). No ROB-1-positive strains were detected.

The MIC_{50}s of ampicillin were 1 mg/L for low-BLNAR and 2 mg/L for BLNAR, four to eight times higher than the value of 0.25 mg/L for BLNAS. In contrast, the MIC_{50}s of cefotaxime and ceftriaxone for the resistant strains were clearly increased. MIC_{50} of cefotaxime was 0.063 mg/L for low-BLNAR and 0.5 mg/L for BLNAR, 32 times higher than the value of 0.016 mg/L for BLNAS. Similarly, the MIC_{50} of ceftriaxone for BLNAR was 0.125 mg/L, 32 times higher than the value of 0.004 mg/L for BLNAS.

The MICs of meropenem for the resistant strains were affected a little by the *ftsI* gene mutations. The MIC_{50} of meropenem for low-BLNAR and BLNAR was 0.125 mg/L, four times higher than the value of 0.031 mg/L for BLNAS.

Yearly changes of resistance

Table 2 shows the yearly changes in resistance types classified genotypically among the strains between 2000 and 2004. BLNAR strains were not isolated during half a year in 1999, though the prevalences of low-BLNAR and BLPAR were 29.2% and 31.7%, respectively. However, BLNAR strains were isolated in 2000, since when their prevalence has increased, accounting for 34.5% of isolates in 2004. In contrast, the incidence of BLNAS and BLPAR strains gradually decreased to 22.4% and 7.8%, respectively, in 2004. The incidence rates of low-BLNAR, BLPACR-I and BLPACR-II strains were broadly flat during the 5 years.

Amino acid substitution in the ftsI gene

Table 3 shows the amino acid substitutions in the *ftsI* genes in BLNAR (n = 138) and BLPACR-II (n = 12) strains classified into nine subgroups on the basis of variation of substitution patterns. BLNAS strain MT196 was taken as the standard ampicillin-susceptible strain. In the BLNAR strains, the six amino acid substitutions shown in Table 3 were important for the development of resistance. Of these substitutions, N526K and R517H were the substitutions which are the base of phenotypic resistance, and three amino acid substitutions were identified surrounding the conserved SSN motif. S385T was common to the II-IV and VI-IX subgroups, and M377I and L389F substitutions were also identified. Strains in subgroup IX with these three substitutions surrounding the SSN motif were the most prevalent.

With regard to the correlation of the number of substitutions and antibiotic susceptibility, the MIC_{50} of ampicillin for the BLNAR with three substitutions of S385T, M377I and L389F was only either two or four times higher than those for the low-BLNAR strains. However, the MIC_{50}s of cefotaxime and ceftriaxone for BLNAR were clearly increased by the additional amino acid substitutions.

Transformation

Ampicillin-susceptible strain Rd was transformed with PCR-amplified *ftsI* genes from isolates of each of the nine subgroups of BLNAR strains. The transformation frequency was $1–3/10^7$. As shown in Figure 1, the MICs of cefotaxime for each transformant increased with additional substitutions in the *ftsI* gene. The cefotaxime MICs for transformants were as follows: N526K, 0.063 mg/L; S385T + N526K, 0.25 mg/L; M377I + S385T + L389F + N526K, 0.5 mg/L. The cefotaxime MICs for transformants with the four amino acid substitutions of subgroup IX were 64 times higher than the value of 0.008 mg/L for the parent Rd strain. The ampicillin MIC (1 mg/L) for transformants of subgroup IX was only four times higher than the value of 0.25 mg/L for the parent strain Rd. The meropenem MIC for transformants of subgroup IX was 0.063 mg/L, the same as that for the parent Rd strain. The correlations between mutation in the *ftsI* gene and susceptibility to ampicillin and meropenem were poor.

Table 3. Amino acid substitutions identified in the *ftsI* genes from BLNAR and BLPACR-II *H. influenzae* strains

Resistance class	Subgroup	No. of strains	Amino acid substitutions						MIC_{50} (mg/L)			
			M377	S385	L389	A502	R517	N526	AMP	CTX	CRO	MEM
BLNAS[a]			–	–	–	–	–	–	0.125	0.016	0.004	0.031
Low-BLNAR[b]			–	–	–	–	H	–	1	0.063	0.008	0.25
Low-BLNAR[c]			–	–	–	–	–	K	1	0.063	0.031	0.25
BLNAR	I	1 (0.7%)	–	–	–	V	H	–	1	0.25	0.031	0.063
BLNAR	II	4 (2.9%)	–	T	–	–	H	–	0.5	0.063	0.016	0.063
BLNAR	III	14 (10.1%)	I	T	–	–	H	–	1	0.125	0.063	0.125
BLNAR	IV	8 (5.8%)	I	T	F	–	H	–	4	1	0.125	0.125
BLNAR	V	2 (1.4%)	–	–	–	V	–	K	2	0.125	0.063	0.25
BLNAR	VI	11 (8.0%)	–	T	–	–	–	K	2	0.25	0.063	0.25
BLNAR	VII	4 (2.9%)	I	T	–	–	–	K	1	0.125	0.031	0.063
BLNAR	VIII	2 (1.4%)	–	T	F	V	–	K	2	0.125	0.063	0.125
BLNAR	IX	92 (66.7%)	I	T	F	–	–	K	2	0.5	0.125	0.25
BLPACR-II	II	1	–	T	–	–	H	–	32	1	0.25	0.125
BLPACR-II	III	1	I	T	–	–	H	–	64	0.125	0.031	0.125
BLPACR-II	VI	1	–	T	–	–	–	K	16	1	0.125	0.063
BLPACR-II	IX	9	I	T	F	–	–	K	64	0.5	0.125	0.25

AMP, ampicillin; CTX, cefotaxime; CRO, ceftriaxone; MEM, meropenem.
[a]Strain #, MT196.
[b]Strain #, H17.

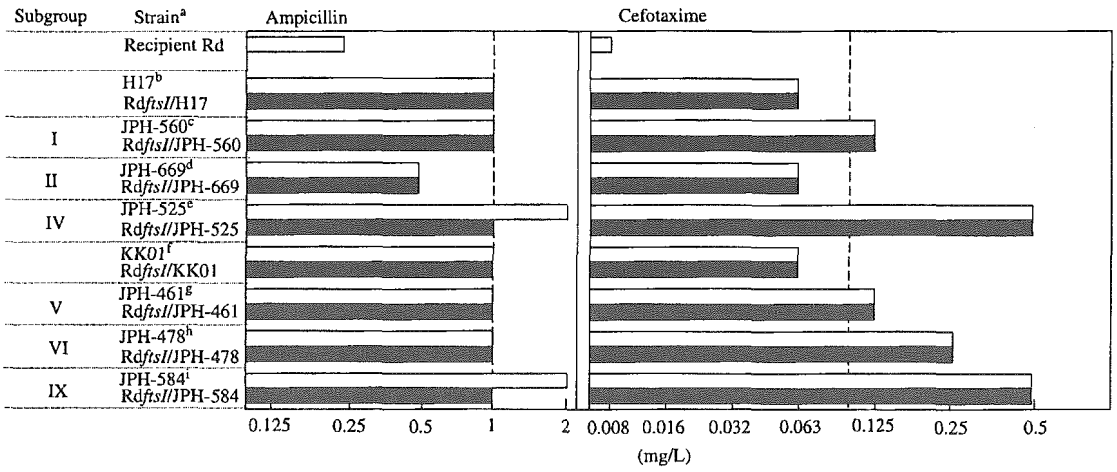

Figure 1. Comparison between susceptibility to β-lactam antibiotics of donors and that of transformants. [a]*H. Influenzae* Rd (ATCC 51097) was transformed with amplified *ftsI* gene DNA fragments. Strains having substitution in the *ftsI* are as follows: [b]R517H; [c]A502V and R517H; [d]S385T and R517H; [e]M377I, S385T, L389F and R517H; [f]N526K; [g]A502V and N526K; [h]S385T and N526K; [i]M377I, S385T, L389F and N526K.

Discussion

Low-BLNAR strains of *H. influenzae* were first described in the USA in the 1980s,[6,7] but were not considered an important clinical problem. Instead, TEM-1- and ROB-1-type BLPAR strains have been prevalent to 26% and 10%, respectively, in the USA in 1999,[8] and, accordingly, amoxicillin/clavulanic acid has been recommended as a first-line oral antibiotic therapy.[16–18]

On the other hand, in Japan, TEM-1-type BLPAR appeared in the 1980s and had a prevalence of 15–20% until 1995, after which its prevalence gradually declined to 3% in 1999.[8] The reason for the decrease in prevalence was the development and predominant use of oral third-generation cephalosporin antibiotics which are stable with respect to β-lactamase.

According to retrospective analysis of the *ftsI* gene in *H. influenzae*, low-BLNARs with amino acid substitution

R517H or N526K had already emerged in 1985. The low-BLNARs were unlikely to pose clinical problems because their ampicillin and cephalosporin susceptibilities decreased only 2- to 4-fold. BLNAR strains, for which the increases in the MICs of cephalosporins are greater, appeared in 1996 and increased rapidly among isolates from paediatric outpatients with RTIs or acute otitis media. Unfortunately, BLNAR strains of Hib causing meningitis appeared in 2000.[1]

The antimicrobial activity of cephalosporins against *H. influenzae* is due to their high affinity for PBP3. Thus, under the selective pressure imposed by oral cephalosporin usage, strains with mutations in the *ftsI* gene which reduce the binding affinity of PBP3 are selected.

As shown in Table 3, of the nine subgroups identified among the BLNAR and BLPACR-II isolates of *H. influenzae*, resistant strains belonging to subgroup IX with three amino acid substitutions surrounding the SSN motif and N526K in the *ftsI* gene were predominantly isolated. These strains in particular showed decreased susceptibility to intravenous cephalosporins, such as cefotaxime and ceftriaxone, which are used for meningitis patients. The results of transformation experiments also supported the fact that the wide distribution of the β-lactam MIC for Hib-type BLNARs reflects the variety of *ftsI* mutations. These results point to the possible emergence of more highly resistant Hib-type BLNARs possessing new amino acid substitutions in the future.

For clinical efficacy in the treatment of meningitis, it is thought that the concentration of cephalosporins in the CSF needs to be 10–20 times greater than the MIC for the infection strain. At the present time, combination chemotherapy has shifted from ampicillin and cefotaxime to cefotaxime and meropenem or ceftriaxone and meropenem for meningitis patients with BLNAR in Japan. The peak concentrations in CSF achieved following intravenous administration range from 2.6–13.2 mg/L for cefotaxime (at a dosage of 50 mg/kg every 6 h), 7.7 mg/L for ceftriaxone (50 mg/kg every 12 h) and 0.3–2.8 mg/L for meropenem (40 mg/kg every 8 h).[19,20] The CSF levels of these agents ranged from 5–26 times the MIC_{50} of cefotaxime for BLNAR, 62 times the MIC_{50} of ceftriaxone and 3–22 times the MIC_{50} of meropenem. Monotherapy with these agents for meningitis caused by BLNAR is suspected to be insufficient clinically in some cases.

Data were not shown here, but some meningitis patients have failed monotherapy with cefotaxime or ceftriaxone, and combination therapy was required with β-lactams having different target for PBP, such as cefotaxime and meropenem or ceftriaxone and meropenem. A questionnaire survey of patients with meningitis indicated that the incidence of severe sequelae was 19.6% (K. Hasegawa, R. Kobayashi, E. Takada, A. Ono, N. Chiba, M. Morozumi, S. Iwata, K. Sunakawa and K. Ubukata, unpublished results). The details will be described in another paper.

Furthermore, according to the NSBM study over 6 years, cases of meningitis caused by BLNAR strains have increased, with the prevalence of these strains being particularly high in patients ≤1 year of age who were immuno immature. An increase in the number of the day nursery children seems to accelerate the increase of infectious diseases caused by these resistant strains. Government approval of the introduction of Hib vaccine should prevent severe Hib infections, including those caused by BLNAR and BLPACR-II strains.

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Transparency declarations

None to declare.

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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

Naoko Chiba[1], Reiko Kobayashi[1], Keiko Hasegawa[1], Miyuki Morozumi[1], Eiichi Nakayama[1,2], Takeshi Tajima[2], Satoshi Iwata[3] and Kimiko Ubukata[1]* on behalf of the Acute Respiratory Diseases Study Group

[1]*Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo, 108-8641, Japan;* [2]*Department of Pediatrics, Hakujikai Memorial Hospital, Adachi-ku, Tokyo, 123-0864, Japan;* [3]*Department of Pediatrics, Tokyo National Medical Center, Higashigaoka, Meguro-ku, Tokyo, 152-8902, Japan*

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***Objectives*: Antibiotic susceptibilities, genes mediating β-lactam and macrolide resistance, and serotypes were analysed for strains of *Streptococcus pneumoniae*.**

***Methods*: A total of 392 strains of *S. pneumoniae* were isolated from paediatric patients with community-acquired pneumonia between May 2002 and 2004. All strains were classified into six genotype patterns according to the mutations found in the *pbp1a*, *pbp2x* and *pbp2b* genes identified by PCR. These results are represented by adding 'g', indicating genotypic identification.**

***Results*: Thirty-nine per cent of the isolates showed mutations in either one or two PBP genes (gPISP, where PISP stands for penicillin-intermediate resistant *S. pneumoniae*) and 52.3% had mutations in three genes (gPRSP, where PRSP stands for penicillin-resistant *S. pneumoniae*). The majority of the strains had a macrolide resistance gene: *mef*(A), (30.6%); *erm*(B), (48.5%); or both *mef*(A) and *erm*(B), (7.7%). The most frequent serotypes of these strains were: 6B (23.2%), 23F (17.6%), 19F (17.3%), 14 (10.5%) and 6A (8.2%). Serotypes of the seven-valent conjugate vaccine covered 70.9% of all isolates, and 89.8% of gPRSP. Serotypes of the strains with cefotaxime MICs of ≥2 mg/L were almost all of a vaccine type.**

***Conclusions*: The results suggest that introduction of conjugate vaccines into infants and children is necessary for the prevention of pneumococcal infections in Japan.**

Keywords: *S. pneumoniae*, antibiotic resistance genes, PBPs, respiratory tract infections

Introduction

Streptococcus pneumoniae is a major pathogen associated with respiratory tract infections (RTIs), acute otitis media, septicaemia, and meningitis acquired in the community.

In Japan, penicillin non-susceptible *S. pneumoniae* has been increasing rapidly since around 1990. According to a nationwide surveillance study that was conducted between 1998 and 2000, the prevalence of penicillin-intermediate resistant *S. pneumoniae* (PISP) and penicillin-resistant *S. pneumoniae* (PRSP) was 34.4 and 49.0%, respectively.[1] In parallel, macrolide resistance increased to 70%.[2]

β-Lactam resistance in *S. pneumoniae* is mediated mainly by multiple genes encoding the PBP1A, PBP2X and PBP2B enzymes (where PBP stands for penicillin-binding protein), which are involved in peptidoglycan synthesis.[3] We have developed a rapid identification method using PCR to detect mutations in these genes.[4]

Conjugate vaccines have recently attracted attention for prevention of various pneumococcal infections[5,6] and large-scale clinical

*Corresponding author. Tel: +81-3-5791-6385; Fax: +81-3-5791-6386; E-mail: ubukatak@lisci.kitasato-u.ac.jp

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trials of the 7- or 11-valent vaccine were conducted in several countries. As a result, some countries have already approved the use of this vaccine in children.

In the present paper, we describe antibiotic susceptibility and identification of resistance genes for *S. pneumoniae* from paediatric patients with pneumonia, and estimate the coverage rates of 7- and 13-valent conjugate vaccines in Japan.

Materials and methods

Strains

The acute respiratory diseases (ARD) study was made possible by participation of paediatric physicians belonging to 10 medical institutions between May 2002 and 2004. A total of 392 strains of *S. pneumoniae* were isolated from clinical samples of infants and children (n = 1121) with community-acquired pneumonia (CAP). Identification of the isolates as *S. pneumoniae* was confirmed by PCR for the autolysin (*lytA*) gene in our laboratory (Kitasato Institute for Life Sciences, Kitasato University).

Susceptibility testing

MICs of β-lactam and macrolide antibiotics were determined by an agar dilution method using Mueller–Hinton agar (MH; Difco Laboratories) supplemented with 5% defibrinized sheep blood. Antibiotics employed in this study were: penicillin and ampicillin (Meiji Seika Kaisha Ltd, Tokyo, Japan); cefotaxime and telithromycin (Aventis Pharma Ltd, Tokyo, Japan); and meropenem (Sumitomo Pharmaceuticals Co., Ltd, Osaka, Japan). *S. pneumoniae* ATCC 49619 was used as a quality control strain for susceptibility testing.

Serotyping

Serotypes of *S. pneumoniae* strains were determined by the Quellung reaction using antiserum purchased from the Statens Serum Institut (Copenhagen, Denmark).

Identification of antibiotic resistance genes by PCR

Oligonucleotide primers for detection of three PBP genes and macrolide resistance genes, *mef*(A) and *erm*(B), were used as previously described.[2]

Each reaction mixture contained (i) primers for detecting the *lytA* and *pbp1a* genes, (ii) primers for detecting the *pbp2x* and *pbp2b* genes, or (iii) primers for detecting the *mef*(A) and *erm*(B) genes. PCR conditions were also described previously.[4]

Sequencing

A total of 28 strains showing MICs of ≥4 mg/L for ampicillin or ≥2 mg/L for cefotaxime were termed tentatively high-resistant PRSP (H-PRSP), and the DNA region corresponding to the transpeptidase domain in each PBP gene was amplified. The nucleotide sequences were determined with an ABI PRISM 377 DNA sequencer.

PFGE analysis

PFGE analysis was carried out by a modification of the method described previously.[7]

For restriction endonuclease digestion, the plugs were incubated in restriction enzyme buffer with 20 U of *ApaI* at 37°C for 16 h. Electrophoresis was performed with a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA, USA). Separation of fragments was carried out at 5.7 V/cm at 14°C for 18 h.

Results

All 392 strains were firstly classified into the following six genotypes by PCR for *pbp1a*, *pbp2x*, and *pbp2b* genes: (i) gPSSP with three normal genes (n = 33, 8.4%); (ii) gPISP (*pbp2x*) with abnormal *pbp2x* (n = 72, 18.4%); (iii) gPISP (*pbp2b*) with abnormal *pbp2b* (n = 2, 0.5%); (iv) gPISP (*pbp2x* + *2b*) with abnormal *pbp2x* and *pbp2b* (n = 26, 6.6%); (v) gPISP (*pbp1a* + *2x*) with abnormal *pbp1a* and *pbp2x* (n = 54, 13.8%); (vi) gPRSP with three abnormal PBP genes (n = 205, 52.3%).

The MIC ranges and MIC_{50}s of four β-lactam antibiotics against gPRSP were as follows: penicillin, 0.5–4 and 2 mg/L; ampicillin, 0.25–8 and 2 mg/L; cefotaxime, 0.25–8 and 0.5 mg/L; meropenem, 0.063–1 and 0.5 mg/L.

Although strains of gPISP (*pbp2x*) exhibited negligible resistance to ampicillin with a 2-fold increase over the MIC_{50} of gPSSP (0.031 mg/L), the MIC_{50} of cefotaxime was 16-fold higher than that of gPSSP (0.016 mg/L). MIC_{50}s of ampicillin and cefotaxime also varied in gPISP (*pbp2x* + *2b*) (0.5 and 0.25 mg/L) and gPISP (*pbp1a* + *2x*) (0.25 and 1 mg/L). Cefotaxime MICs against gPISP (*pbp1a* + *2x*) increased markedly, and were also affected by abnormal *pbp1a* genes. Susceptibilities to ampicillin were equally influenced by abnormal *pbp1a*, *pbp2x* and *pbp2b* genes.

Along with the above results, it was noteworthy that some strains showed an ampicillin MIC of ≥4 mg/L (n = 13) or a cefotaxime MIC of ≥2 mg/L (n = 15). Five PBP genes in these H-PRSP strains were analysed in detail, as described below.

For macrolide resistance, the numbers of strains possessing *mef*(A), *erm*(B) or both genes were 120 (30.6%), 190 (48.5%) and 30 (7.7%), respectively. The number of susceptible strains without any resistance gene was as low as 52 strains (13.3%). The MICs of telithromycin for the strains with *erm*(B) or *mef*(A) were from 0.063 to 0.125 mg/L, except for four (1.0%) strains with MICs of 2–4 mg/L.

Capsular serotypes of tested strains are listed in Table 1 according to the genotypic patterns for PBP genes.

The most frequent serotypes were: 6B (23.2%), 23F (17.6%), 19F (17.3%) and 14 (10.5%). The disproportionate distributions of serotypes of gPSSP, gPISP and gPRSP were confirmed (χ^2 = 595.9100, P = 0.0000). Various serotypes were also noted in gPISP (*pbp2x*) strains, of which serotypes 6A, 6B and 3 were most prevalent. Serotypes 23A and 14 showed the greatest prevalence in gPISP (*pbp2x* + *2b*) and gPISP (*pbp1a* + *2x*) strains, respectively. Five serotypes (19F, 6B, 23F, 6A and 14) accounted for 96.1% of gPRSP, while there were only three strains each of serotypes 23A and 35.

Incidentally, 7- and 13-valent conjugate vaccines covered 70.9 and 84.9% of all strain serotypes tested, respectively. However, being confined to gPRSP, the coverage rates of 7- and 13-valent conjugate vaccines were as high as 89.8 and 96.1%, respectively.

DNA sequences of transpeptidase regions in *pbp1a*, *pbp1b*, *pbp2a*, *pbp2x* and *pbp2b* genes were determined for H-PRSP strains. The results were compared with the sequences of gPRSP and the R6 strain.

Table 2 shows the results obtained from 16 strains that had amino acid substitutions different from those found in the major gPRSP. Among strains with an ampicillin MIC of ≥4 mg/L, 10 amino acid substitutions near the conserved motif Lys^{614}-Thr^{615}-Gly^{616} (KTG) in the *pbp2b* gene, as described by Kosowska *et al.*[8] were observed in only one strain (ARD-963) with an MIC of

Table 1. Serotype distribution according to genotype patterns classified by PCR for *pbp* genes in *S. pneumoniae* isolates

Genotype	Serotype															Total
	1	3	4	6A	6B	9	11	14	15	18	19A	19F	23A	23F	other	NT	
gPSSP	2		2	1		2	5	1	2	1	6	1	1	1	8		33
gPISP (*pbp2x*)		8		15	14	4	1	7	3		5	1		5	9		72
gPISP (*pbp2b*)					1								1				2
gPISP (*pbp2x* + 2*b*)				1	6			2	1			1	11	4			26
gPISP (*pbp1a* + 2*x*)				2	12		2	27	6		2			2		1	54
gPRSP (*pbp1a* + 2*x* + 2*b*)				13	58			4				65	3	57	3	2	205
Total (%)	2	8	2	32	91	6	8	41	12	1	13	68	16	69	20	3	392
	(0.5)	(2.0)	(0.5)	(8.2)	(23.2)	(1.5)	(2.0)	(10.5)	(3.1)	(0.3)	(3.3)	(17.3)	(4.1)	(17.6)	(5.1)	(0.7)	(100)

8 mg/L which were different from those found in other strains; substitutions of Ala^{591}→Ser, Gly^{596}→Pro, Asn^{605}→Asp, Leu^{608}→Thr, Ala^{618}→Gly, Asp^{624}→Gly, Gln^{627}→Glu, Thr^{629}→Asn, Ser^{639}→Thr and Asp^{640}→Glu.

Of 15 strains with a cefotaxime MIC of ≥2 mg/L, 13 strains possessed amino acid substitutions in the *pbp2x* gene that changed Ser^{337}-The^{338}-Met^{339}-Lys^{340} (STMK) to Ser-Ala-Phe-Lys (SAFK). In all of the tested strains, no unknown amino acid substitutions were observed in the *pbp1a*, *pbp1b* and *pbp2a* genes.

Figure 1 shows the PFGE patterns of 16 H-PRSP strains listed in Table 2. PFGE was performed according to each serotype of these strains. Serotype 19F ($n = 6$) was most common, followed by two strains each of 14, 23F, 6A, 6B and non-typeable. Comparisons based on a dendrogram indicated a high similarity among strains of serotypes 19F and 14, but apparent differences in all other pairs of serotype strains.

Discussion

Resistance to various chemotherapeutical agents in *S. pneumoniae*, a leading cause of respiratory infections, has become a major worldwide problem. In Japan, according to the results based on our PCR methods, the causative gPISP and gPRSP in meningitis reached 37.3 and 41.5%, respectively in 2002.[4] Nearly 70% of these isolates also possessed *mef*(A) or *erm*(B) genes mediating macrolide resistance. Several reasons for the rapid increase in resistance were considered. One reason was that almost all of the oral cephalosporin antibiotics prescribed to outpatients obtain low serum concentrations and tissue distribution. Basically, circumstances differ from those in Europe and the US, where amoxicillin/clavulanic acid, which obtains high serum concentrations, is recommended as the first choice oral antibiotic for paediatric outpatients with RTIs. It follows that these differences in the prescribing patterns are reflected in the PBP gene alterations in PRSP and PISP. Non-susceptible strains with an abnormal *pbp2x* gene, resulting in intermediate-resistance to cephalosporin antibiotics, are isolated at a high rate in Japan, while strains with an abnormal *pbp2b* are more prevalent in the US.[9]

The relatively high population density in Japan is a secondary factor, which results in easy transmission of resistance among young children.

On a global basis, with the goal of preventing pneumococcal infections in children, large-scale clinical trials of 7-valent conjugate vaccine have been conducted in a number of countries, and vaccination programmes have already become compulsory in some countries.[5] However, the *S. pneumoniae* serotype distributions are known to vary between developing and developed countries, and with patient's age and variety of infection.

In Japan, a clinical trial of a 7-valent conjugate vaccine for children has been in progress since 2004. No recent epidemiological data regarding the serotypes in RTIs, which are fundamental for vaccine introduction, are available except the data of the Nationwide Surveillance for Bacterial Meningitis.

As described in the results, the proportion of gPRSP was as high as 52.3%, while the proportion of gPISP was confirmed as 39.3%, and the majority of them with vaccine serotypes. The 7-valent conjugate vaccine covered 70.9% of all the isolates, but 89.8%, if limited to gPRSP including H-PRSP.

Several data have been reported regarding a change in serotype after vaccination, and the increase in types 3 and 6A is of particular

Table 2. Serotype, antibiotic susceptibility and amino acid substitutions for three *pbp* genes in *S. pneumoniae* showing high MICs of ampicillin and cefotaxime

						Amino acid substitution								
		MIC (mg/L)				PBP1A			PBP2X			PBP2B		
Strain no.	Serotype	PEN	AMP	CTX	MEM	STMK	SRNVP	KTG	STMK	SSNVGM	LKSG	SVVK	SSNT	KTGTA
Major gPRSP		0.5–2	1–4	0.5–1	0.125–0.5	-A--	----T	---	-A--	------	V---	----	---A	-----
ARD-963[a]	19F	2	8	0.5	1	-S--	----T	---	-A--	------	V---	----	---A	----G
ARD-691	19F	2	4	4	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-850	19F	2	2	2	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-2337	19F	2	2	4	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-2098	19F	2	4	2	0.5	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-1346	19F	2	4	4	0.5	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-470	14	1	0.5	2	0.063	-A--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-986	14	1	1	4	0.063	-A--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-1152	23F	2	4	8	0.5	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-1268	23F	2	4	2	0.5	-S--	----T	---	-A--	------	V---	----	---A	-----
ARD-213	6A	2	4	4	0.5	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-1850	6A	2	2	2	0.25	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-15	6B	2	2	2	0.25	-S--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-724	6B	1	1	2	0.125	-A--	----T	---	-AF-	-----T	V---	----	---A	-----
ARD-241	NT	2	2	2	0.25	-S--	----T	---	-AF-	------	V---	----	---A	-----
ARD-1334	NT	1	0.25	2	0.063	-S--	----T	---	-G--	------	V---	----	----	-----

PEN, penicillin; AMP, ampicillin; CTX, cefotaxime; MEM, meropenem.
[a]Ten amino acid substitutions near the conserved motif KTG in *pbp2b* gene were identified.

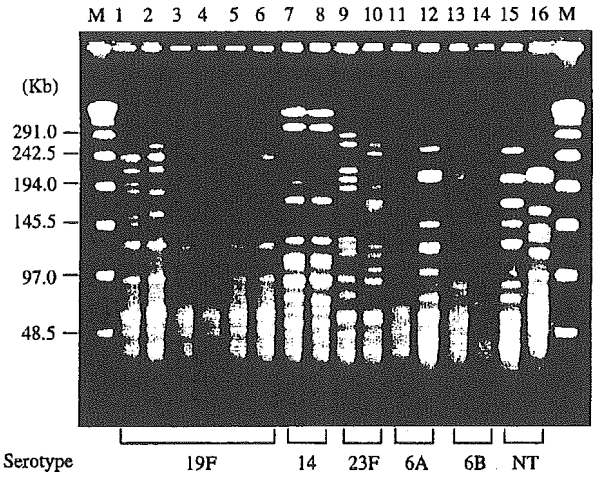

Figure 1. PFGE patterns of chromosomal DNA from H-PRSP digested with *Apa*I restriction enzyme. These strains show ampicillin MICs ≥4 mg/L, or cefotaxime MICs ≥2 mg/L and possess several additional amino acid substitutions in *pbp2x* ($n = 15$) or *pbp2b* ($n = 1$) genes compared with the common PRSPs. Lanes: M, λ ladder molecular size marker; 1, ARD-963; 2, ARD-691; 3, ARD-850; 4, ARD-2337; 5, ARD-2098; 6, ARD-1346; 7, ARD-470; 8, ARD-986; 9, ARD-1152; 10, ARD-1268; 11, ARD-213; 12, ARD-1850; 13, ARD-15; 14, ARD-724; 15, ARD-241; 16, ARD-1334.

concern.[10] A rapid change to a 13-valent conjugate vaccine is required.

Finally, to ensure the effectiveness of the vaccination, it would be ideal to add serotypes 10 and 22, which are common in severe infections in adults, to serotypes 6A, 3 and 23A.

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Decreased Serum Opsonic Activity against *Streptococcus pneumoniae* in Human Immunodeficiency Virus–Infected Ugandan Adults

Hidehiko Takahashi,[1] Kazunori Oishi,[1] Hiroyuki Yoshimine,[1] Atsushi Kumatori,[2] Kazuhiko Moji,[3] Kiwao Watanabe,[1] Hawa Nalwoga,[4] Sitefano Buguruka Tugume,[5] Anthony Kebba,[5] Roy Mugerwa,[4] Peter Mugyenyi,[5] and Tsuyoshi Nagatake[1]

Departments of [1]Internal Medicine and [2]Biochemistry and [3]Research Center for Tropical Infectious Diseases, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan; and [4]Makerere University and [5]Joint Clinical Research Centre, Kampala, Uganda

Type-specific immunoglobulin G (IgG) to pneumococcal capsular polysaccharide (CPS) and opsonic activity against *Streptococcus pneumoniae* were evaluated in serum samples from 36 Ugandan adults with community-acquired pneumonia and 58 asymptomatic Ugandan adults with or without human immunodeficiency virus type 1 (HIV-1) infection. The levels of serum IgG to CPS were significantly higher in HIV-1–infected subjects than in HIV-uninfected subjects. Serum samples from HIV-1–infected subjects that had lower IgG titers demonstrated higher opsonic activity against type 3 (titers of 7) and type 9 (titers of 7–11) pneumococcal strains. Plasma HIV-1 load also correlated inversely with serum opsonic activity against these strains, and peripheral blood $CD4^+$ lymphocyte numbers also tended to correlate with serum opsonic activity in asymptomatic HIV-1–infected adults. Our findings suggest that the opsonic activity of type-specific IgG is impaired in the serum of HIV-1–infected African adults, which may expose them to a serious risk of invasive pneumococcal infections.

More than two-thirds of the 40 million people living with HIV in the world reside in sub-Saharan Africa [1]. *Streptococcus pneumoniae* causes invasive infections in HIV-infected subjects in this area [2–5]. Furthermore, we, as well as other investigators, have documented that *S. pneumoniae* is the most common pathogen causing community-acquired pneumonia (CAP) in Ugandan and Kenyan adults living with HIV [6, 7]. A substantial activation and dysfunction of B cells that are associated with HIV infection may predispose these individuals to invasive pneumococcal infections [8–11].

The host defense against pneumococcal pneumonia depends largely on alveolar macrophages and the opsonization of specific IgG to pneumococcal capsular polysaccharide (CPS) and complement, followed by phagocytic killing [12, 13]. The phagocytic system in HIV-infected individuals has been a subject of extensive investigation, and several functional defects in phagocytic cells in such persons have been reported [14–16]. The mechanisms of impaired humoral immunity to *S. pneumoniae* in HIV-infected individuals, however, remain unclear.

We hypothesize that an impaired host defense against invasive pneumococcal infection could be attributed to a decreased type-specific IgG response or to a decrease in opsonic activity of type-specific IgG in the circulating serum of HIV-infected subjects. To further explore this hypothesis, we examined the levels of type-specific IgG, as well as the opsonic activity against *S. pneumoniae*,

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Reprints or correspondence: Dr. Kazunori Oishi, Dept. of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan (oishi-k@net.nagasaki-u.ac.jp).

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