to utilize lactate and to produce butyrate [60]. In a clinical setting, fecal butyrate levels are reportedly increased by yogurt consumption [61]. In addition, butyrate plays an important role in intestinal barrier function [62]. Thus, we used butyrate as a positive control for the suppression of *E. coli*-induced chemokine expression and HSP induction.

Taken together, these findings suggest that further study is warranted to clarify the mechanisms by which some LAB strains significantly suppress chemokine production, while others do not. If these precise mechanisms can be clarified, this knowledge may help to establish new preventative strategies for allergic diseases.

In the present study, we used both live and heat-killed bacteria. Since Caco-2 cells were seriously damaged by live bacteria at high bacterial concentrations of more than 1×10^8 CFU/ml, we were unable to examine the chemokine expressions of live bacteria in Caco-2 cells. However, the effect of live bacteria should be examined further [63]. Because the bacteria that we used in most experiments were heat-killed, the adhesion of the bacteria is unlikely to be involved in the induction or suppression of chemokine expression [64, 65].

In conclusion, we found that the induction of chemokines by LAB differs depending on the strain of LAB and that induction was inversely associated with the inhibition of commensal *E. coli*-induced chemokine expression in IECs. The mechanisms by which different LAB produce different results in vitro requires further investigation; however, *LGG* and *L. casei* markedly suppressed *E. coli*-induced chemokine expression, presumably through the suppression of the TLR-mediated signal transduction pathway. The significance of this inhibition to the onset of allergic diseases requires further investigation; however, the induction of chemokines in Caco-2 cells by intestinal bacteria may be useful as a marker for predicting the effect of LAB in vitro. In a very recent review published in the *Journal of Allergy and Clinical Immunology*, Prescott et al. [66] suggested that the varied clinical effects of LAB administration may be explained by differences in the LAB strains that were used in the trials. Our findings may add a new method for selecting potentially effective strains of LAB.

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NOTE

Blood kinetics of four intraperitoneally administered therapeutic candidate bacteriophages in healthy and neutropenic mice

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ABSTRACT

Due to multiple-drug resistant bacteria, phage therapy is being revisited. Although most animal experiments focus on therapeutic efficacy, the blood clearance kinetics of phages have not been well described. For further development of an efficient therapeutic strategy, information on phage blood kinetics is important. In this study, time-course concentration changes in peripheral blood of healthy and neutropenic mice were measured using four therapeutic phages (ϕMR11, KPP10, ϕEF24C, and KEP10). The results showed a two- to three-day rapid phage clearance, which fits a two-compartment model.

Key words bacteriophage, blood kinetics, phage therapy.

The ongoing development of bacterial drug resistance has disabled conventional antibiotic therapy. Bacteriophage (phage) therapy, which has a long history of use in Eastern European countries, has recently been revived as an alternative therapeutic in the West (1, 2). Some phage products are commercially available, but as drugs, they are still under development (3). Although most reports on phage therapy have described therapeutic efficacy, blood phage kinetics, one of the important criteria to determine optimal therapeutic strategy, has not been well described (2, 4).

Of isolated phages, 96% are tailed phage (order *Caudovidales*), which are physically and genetically diverse (5). The tailed phage is typically used as a therapeutic agent against bacterial infections. We have recently characterized four therapeutic tailed phages against different bacteria (6–10). Table 1 describes the four therapeutic phage prototypes, ϕMR11, KPP10, ϕEF24C, and KEP10. Although single administration of these phages at low concentrations via peritoneum has been shown to rescue bacterially infected mice, no blood kinetics have been shown in detail. In addition, although the reticuloendothelial system is known to eliminate circulating phage (4), the potential net influence on phage clearance by innate immunity is not shown. In this study, the time course of phage concentration was measured in healthy and neutropenic mice.

Culture medium and its constituents were purchased from Becton Dickinson (Sparks, MD, USA) and Nacalai Tesque (Tokyo, Japan), respectively, unless otherwise stated. The host bacteria strains and appropriate culture media used in this study have been described previously (also see Table 1). The phage were incubated with the respective bacteria strains in 400 mL culture media at 37°C. Phage purification essentially followed methods described previously, with some modifications. Briefly, after debris

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List of Abbreviations: ANOVA, analyses of variances; HIMC, heart infusion broth supplemented with 20 mM $MgCl_2$ and 20 mM $CaCl_2$; pfu, plaque-forming units; SMC, saline with 20 mM $MgCl_2$ and 20 mM $CaCl_2$.

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Table 1 Four therapeutic phage candidates

Therapeutic phage candidate	Host bacterium	Culture Medium	Phage taxonomy: *Family* (morphotype)	Morphological description	Size		Genomic information availability (GenBank accession No.)	Reference(s)
					Head diameter (nm)	Tail length (nm)		
ϕMR11	*Staphylococcus aureus* SA37	TSBM	*Siphoviridae* (B1)	Icosahedral head, and non-contractile tail	56	175	Available (AB370268)	6
KPP10	*Pseudomonas aeruginosa* D4	LB	*Myoviridae* (A1)	Icosahedral head, contractile tail	72	116	Available (AB472900)	10
ϕEF24C	*Enterococcus faecalis* EF24	TSB	*Myoviridae* (A1)	Icosahedral head, contractile tail	93	204	Available (AP009390)	8, 9
KEP10	*Escherichia coli* ECU30	LB	*Myoviridae* (A2)	Elongated head, contractile tail	112 (length), 83 (width)	104	Partially available (AB326953 and AB326954)	7

LB, Luria-Bertani medium; TSB, tryptic soy broth; TSBM, tryptic soy broth supplemented with 20 mM $MgCl_2$ and 20 mM $CaCl_2$.

removal by centrifugation (10 min, 8000 *g*, 4°C) and addition of polyethylene glycol 6000 (final, 10%; Sigma-Aldrich, St Louis, MO, USA) and NaCl to lysate (final, 0.5 M) phage solution, the phage were precipitated by centrifugation (30 min, 8000 *g*, 4°C). The phage pellets were treated with DNase I (Type II; Sigma-Aldrich) and RNase A (Type IA; Sigma-Aldrich) (both 50 μg/mL). The phage solution was purified by CsCl-step-gradient ultracentrifugation (CsCl, $\rho = 1.7$, 1.5 and 1.3) (ϕEF24C: 50 000 *g*, 4°C, 2 hr; ϕKEP10, KPP10, and ϕMR11: 100 000 *g*, 4°C, 1 hr). After placing the collected phage between $\rho = 1.7$ and 1.3 of CsCl, the phage were purified again by ultracentrifugation. The purified phage were dialyzed against SMC (saline with 20 mM $MgCl_2$ and 20 mM $CaCl_2$) (4°C, 30 min) and HIMC (heart infusion broth supplemented with 20 mM $MgCl_2$ and 20 mM $CaCl_2$) (4°C, 30 min), respectively. Phage titer was measured by a plaque formation assay, in which phage and host bacteria were inoculated on double-layered agar plates. Phage were stored at 4°C.

All animal experiments were conducted with the approval of the Animal Experiment Committee of Kochi University. In this study, female BALB/c mice (8 weeks; weight 18 ± 0.5 g) were used. Untreated mice were used as healthy controls. To induce neutropenia in the mice, 200 mg/kg and 150 mg/kg of cyclophosphamide (Sigma-Aldrich) were intraperitoneally administered to mice on days 1 and 4, respectively. Blood cells were counted by Sysmex K4500 (Kobe, Japan), and Wright-Giemsa-stained cells were semi-quantitatively enumerated by light microscopic observation. Severe neutropenia (neutrophils less than 100 cells/μL) was seen from days 6 to 8 (data not shown).

The purified phage were diluted by HIMC to 5.0×10^{11} pfu/mL. 0.2 mL of phage (1.0×10^{11} pfu) was then administered into the abdominal cavity of either healthy or cyclophosphamide-treated mice on day 6 ($n = 9$ or 10 per group). Five μL of blood was sampled from the tail by cutting with a razor, and active phage were sequentially enumerated by a plaque formation assay of the sample blood 2, 4, 8, 12, 24, 48 and 72 hrs after phage administration. Due to rapid phage clearance from the blood, the ϕEF24C measurement is shown only up to 48 hrs.

Overall, our therapeutic phages rapidly decreased over the first 8 to 12 hrs and then gradually decreased and disappeared within three days, a clearance pattern that seemed to fit a two-compartment model (Fig. 1). The initial rapid decrease from 8 to 12 hrs and the following gradual decrease were considered as the alpha phase (distribution of drug to organs) and the beta phase (elimination of drug), respectively. Based on these pharmacokinetic assumptions, the appropriate mathematical formulas were manually calculated from the mean values (see the legend of Fig. 1).

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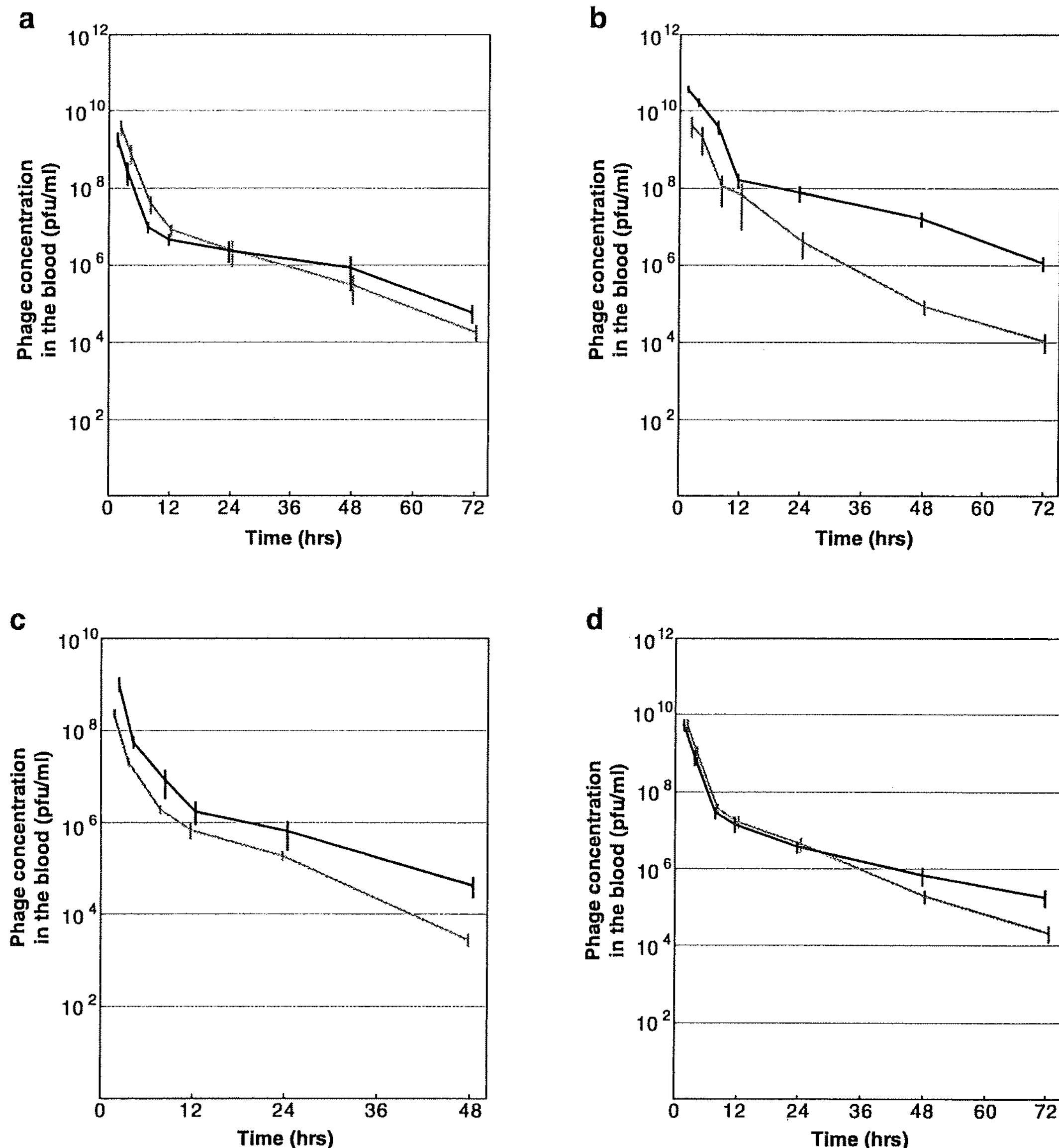

Fig. 1. Time-course concentration changes in active phage in the peripheral blood of healthy and neutropenic mice. Mean of active phage concentration in neutropenic or healthy mice is graphed, shown in black or grey lines, respectively. Vertical bar indicates standard error of mean. Approximate mathematical formulas of a two-compartment model in neutropenic or healthy mice were also calculated. (a) Phage ϕMR11 ($C = 3.72 \times 10^9 \times e^{-0.753t} + 2.35 \times 10^7 \times e^{-0.0997t}$ (in healthy mice); $C = 1.85 \times 10^9 \times e^{-0.868t} + 6.95 \times 10^6 \times e^{-0.0449t}$ (in neutropenic mice)). (b) Phage KPP10 ($C = 2.45 \times 10^9 \times e^{-0.707t} + 2.18 \times 10^7 \times e^{-0.114t}$ (in healthy mice); $C = 3.52 \times 10^{10} \times e^{-0.367t} + 3.02 \times 10^8 \times e^{-0.0626t}$ (in neutropenic mice)). (c) Phage ϕEF24 ($C = 2.14 \times 10^8 \times e^{-0.760t} + 4.07 \times 10^6 \times e^{-0.156t}$ (in healthy mice); $C = 5.64 \times 10^8 \times e^{-0.751t} + 5.40 \times 10^6 \times e^{-0.104t}$ (in neutropenic mice)). (d) Phage KEP10 ($C = 5.41 \times 10^9 \times e^{-0.825t} + 4.82 \times 10^7 \times e^{-0.113t}$ (in healthy mice); $C = 5.05 \times 10^9 \times e^{-0.866t} + 2.88 \times 10^7 \times e^{-0.0824t}$ (in neutropenic mice)).C, phage concentration; e, Napier's number or base of natural logarithm; t, time.

Differences in phage kinetics in healthy and neutropenic mice, and differences in phage kinetics in healthy mice, were statistically compared by two-way repeated-measures ANOVA, using statistical software SPSS 12.0J (SPSS Japan, Tokyo, Japan). The criterion for statistical significance was set at $P \leq 0.05$. Due to the limited sample size and the effect on Mauchly's test of sphericity, the Greenhouse–Geisser epsilon was calculated and utilized to adjust the degrees of freedom to avoid assumptions made about the variance-covariance matrices of the dependent variables.

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Firstly, blood concentration changes of each active phage between healthy and neutropenic mice were not significantly different ($P > 0.05$), implying that cyclophosphamide-induced immunodepression did not influence phage clearance in this experimental setting. In addition, the stability of phage in mouse blood, HIMC, and PBS showed a similar degree of phage reduction to the beta phase of a two-compartment model (data not shown). This also supports the phages not being influenced by innate immunity. Secondly, the only pair of phages between which there was a significant difference ($P < 0.05$) was KEP10 and ϕEF24C. Thus, various phage molecular features did not seem to influence phage blood clearance.

Considering past studies together with this study, the prototype therapeutic phages seem to be rapidly cleared. Generally, longer persistence of a drug *in vivo* is considered to be better (4). In another study, long-circulating mutant phages were isolated by several passages through the immune system (11–13). However, as phages do not act like a chemical drug (i.e. phage can propagate until target bacteria are eliminated), it may be better to rapidly clear phage once treatment has been completed (14, 15). In this study, the blood clearance of our candidate phages has briefly been described using healthy and neutropenic mice not previously exposed to phage. We hope that these results help further pharmaceutical study on phage therapy.

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Case Report

Neonatal bacterial meningitis caused by *Streptococcus gallolyticus* subsp. *pasteurianus*

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This report describes a case of neonatal bacteraemia and meningitis due to *Streptococcus gallolyticus* subsp. *pasteurianus*. Based on the identification kit results, this species may have been reported as *Streptococcus bovis* or *S. bovis* biotype II. The accurate identification of this organism is mandatory for evaluating the aetiology of neonatal meningitis.

Case report

A 5-day-old female was admitted to Fukuoka Red Cross Hospital. She was born at term, weighing 3192 g, and the culture of a maternal prenatal vaginal swab was negative for group B streptococcus. The labour was uneventful, without premature rupture of the membrane. The patient had a fever of 38.4 °C on the fourth day after birth. The results of blood examination revealed that there were 3600 leukocytes ml^{-1} and that the C-reactive protein level was less than 1 mg l^{-1}. The fever persisted the next day, and the patient was then admitted to the hospital. The patient's anterior fontanel bulged slightly, and her overall activity was poor. A sepsis work-up and lumbar puncture were performed. The results of the blood examination were as follows: 13 900 leukocytes ml^{-1}; 12.8 g haemoglobin dl^{-1}; 279 000 platelets ml^{-1}; and 65 mg C-reactive protein l^{-1}. The cerebrospinal fluid was cloudy with 12 971 leukocytes ml^{-1} (12 800 polymorphonuclear cells ml^{-1} and 171 mononuclear cells ml^{-1}). The cerebrospinal glucose level was 21 mg dl^{-1} and the protein level was 3.32 g dl^{-1}. No antigens for *Haemophilus influenzae* type b, *Streptococcus pneumoniae, Neisseria meningitidis* group A, B, or C, or *E. coli* K1 were detected (Slidex-Méningite-Kit5; bioMérieux) in the cerebrospinal fluid. Treatment with cefotaxime (200 mg·kg^{-1} per day), panipenem–betamipron (120 mg·kg^{-1} per day) and intravenous γ-globulin (300 mg·kg^{-1} per day for 2 days) was started.

Cultures of both the cerebrospinal fluid and blood showed Gram-positive cocci, which were initially reported as *Streptococcus* species (non-enterococcus). The cerebrospinal fluid isolate was susceptible to penicillin G (MIC 0.06 μg ml^{-1}), cefotaxime (MIC 0.06 μg ml^{-1}) and imipenem (MIC $\leqslant$0.008 μg ml^{-1}). Panipenem–betamipron treatment was discontinued, and treatment with cefotaxime alone continued for 14 days. Culture of the cerebrospinal fluid was negative on day 3 of hospitalization. Non-contrast head computed tomography scans, which were obtained on day 11 of hospitalization, revealed no intracranial haemorrhage or subdural abscess. The patient was discharged without sequelae.

The isolate possessed Lancefield's D antigen (Streptex; Remel). According to an API 20 Strep test (bioMérieux), the isolate was identified as *S. bovis* biotype II/2. Because the *S. bovis* complex has been recently reclassified (Schlegel *et al.*, 2003; and see below), we tested for gallate hydrolysis (tannase) activity of the isolate according to the reference by Osawa *et al.* (1995). Various biochemical activities of the isolate are described in Table 1 with comparison to those of *S. gallolyticus* subsp. *gallolyticus, S. gallolyticus* subsp. *pasteurianus* and *S. gallolyticus* subsp. *macedonicus.* The biochemical characteristics of the isolate coincided well with those of *S. gallolyticus* subsp. *pasteurianus.* A positive result was obtained with the recently developed PCR test for detecting *sod*A of *S. gallolyticus* (data not shown) (Sasaki *et al.*, 2004). The 5′ side of the isolate's 16S rRNA gene sequence revealed 99.4 % (354/356 bp) and 100 % (356/356 bp) homology with those of *S. gallolyticus* subsp. *gallolyticus* (ATCC 43143) and *S. gallolyticus* subsp. *pasteurianus* (ATCC 43144), respectively. From the results of these biochemical and molecular tests, the isolate was identified as *S. gallolyticus* subsp. *pasteurianus.*

Discussion

S. gallolyticus subsp. *pasteurianus* belongs to the group D streptococci, and was previously recognized as *S. bovis*

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Table 1. Biochemical characteristics of the isolate from this case and the three subspecies of *S. gallolyticus*

The characteristics of three subspecies of *S. gallolyticus* refer to a reference by Schlegel *et al.* (2003).

Characteristic	Our isolate	*S. gallolyticus* subsp. *gallolyticus*	*S. gallolyticus* subsp. *pasteurianus*	*S. gallolyticus* subsp. *macedonicus*
Hydrolysis of:				
Aesculin	+	+	+	−
Gallate (tannase activity)	−	+	−	−
Production of:				
β-Glucosidase	+	+	+	−
β-Glucuronidase	−	−	+	−
α-Galactosidase	+	+	v	v
β-Galactosidase	+	−	+	+
Acidification of:				
Starch	−	+	−	+
Glycogen	−	+	−	−
Inulin	−	+	−	−
Lactose	+	+	+	+
Mannitol	−	+	−	−
Raffinose	+	+	v	−
Trehalose	+	+	+	−

+, $\geqslant$80 % activity compared to positive control reaction; −, $\leqslant$20 % activity compared to positive control reaction; v, 21–79 % activity compared to positive control reaction.

biotype II/2. *S. bovis* is delineated into two biotypes according to their ability (biotype I) or inability (biotype II) to ferment mannitol (Facklam, 1972; Parker & Ball, 1976). *S. bovis* (biotype II) is further divided into biotypes II/1 and II/2 on the basis of phenotypic testing with the Rapid Strep system (bioMérieux) (Coykendall & Gustafson, 1985). It has been well documented that *S. bovis* (biotype I) is associated with colonic neoplasia and bacterial endocarditis in adults (Ruoff *et al.*, 1989; Herrero *et al.*, 2002). In contrast, *S. bovis* (biotype II) is associated with invasive infection in neonates and infants (Grant *et al.*, 2000; Cheung *et al.*, 2000; Gavin *et al.*, 2003; Nagai *et al.*, 2008), as well as adult bacteraemia both in Western and Eastern countries (Clarridge *et al.*, 2001; Lee *et al.*, 2003). Among the reported cases of neonatal invasive infection due to *S. bovis* (Gerber *et al.*, 2006; Nagai *et al.*, 2008), *S. bovis* biotype II/2 was described in two cases (Gavin *et al.*, 2003; Nagai *et al.*, 2008). No reports have described the aetiological organism of invasive infections as *S. gallolyticus* subsp. *pasteurianus*.

The taxonomic status of the *S. bovis* group has been evolving in the last few decades. Farrow *et al.* (1984) demonstrated that the *S. bovis/Streptococcus equinus* complex comprised six DNA groups. It was shown that *S. bovis* biotype II/2 belonged to the DNA group 2 of Farrow's classification (Schlegel *et al.*, 2003; Poyart *et al.*, 2002). According to the biochemical characteristics, the members in this DNA group have been reclassified and renamed *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus* or *S. gallolyticus* subsp. *macedonicus* (Schlegel *et al.*, 2003). These subspecies have similar 16S rRNA gene sequences and cannot be discriminated from each other solely by 16S rRNA gene sequence (Clarridge *et al.*, 2001; Schlegel *et al.*, 2003). Instead, the aesculin- and gallate-hydrolysis activity measurement works for identifying these subspecies, though the latter is not included in the identification kit (Table 1) (Osawa & Sasaki, 2004). According to the new classification, *S. bovis* biotype I and *S. bovis* biotype II/2 correspond to *S. gallolyticus* subsp. *gallolyticus* and *S. gallolyticus* subsp. *pasteurianus*, respectively (Schlegel *et al.*, 2003).

The isolate from our case was susceptible to penicillin G, cefotaxime and panipenem, and resistant to erythromycin and minocycline. *Enterococcus* spp. have phenotypic characteristics similar to those of *S. gallolyticus* subsp. *pasteurianus*, i.e. they are non-haemolytic, positive for Lancefield's D antigen and positive for aesculin hydrolysis. Penicillin G is considered to be an efficient treatment for neonatal infections caused by *S. gallolyticus* subsp. *pasteurianus*, while vancomycin and/or aminoglycosides may be considered for the treatment of neonatal infections caused by *Enterococcus*. Thus, the accurate identification of the isolate is crucial for selecting appropriate antibiotic therapy.

This report describes a case of neonatal bacterial meningitis due to *S. gallolyticus* subsp. *pasteurianus*. The importance of this organism as a causative agent of invasive infection in neonates should be emphasized.

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NOTE

Presence of multiple copies of capsulation loci in invasive *Haemophilus influenzae* type b (Hib) strains in Japan before introduction of the Hib conjugate vaccine

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ABSTRACT

Despite the effectiveness of the Hib vaccine, multiple amplification of the *cap*b locus contributes to vaccine failure. However, there has been no report on the effect of Hib locus amplification in Japan. We examined 24 Hib strains from Japanese children with invasive diseases due to Hib. Although all strains showed the same *cap*b sequence, Southern blot analysis showed that four strains (16.7%) harbored multiple copies (more than two) of the *cap*b locus. Careful analysis of the locus in circulating Hib strains is necessary now that the Hib vaccine has been introduced into Japan.

Key words capsular polysaccharide, *Haemophilus influenzae* type b, Hib conjugate vaccine.

Hib occasionally causes invasive bacterial diseases such as meningitis, epiglottitis and sepsis, especially among young children. Hib conjugate vaccines, which consist of capsule polysaccharide conjugated with carrier protein, are very effective and safe. Since the Hib conjugate vaccine was introduced in Europe and America in the 1990s, the incidence of invasive Hib disease has decreased dramatically in many countries (1). However, despite the efficacy of the Hib vaccine, an increased number of cases of the rare invasive Hib diseases (i.e. cases of true vaccine failure) have now been reported in Europe in fully vaccinated children (2–5). Although possibly contributory host factors such as lower avidity of the anti-Hib antibody are known to occur (6, 7), amplification of the capsulation locus may also have contributed to vaccine failure (8, 9).

Type b polysaccharide capsules, polymers of PRP, are cell-surface components that serve as major virulence factors against host defense mechanisms. The genes involved in Hib capsule expression are found within the *cap*b locus, an 18-kb DNA segment of the chromosome (10). Most invasive Hib strains contain a partial duplication of the *cap*b locus which consists of one intact copy of the locus, and a second copy with a 1.2-kb deletion region containing the *bex*A gene and an IS1016 insertion element that flanks the locus (10). Polysaccharide capsule production relates to the number of copies of the locus (11). Recently, Cerquetti *et al.* reported that amplification of the *cap*b locus to as many as three to five copies is associated with vaccine failure (8, 9). In addition, Schouls *et al.* found two variants of the capsular gene cluster, designated type I and type II, which were assessed by considerable sequence divergence in the *hcs*A and *hcs*B genes of the *capb* locus. They found that type I strains carry approximately twice as much capsular polysaccharide on the cell surface as type II strains (12).

In Japan, the Hib conjugate vaccine was licensed in January 2007, and introduced in December 2008; however, the vaccination plan has not yet been fully implemented. Although 55% of bacterial meningitis cases in children in Japan were caused by Hib (13), there has been no national

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List of Abbreviations: capsulation b, *capb*; CSF, cerebrospinal fluid; DIG, digoxigenin; Hib, *Haemophilus influenzae* type b; PFGE, pulsed-field gel electrophoresis; PRP, polymers of ribose ribitol phosphate.

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Table 1. Sequence type and number of copies of the *cap*b locus of the 21 *Haemophilus influenzae* strains examined in this study

No. of cases	No. of strains	Detected date (Year/month)	Age (months)	specimen	disease	Ampicillin susceptibility	PFGE pattern	the *cap*b locus		
								Sequence type	Size of band	No. of copies
1	C1650	2004/11	14	blood	bacteremia	R[†]	H	I	45 kb	2
2	K4646	2005/7	9	blood	meningitis	R	G	I	81 kb	4
3	K5003	2005/11	53	blood	meningitis	S[‡]	A1	I	45 kb	2
4	K5154	2006/1	17	CSF	meningitis	S	D	I	45 kb	2
5	K5221	2006/1	5	CSF	meningitis	S	B	I	45 kb	2
6	K5331	2006/2	24	CSF	meningitis	S	E	I	45 kb	2
7	K5545	2006/4	12	blood	cellulitis	-	A1	I	45 kb	2
8	K5625	2006/5	31	CSF	meningitis	R	F	I	45 kb	2
9	K5905	2006/9	19	CSF	meningitis	S	A1	I	45 kb	2
10	K6066	2006/11	7	CSF	meningitis	S	B	I	63 kb	3
11	K6168	2006/12	56	CSF	meningitis	R	B	I	45 kb	2
12	K6519	2007/8	20	CSF	meningitis	S	A1	I	45 kb	2
13	K6803	2007/10	29	blood	epiglottitis	S	A1	I	45 kb	2
14	K6886	2007/12	21	CSF	meningitis	S	A1	I	45 kb	2
15	K6892	2007/12	9	CSF	meningitis	R	A1	I	45 kb	2
16	K6930	2008/1	63	blood	bacteremia	R	A1	I	45 kb	2
17	K6934	2008/1	2	CSF	meningitis	R	A1	I	45 kb	2
18	K7112	2008/3	15	blood	meningitis	S	A1	I	45 kb	2
19	K7448	2008/7	8	CSF	meningitis	S	C	I	45 kb	2
20	K7450	2008/7	7	CSF	meningitis	S	A1	I	45 kb	2
21	K7522	2008/9	14	CSF	meningitis	S	A1	I	45 kb	2
22	K7639	2009/4	4	blood	meningitis	S	A2	I	81 kb	4
23	K7641	2009/4	12	CSF	meningitis	S	A1	I	45 kb	2
24	K7721	2009/5	4	blood	bacteremia	S	I	I	63 kb	3

[†]resistant, [‡]susceptible.

survey of strains isolated from patients with invasive Hib diseases including meningitis. Furthermore, there are no reports on the amplification or sequence divergence of the *cap*b locus. The principle aim of this study was to analyze the number of *cap*b copies, and to assess sequence divergence in the *hcs*A and *hcs*B genes of Hib strains isolated from children with Hib diseases in our district before the introduction of the Hib conjugate vaccine.

A total of 24 Hib strains isolated between November 2004 and May 2009 from 24 children with invasive Hib diseases who had not received Hib conjugate vaccine in Kagoshima Prefecture, Japan, were collected and examined. Of these strains, 15 were isolated from CSF and 9 from blood. The strains were epidemiologically unrelated and individually stored at −80°C. All isolates were identified as serotype b by PCR capsular genotyping (14). PFGE was performed using a CHEF-DR 3 apparatus (Nippon Bio-Rad Laboratories, Tokyo, Japan) according to previously reported methodology (15). Briefly, DNA was digested by *Sma*I and separated on 1% agarose gels by PFGE under the following conditions: current range, 100 to 130 mA at 14°C for 16 hr; initial switch time, 5.3 s, linearly increasing to a final switch time of 49.9 s; angle, 120°; field strength, 6 volts/cm. The gels were stained with ethidium bromide and photographed. A lambda with a size range of 48.5 kb to 1 Mb (BME, Rockland, ME, USA) was used as a size marker. For interpretation of banding patterns separated by PFGE, we referred to the criteria of Tenover *et al.* (16).

Two variants of the *cap*b locus DNA sequence, type I and type II, were determined by PCR using two primer sets targeting the *hcs*A gene which could discriminate between the two capsular genotypes as described in a previous report (12). The DNA sequences of the PCR products were determined by an ABI Prism 310 sequencer (Applied Biosystems Japan, Tokyo, Japan).

The number of *cap*b locus copies was detected by Southern blotting analysis according to previously reported methods (8). Because *Kpn*I and *Sma*I restriction sites flank the *cap*b locus, extracted DNA in an agarose plug was digested with these enzymes, separated by PFGE, and transferred to a nylon membrane. A Hib capsule-specific 480-bp probe was constructed by PCR (14) and labeled with DIG using a DIG high prime DNA labeling kit (Roche Diagnostics, Mannheim, Germany). The membrane was hybridized with the probe and visualized by

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chemiluminescent detection using a DIG detection kit (Roche Diagnostics). The *Kpn* I/*Sma* I fragment of a two copy strain was expected to be 45-kb, because it includes two repeats of the locus (18 + 17 kb) plus additional segments (~10 kb) upstream and downstream of the *cap* region (17). Three-, four-, and five-copy fragments showed increased size in 18-kb increments for each additional copy (63, 81, and 99-kb, respectively) (8).

A summary of results is shown in Table 1. The type I-associated *hcs*A gene was found in all of the strains examined. The DNA sequences of all the PCR products were completely identical. PFGE analysis showed nine distinctive restriction patterns (A to I) among the 24 isolates. Fourteen strains with the A pattern were divided into A1 subtype (13 strains) and the closely-related A2 subtype (one strain). Southern blotting analysis demonstrated that 20 strains showed a two-copy arrangement of the *cap*b locus (45-kb), two strains showed three copies (63-kb), and the other two showed four copies (81-kb) (Fig. 1). The incidence of multiple-copy strains (>two copies) among examined strains was 16.7% (4/24). All of the strains with the dominant PFGE pattern (A1) possessed two copies, while one with the closely-related A2 subtype harbored four copies. The other three strains with multiple copies showed minor PFGE patterns (B, G or I). All the patients infected by strains with multiple copies were treated successfully without neurological or physical sequelae.

Amplified *cap*b sequences were detected more frequently among strains from children with true vaccine failure than among those from unvaccinated children (24% vs. 10%) in the United Kingdom (8). Furthermore, the proportion of strains with multiple copies of the *cap*b locus increased over time in Italy (9). Amplification of the *cap*b locus is associated with decreased susceptibility to complement-mediated lysis and decreased complement-mediated opsonization (11). Thus, amplification of the *cap*b locus may result in the overcoming of host defenses and contribute to vaccine failure. We have found that Hib strains with multiple (three or four) copies of the *cap*b locus were present in Japan before the introduction of the Hib conjugate vaccine. The incidence of 16.7% (4/24) of multiple-copy strains found in our study is slightly higher than that found in the UK between 1991 and 1992 before routine immunization was introduced (10.1%, 9/89) (8). In our study, most of the multiple-copy strains showed rare PFGE patterns. Thus these strains might be selected and involved in vaccine failure after the introduction of Hib conjugate vaccination in Japan.

Sequence typing of the *capb* locus is based on the considerable sequence divergence in the *hcs*A and *hcs*B genes, which are involved in the transport of capsular polysaccharides across the outer membrane (18). Schouls *et al.* have reported that type II strains display less expression of capsular polysaccharide than do type I, and were isolated only during the pre-vaccination era in the Netherlands (12). The greater polysaccharide expression may have provided a selective advantage for type I strains, resulting in the rapid elimination of type II. In addition, there have been remarkable differences in the geographic distribution of type I and type II; with a higher incidence in the United States (73%) than the Netherlands (5%) of type II among Hib strains isolated from patients (12). While we did not find type II strains in this study, more Hib strains should be evaluated to clarify the exact incidence.

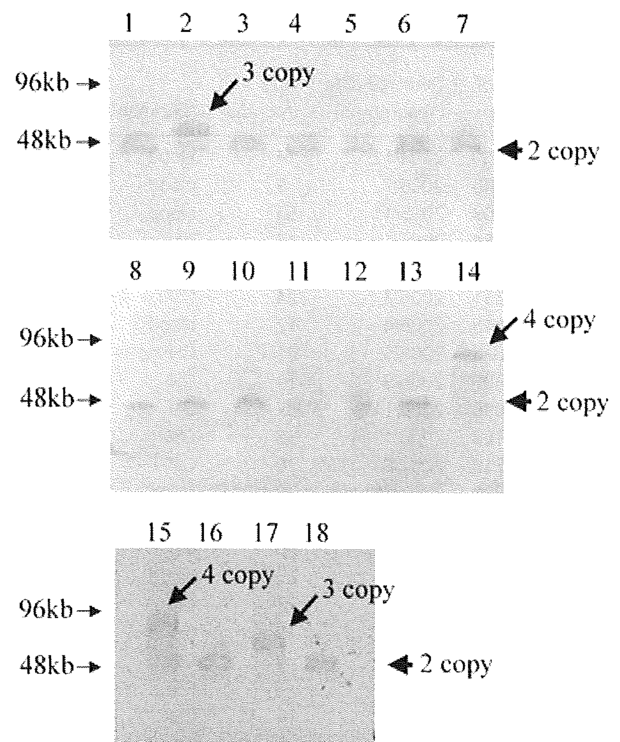

Fig. 1. Examples of Southern blot analysis of DNA from *Haemophilus influenzae* type b strains digested with KpnI/SmaI, separated by PFGE, and hybridized with the 480-bp DIG-labeled *cap*b probe. Strain K6066 in lane 2 and strain K7721 in lane 17 showed three-copy arrangement of the *cap*b locus (ca. 63-kb). Strain K4646 in lane 14 and K7639 in lane 15 had four-locus copies (ca. 81-kb). Other strains had two copies (ca. 45-kb).

To our knowledge, this is the first study to investigate *cap*b locus copy number in invasive Hib strains isolated in Japan. We found that multiple-copy strains were in existence in Japan before the introduction of Hib conjugate vaccine. Molecular epidemiological surveillance of invasive Hib strains after the introduction of vaccines will allow prompt detection of any changes in bacterial properties. In addition, because higher antibody concentrations may be required to protect against Hib disease caused by strains with multiple copies of the *cap*b locus, we strongly

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recommend the complete implementation of Hib vaccination in young children in Japan.

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