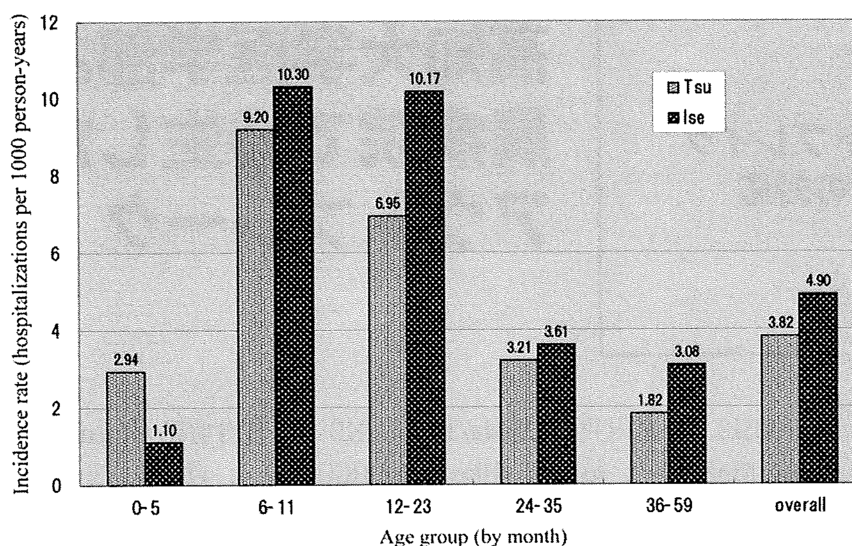

Figure 4. Incidence rate of hospitalizations for rotavirus acute gastroenteritis, by age, in 2 cities (Tsu and Ise) in Mie Prefecture, Japan, 2003–2007.

tavirus. For the same reason, we were not able to obtain rotavirus-positive stool specimens to characterize strains.

In conclusion, our findings confirm a substantial health burden of hospitalization for rotavirus AGE among Japanese children, and our data should help pediatricians and policy makers to assess the potential benefits of implementation of rotavirus vaccination in Japan. To overcome some of the limitations of retrospective surveillance, to better understand the current burden of rotavirus disease, and to characterize rotavirus strains, we have initiated active prospective surveillance at the 2 study hospitals, which began in October 2007.

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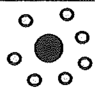

アウトブレイクへの対応

高知大学医学部および附属病院で発生した百日咳アウトブレーク

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◆はじめに

高知大学医学部附属病院感染対策チーム（infection control team：ICT）は，他大学における百日咳集団発生と休講措置の報道を受け，水際阻止対策を講じていた．百日咳抗体価高値の医学部学生が認められた直後から，感染状況の把握と感染経路遮断を目的に対策を徹底した結果，終息を向かえることができた．国立感染症研究所の支援調査を受けて実施した疫学調査の結果を合わせて報告する．

◆経過と対応

1．水際阻止対策

2007年5月25日他大学の百日咳集団発生が報道された．高知大学医学部では，百日咳の高知浸透を念頭に置き，百日咳症例を早期発見するための水際阻止対策を講じることとした．当時持ち得た情報では，成人百日咳は「①症状が軽く，典型的でない場合が多いが，小児への感染源として重要．②感染経路は飛沫感染と接触感染．③感染性のある期間は3週間であるが，抗菌薬を内服すれば5日目まで．④年長児，成人例では基礎免疫が残存する例が少なくないので，ペア血清による血清診断も困難．⑤確定診断には百日咳菌の培養（咳嗽飛沫を直接あるいは鼻咽腔ぬぐい液を専用培地に接種），あるいは鼻咽腔ぬぐい液でポリメラーゼ連鎖反応（polymerase chain reaction：PCR）が必要」であった．

当院では，百日咳抗体価の血清診断は外部委託のため検査結果を得るまでに5日から7日間を要することと，成人例を対象とするので有用な方法と考えられなかった．検査室の百日咳専用培地は毎年10枚程度を購入するのみであったので，集団発生に備え追加発注を行ったが，10日間の培養期間が必要とされた．また，PCR検査は国立感染症研究所か特定の都道府県衛生所に依頼するしかなかった．百日咳症例の早期発見のために検査室スタッフが研修し，独自にPCR検査を行える体制を院内に構築した．

準備の整った6月12日，百日咳注意報として感染対策委員長（病院長）から「職員ALLメール」で成人百日咳を想定した診療を全診療科の医師に依頼した．6月26日，「学生食堂で咳をする学生が多い」との情報提供があり，学生に自発的な受診を求めるポスターを掲示した．これに呼応した5人の学生が受診したが，いずれもPCR陰性であった．

7月18日夕刻，小児科からICTに，「7月11日に1ヵ月以上続く咳と肋骨骨折のため受診した医学部学生が百日咳抗体価を聞くために本日再診したが抗体価が高値であった」との一報が入った．以降実施した対策を表1にまとめた．

2．学　生

直ちに感染拡大状況の把握・感染経路遮断のために病院長，副病院長，医学部長，医学部学務委員長，ICD，ICNで緊急会議を開催し，① 翌19日の病院

表1　高知大学医学部における百日咳アウトブレークへの対応

日付	対応
7月18日	・百日咳抗体価高値の医学部学生が1名いることが判明 ・緊急会議（病院長，副病院長，医学部長，医学部学務委員長，ICD，ICN）
7月19日	・「検体採取は40歳以上のスタッフで行おう」と判断† ・学生146名にPCR検査実施（日中），24/28で陽性と判明（夕刻） ・結果的に，学生の最終発症日となる ・鼻腔からの検体と咽頭からの検体のPCR陽性率を検証‡
20日	・学生から院内臨床実習で接触した患者さんを聞き取り，症状調査，必要に応じてPCR ・院外実習施設へ百日咳集団発生を連絡 ・面接，検体採取に関与した職員へのクラリス®配付（58人） ・福祉保健所に報告，入院患者に掲示 ・学生に課外活動，対外試合，グループ学習，帰省の禁止，外出の自粛を要請¶ ・有症状者，予防内服希望者の学生にクラリス®処方 ・時間外の患者さんからの問い合わせ対応マニュアル作成 ・報道発表
21日	・臨床実習で学生と接触したハイリスク患者（30人）へのクラリス®予防投与
23日	・職員（抜けない咳，最近始まった咳・鼻水のある人，心配な人）にPCR検査実施（212人）※
25日	・23日の職員PCRの結果82人中59人が陽性（72%） ・職員有症状者，ハイリスク部署勤務者，および予防内服希望者にクラリス®配付 ・PCR陽性職員には，①クラリス®10日間内服，②2週間以内に咳が始まった人は5日間自宅待機，③無症状者はマスク着用，ただしハイリスク患者には接触禁止を指示 ・PICU医師，看護師全員クラリス®予防内服 ・23日のPCR陽性者に依頼して再検査：陽性一致率72.2%（13/18）♮ ・全職員にメールでマスク着用と手洗いを再度徹底，ミーティングの自粛を依頼
26日	・手術室医師，看護師全員クラリス®予防内服 ・国立感染症研究所へFETPチームの派遣を非公式に打診 ・感染対策委員長（病院長）から全職員に口頭説明会 ・外注業者責任者に現状説明＊
27日	・全職員の症状アンケート開始 ・23日のPCR陽性者20人に依頼して血清抗体価測定
	・院内保育所児童のワクチン接種歴確認と症状聞き取り＊＊ ・県を通して国立感染症研究所へFETPチームの派遣を公式に依頼
30日	・院内保育所児童の検診
8月1-2日	・学生症状調査開始（携帯電話アンケートを用いた詳細調査：回収率100%）
2日	・FETPチーム来高
3日	・無症状学生に帰省許可，グループ行動は禁止．非治癒，新たな感染疑いの学生には面接とPCR検査 ・「7月19日無症状でPCR陰性→予防内服せず→8月1日のアンケートでも無症状」の学生に依頼して試験的にPCR検査を実施§
8-10日	・FETPチームと合同で学生および職員に詳細アンケート実施
12日	・最終発症日（職員）となる
20日	・条件（マスクなどの感染対策の遵守，現場の実習責任者による日々の学生の体調管理）を設定して臨床実習開始
9月1日	・学生の学外活動（他施設における実習，対外試合など）の再開を許可
23日	・終息宣言（最終発症日から最長潜伏期間の2倍［42日間］の期間監視後）

† 年齢による抗体価の高低はあるが，菌が生着しない年齢層はないので，結果的には意味のある判断ではなかった．

‡ 34人のうち鼻腔29人，咽頭24人が陽性，いずれか陽性は33人，両方とも陽性だったのは20人．鼻腔，咽頭両検体からの陽性率はほぼ同じと考えられる．

¶ 麻疹の集団発生で帰省して感染を拡大した事例を参考に，帰省も禁止した．

※ 「学生はともかく，“大人”は大丈夫だろう」という予断が職員の検査を後回しにすることになった．

♮ 信じがたい，驚異的な陽性率であったので，検体採取時の偽陽性を否定するため行った．

＊ ＊＊外注業者や院内保育所職員への対応は結果的に後手になってしまった．

§ 6人中2人陽性であった．

実習は中止，② 学生全員にアンケート調査を行う，③ アンケート項目のうち，咳または鼻水がある，またはあった学生には面談を実施する，④ 面談で「症状がある」と認定できる学生にPCR検査への協力を依頼する，⑤「職員ALLメール」で注意喚起を行うことを決定した．併せて公表マニュアルに沿って，公的機関への通知やマスコミ対応の準備に入った．翌19日（Day1）だけで，学生816人中711名（87%）がアンケート調査に回答し，296名が面談に応じ依頼した146名全員がPCR検査に協力した．146人の大半からは「喫煙のせいで咳がある」「花粉症気味」との声があり，医療者の目から見ても百日咳かもしれないと思われるケースは20数例に過ぎなかった．しかし同日夜になって，146サンプルのうち急ぎ検査した28サンプル中24サンプル（85.7%）は，PCR法で百日咳菌陽性であることが判明した．即刻医学部と協議の上1週間の休講を決定した．

翌20日(Day2)には，翌週の教授会審議を待たず，夏休み期間を含む2学期までの医学部休講措置を決定し，学内外への伝播を防止するために課外活動，体外試合，グループ学習，帰省の禁止，外出の自粛を要請し，所轄福祉保健所に報告し，夕刻には報道発表を行った．そして，有症状者，予防内服希望の学生にクラリス®を配布した．

3．患者さん

7月20日(Day2)に，学生から院内臨床実習で接触した患者さんを聞取り，患者さんの症状調査，必要に応じてPCR検査を行うとともに，院外実習施設にも百日咳集団発生を連絡した．翌21日(Day3)には学生全員が排菌あるいは保菌者と想定し，学生と接触したハイリスク患者さんにクラリス®の予防内服をしていただいた．この際，説明する医師宛と患者さん宛の文書を作成し，統一した説明ができるようにした．

4．職　員

7月23日（Day5）から3日間PCR検査を行い，7月25日（Day7）から有症状者，ハイリスク部署勤務者，予防内服希望者にクラリス®を配布した．PCR陽性職員には，① クラリス®10日間内服，② 2週間以内に咳が始まった人は5日間自宅待機，③ 無症状者はマスク着用，ただしハイリスク患者には接触禁止を指示し，全職員にマスク着用と手洗いの徹底，不急不要のミーティングの自粛を依頼した．

表2　PCR検査結果

区分	陽性人数／検査人数（陽性率）	内訳	
学生	74/162 (45.7%)	医学科 (60.4%)	67/111
		看護学科 (13.7%)	7/51
職員	148/212 (69.8%)	臨床系医師 (60.0%)	18/30
		基礎系講座 (72.7%)	32/44
		看護師 (70.9%)	39/55
		コメディカル (72.2%)	13/18
		その他 (70.8%)	46/65

陽性人数／検査人数（陽性率）

5．外注業者，院内保育所

外注業者には7月26日（Day8）に責任者に現状説明を行い，症状出現時は速やかに受診することとマスクの着用を依頼した．院内保育所には，7月27日（Day9）に責任者に現状説明するとともに児童のワクチン接種歴確認と症状聞取りを行い，7月30日（Day12）に児童の検診を行った．

◆調査結果および考察

1．PCR結果

PCR検査の結果を表2に示す．学生は45.7%(162人中74人)，職員は69.8%（212人中148人）の陽性率であった．しかし，検出結果と症状の有無は必ずしも一致せず，陽性者のうち実に60%は無症状であった．また，98検体（PCR陽性63名陰性35名）に対して培養検査を試みたが，菌は1例も分離できなかった．

PCR検査は迅速・高感度な検出法であり，今回は百日咳菌に特異的な2遺伝子を標的部位として実施した．PCR以外の遺伝子診断法としてLAMP法を実施し，今回の結果を検証したが大きな乖離は認めなかった．アウトブレークした百日咳菌はmultilocus sequence typing-1(MLST-1)型で，近県で発生したMLST-2型とは異なっていた．

2．積極的疫学調査結果

PCRの陽性率が高いことから，感染源，感染経

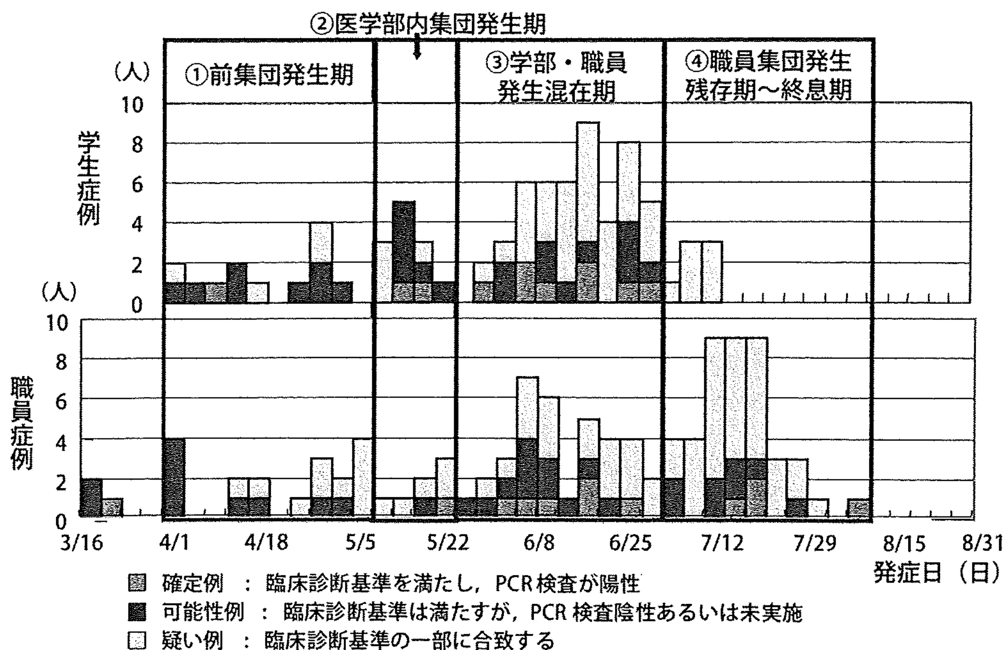

図1　百日咳発生状況と流行曲線からみた集団発生の期間区分

路の特定には2200人を対象とした疫学調査が必要であり，公開性・透明性の点からも外部調査が必要と判断し，7月26日国立感染症研究所にコンタクトを行った．

7月27日に職員第1回，8月1日に学生第1回現状調査を行った．Web上でCDCの診断基準に基づいた質問内容に回答する方法とした．職員の回答には，「6月下旬に外来受診した高校生が長期に続く咳があり，近医で抗菌薬をもらっていた」「中学2年生の子供がいるが，同じクラブの子が百日咳で休んでいる」など重要な情報提供があった．また学生からは，翌日までに816人，100%の回答が得られた．

8月2日から国立感染症研究所（Field Epidemio -logy Training Program Japan：FETP）による支援調査が開始された．FETPには積極的症例探査，現場および関連施設の観察調査，症例群の特徴把握と図式化，感染源/感染経路やリスクファクターに関する仮説の検証を実施していただいた．積極的症例探査は「症例定義」を作成し，8月7日以前の症例に後向き調査，8月8日以降の症例には前向き調査（質問票回収率：学生90.2%，職員93.7%）を行った．症例定義は，4月1日以降に発症し，「①咳が2週間以上続く」and「②吐きそうになる咳or③突然連続して起こる咳or④“woop”のある咳がある」という臨床診断基準を満たすものとした．

4月1日から8月31日までの患者発生状況を学生・職員別に示す（**図1**）．職員では3月中旬から，学生では4月から発生がみられる．前集団発生期には，症例間における学年・部活動・所属部署などの共通点には乏しく，地域流行の持込が複数回あった可能性が示唆された．第2の医学部内集団発生期には，講義室，臨床実習班，部活動など比較的濃厚な接触による感染伝播が考えられた．次の学部・職員発生混在期には，学生間の伝播がさらに拡大し，一部病棟においては臨床実習生から職員に感染が伝播した可能性が考えられた．そして第4の職員集団発生残存期～終息期に移行していた．感染経路は学生間では講義室・部活動での濃厚接触，学生－職員間は臨床実習等を介した感染伝播が推測され，職員間では特定の部署において感染がより拡大した可能性が示唆された．集団発生の要因としては，乳幼児期のワクチン効果の限界をベースにして，地域の不顕性の流行があり，それが大学に持ち込まれた．そして感染性を有する期間における発症者の登校が続き，比較的長時間狭い空間を共有する講義室や職場の環境でアウトブレークが潜行しながら広がった．

しかし学生，職員，医師にこの再興感染症に対する認識がなく，あるいは百日咳の感染や発症についての医学知識がup to dateされていなかったことが上げられる．

入院患者，退院患者には全期間通じて症例定義に合致する症例はなく，外来患者についてはカルテ調査から数例が百日咳と疑われたが，当院における症例との疫学的な関連性はなかった．患者さんに対して業務を行う場合のマスク着用率は，職員同士での着用率よりも有意に高く，患者さんへの感染伝播防止に有効であったと考えられた．

3．抗菌薬内服状況

抗菌薬内服状況を**表3**に示す．学生は610人（81.9%），職員は473人（35.9%）が内服し，うちクラリスロマイシンを5日以上内服した者は学生581人（96.4%），職員396人（84.6%）であった．部署別の発症率と抗菌薬予防服用率を見ると，発症率が高い部署ほど，またPICU，小児科，救急部など感染が起きた際のリスクが高いと想定される部署ほど高い服用率を示した．また，26名の陽性者を対象に服薬1週間後のPCR検査を行い19名の陰性化を確認した．

4．終　息

学生については，対策導入後は新規発症者を認めず，休講と，高い抗菌薬内服率（81.9%）が主たる要因と考えられた．職員については，少数の新規発症者を認めたが，予防内服に加え，就業制限などを強化した結果，終息に向かうことができた．感染対策の強化については咳エチケット，手洗いの徹底など全職員にメールを発信し協力を依頼した．

学生の最終発症日は7月19日，職員の最終発症日は8月12日であった．最終発症者から最長潜伏期間の2倍の期間（42日間）監視を行い，9月23日に終息と判断した．

終息を迎えられたのは，学生・職員の対策への積極的な協力の成果と考えている．

◆おわりに

私達が行った百日咳水際阻止対策は失敗に終わった．早期検知のために予防措置を講じ，第一例発生後は迅速に対応したが，既に4ヵ月も前から百日咳は大学に侵入していた．その背景には，市中感染の蔓延，無症状保菌者の存在，百日咳診断の困難さがあった．

アウトブレークの対応は早期認知と迅速対応が基本である．早期認知のためには，感染症に対する意識の向上，臨床診断に力を注ぐこと，ベースラインの把握が必要である．迅速対応のためには，組織が一体となった対応ができるように，正確な情報の共有と連携，さらには日常的な活動の積み重ねが必須であると実感された．

表3　抗菌薬内服状況

	学生 n＝745	職員 n＝1318
抗菌薬内服者数	610 (81.9%)	473 (35.9%)
クラリスロマイシン	603 (98.9%)	468 (98.9%)
エリスロマイシン	3 (0.5%)	2 (0.4%)
その他	4 (0.7%)	3 (0.6%)
クラリスロマイシン内服日数		
14日以上	324 (53.7%)	7 (1.5%)
10日以上	435 (72.1%)	223 (47.6%)
7日以上	542 (89.9%)	297 (63.5%)
5日以上	581 (96.4%)	396 (84.6%)

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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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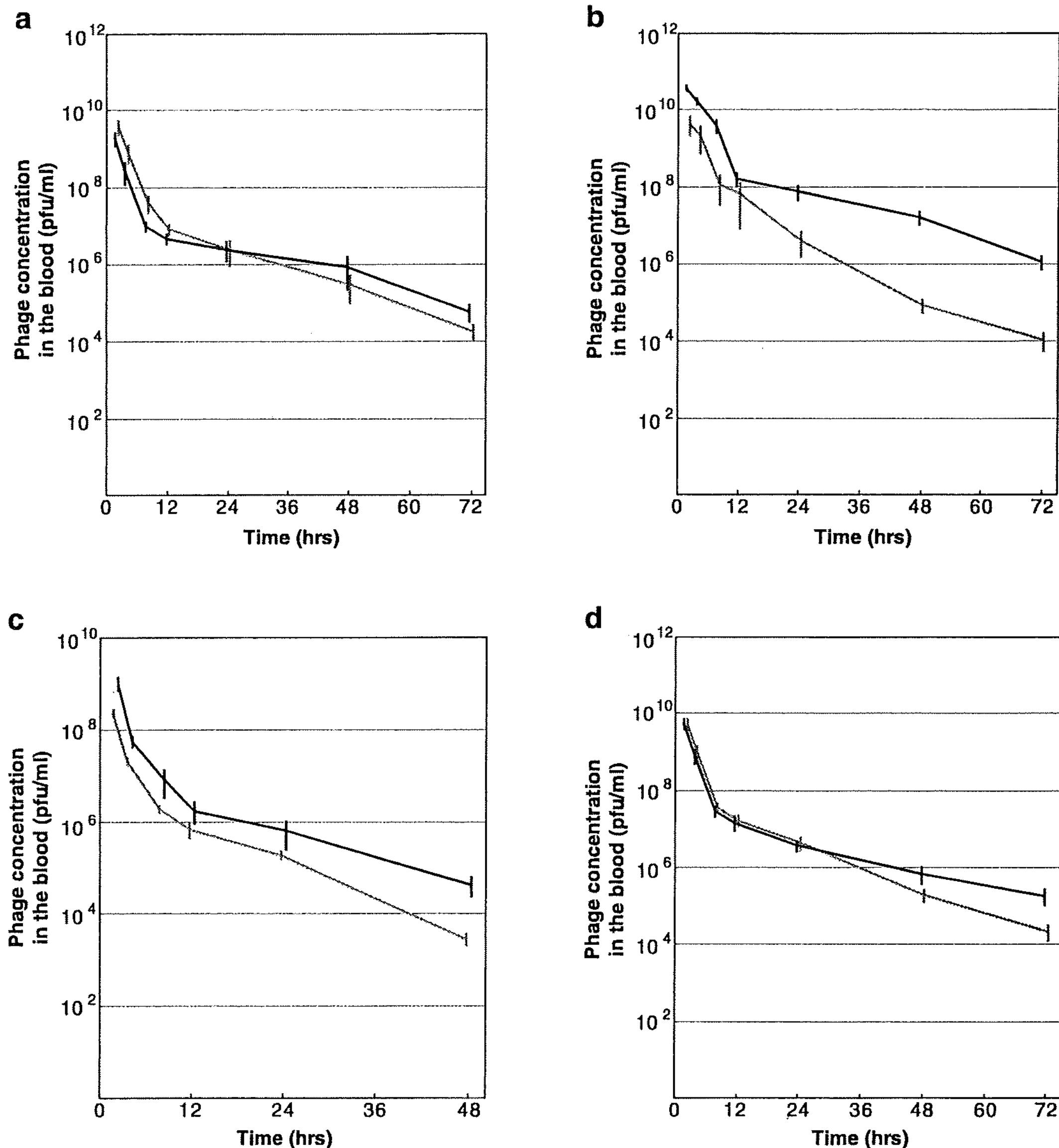

Fig. 1. Time-course concentration changes in active phage in the peripheral blood of healthy and neutropenic mice. Mean of active phage concentration in neutropenic or healthy mice is graphed, shown in black or grey lines, respectively. Vertical bar indicates standard error of mean. Approximate mathematical 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.

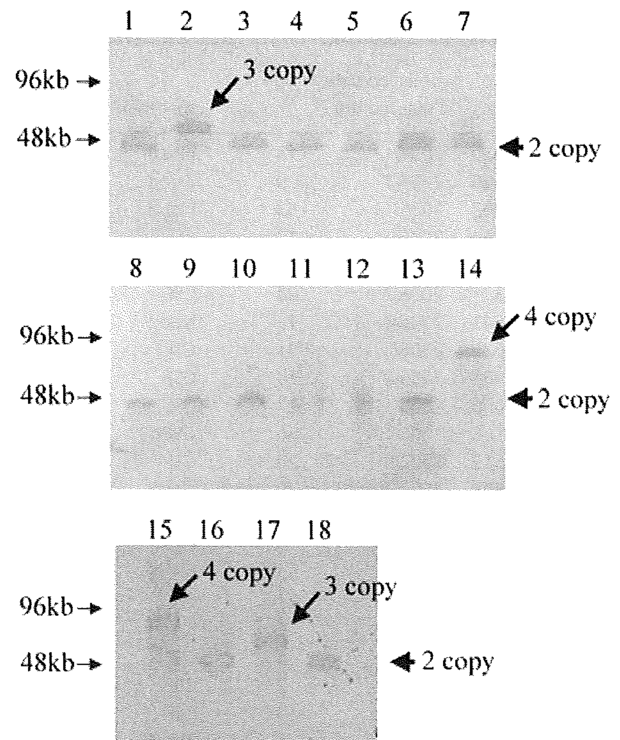

Fig. 1. Examples of Southern blot analysis of DNA from *Haemophilus influenzae* type b strains digested with KpnI/SmaI, separated by PFGE, and hybridized with the 480-bp DIG-labeled *cap*b probe. Strain K6066 in lane 2 and strain K7721 in lane 17 showed three-copy arrangement of the *cap*b locus (ca. 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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