Table 2. Point mutations detected in the QRDRs of *gyrA* and *parC* with the corresponding amino acid substitutions in parent strains and selected mutants (M)

Parent/mutant in steps[a]	*emm* type	Levofloxacin MIC (mg/L)	Point mutation in *gyrA* (codon)	Point mutation in *parC* (codon)	
			residue 81	residue 79	residue 83
1-Parent	1	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
1M-1		2	Ser-81 (TCT)	Phe-79 (TTC)	Asp-83 (GAT)
1M-2		2	N/P	N/P	N/P
1M-3		2	N/P	N/P	N/P
1M-4		32	Tyr-81 (TAT)	Phe-79 (TTC)	Asp-83 (GAT)
1M-5		64	Tyr-81 (TAT)	Phe-79 (TTC)	Asp-83 (GAT)
1M-6		128	Tyr-81 (TAT)	Phe-79 (TTC)	Asp-83 (GAT)
79-Parent	4-1	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
79M-1		8	Tyr-81 (TAT)	Ala-79 (GCC)	Asp-83 (GAT)
79M-2		8	N/P	N/P	N/P
79M-3		8	N/P	N/P	N/P
79M-4		64	Tyr-81 (TAT)	Ala-79 (GCC)	Gly-83 (GGT)
79M-4		64	N/P	N/P	N/P
79M-5		64	N/P	N/P	N/P
79M-6		128	Tyr-81 (TAT)	Ala-79 (GCC)	Gly-83 (GGT)
25-Parent	9	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
25M-1		8	Tyr-81 (TAT)	Ser-79 (TCC)	Asp-83 (GAT)
25M-2		16	Tyr-81 (TAT)	Ser-79 (TCC)	Asp-83 (GAT)
25M-4		32	Tyr-81 (TAT)	Ser-79 (TCC)	Asp-83 (GAT)
25M-4		32	N/P	N/P	N/P
25M-5		128	Tyr-81 (TAT)	Tyr-79 (TAC)	Asp-83 (GAT)
25M-6		128	N/P	N/P	N/P
25M-7		256	Tyr-81 (TAT)	Tyr-79 (TAC)	Asp-83 (GAT)
87-Parent	73-3	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
87M-1		0.5	N/P	N/P	N/P
87M-2		8	Tyr-81 (TAT)	Ala-79 (GCC)	Asp-83 (GAT)
87M-3		64	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
87M-4		128	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
87M-5		128	N/P	N/P	N/P
87M-6		128	N/P	N/P	N/P
87M-7		256	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
19-Parent	75-5	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
19M-1		32	Tyr-81 (TAT)	Ala-79 (GCC)	Asp-83 (GAT)
19M-2		32	N/P	N/P	N/P
19M-3		64	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
19M-4		128	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
3-Parent	28	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
3M-1		2	Ser-81 (TCT)	Phe-79 (TTC)	Asp-83 (GAT)
3M-2		2	N/P	N/P	N/P
3M-3		2	N/P	N/P	N/P
3M-4		32	Tyr-81 (TAT)	Phe-79 (TTC)	Asp-83 (GAT)
3M-5		32	N/P	N/P	N/P
3M-6		32	N/P	N/P	N/P
3M-7		64	Tyr-81 (TAT)	Phe-79 (TTC)	Asp-83 (GAT)
6-Parent	94	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
6M-1		2	Phe-81 (TTT)	Ser-79 (TCC)	Gly-83 (GGT)
6M-2		4	Phe-81 (TTT)	Ser-79 (TCC)	Gly-83 (GGT)
6M-3		8	Phe-81 (TTT)	Ser-79 (TCC)	Gly-83 (GGT)
6M-4		8	N/P	N/P	N/P

Table 2. (*continued*).

Parent/mutant in steps[a]	*emm* type	Levofloxacin MIC (mg/L)	Point mutation in *gyrA* (codon) residue 81	Point mutation in *parC* (codon) residue 79	Point mutation in *parC* (codon) residue 83
6M-5		8	N/P	N/P	N/P
6M-6		32	Phe-81 (TTT)	Ser-79 (TCC)	Gly-83 (GGT)
10-Parent	103	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
10M-1		4	Ser-81 (TCT)	Phe-79 (TTC)	Asp-83 (GAT)
10M-2		4	N/P	N/P	N/P
10M-3		4	N/P	N/P	N/P
10M-4		64	Phe-81 (TTT)	Tyr-79 (TAC)	Asp-83 (GAT)
10M-5		64	N/P	N/P	N/P
10M-6		64	N/P	N/P	N/P
10M-7		128	Phe-81 (TTT)	Tyr-79 (TAC)	Asp-83 (GAT)
13-Parent	89	0.5	Ser-81 (TCT)	Ser-79 (TCC)	Asp-83 (GAT)
13M-1		16	Ser-81 (TCT)	Tyr-79 (TAC)	Asp-83 (GAT)
13M-2		16	N/P	N/P	N/P
13M-3		128	Phe-81 (TTT)	Tyr-79 (TAC)	Asp-83 (GAT)
13M-4		128	N/P	N/P	N/P
13M-5		128	N/P	N/P	N/P
13M-6		128	N/P	N/P	N/P
13M-7		128	N/P	N/P	N/P
13M-8		256	Phe-81 (TTT)	Tyr-79 (TAC)	Asp-83 (GAT)
53-Parent	11	1	Ser-81 (TCT)	Phe-79 (TTC)	Asp-83 (GAT)
53M-1		64	Tyr-81 (TAT)	Ala-79 (GCC)	Asp-83 (GAT)
53M-2		64	N/P	N/P	N/P
53M-3		128	Tyr-81 (TAT)	Ala-79 (GCC)	Asp-83 (GAT)
53M-4		128	N/P	N/P	N/P
53M-5		128	N/P	N/P	N/P
53M-6		256	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
30-Parent	12	2	Ser-81 (TCT)	Phe-79 (TTC)	Asp-83 (GAT)
30M-1		64	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
30M-2		128	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
1314-Parent	6-1	2	Ser-81 (TCT)	Ala-79 (GCC	Asp-83 (GAT)
1314M-1		4	N/P	N/P	N/P
1314M-2		8	N/P	N/P	N/P
1314M-3		4	N/P	N/P	N/P
1314M-4		4	N/P	N/P	N/P
1314M-5		8	Tyr-81 (TAT)	Ala-79 (GCC)	Asp-83 (GAT)
1314M-6		4	N/P	N/P	N/P
1314M-7		4	Tyr-81 (TAT)	Ala-79 (GCC)	Asp-83 (GAT)
1314M-8		8	N/P	N/P	N/P
1314M-9		4	N/P	N/P	N/P
1314M-10		8	N/P	N/P	N/P
1314M-11		8	N/P	N/P	N/P
1314M-12		32	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)
1314M-13		32	N/P	N/P	N/P
1314M-14		64	Tyr-81 (TAT)	Val-79 (GTC)	Asp-83 (GAT)

N/P, not performed.
[a]The number following each mutant (M) indicates the number of induction steps.

for levofloxacin. All mutants reached MICs of ciprofloxacin >32 mg/L after four cycles of induction/selection except strains 6 and 1314 which took six and twelve cycles, respectively. MICs of gatifloxacin were >32 mg/L after three or more cycles of induction, and the mutants also had high MICs of ofloxacin and norfloxacin (data not shown).

With each set of parent-mutants, the MIC of levofloxacin for mutants was increased following induction/selection cycles.

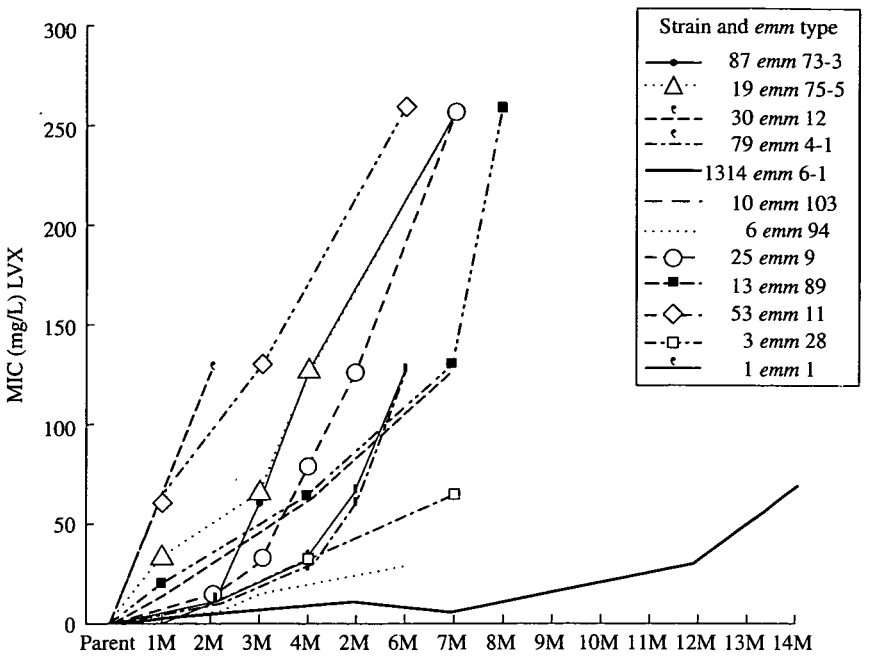

Figure 1. Fluoroquinolone susceptibility changes in selected mutants. Twelve patient strains representing 12 different *emm* types were used as parent strains to be exposed to levofloxacin (LVX) in a stepwise manner. Mutants were generated by repeated cycles of induction and selection processes. MIC was measured in a representative mutant (M) in each cycle. *x*-axis numbers represent the number of cycles of induction and selection.

However, the number of cycles required to generate mutants with significantly high MICs varied among the 12 strains studied, even in strains with the same pre-induction MICs. In nine parent-mutant sets, up to seven cycles of induction/selection cycles were required to yield resistant mutants with an MIC of levofloxacin of 128 mg/L. Mutants from strains 3 (*emm* 28) and 6 (*emm* 94) reached MICs of 64 and 32 mg/L, respectively, after seven and six cycles of induction/selection. Strain 30 (*emm* type 12) yielded mutants with MICs of 128 mg/L after only two steps of exposure, and strains 87 (*emm* type 73-3), 19 (*emm* type 75-5) and 79 (*emm* type 4-1) yielded numerous mutants with MICs of levofloxacin of 64 mg/L after 3–4 cycles of induction/selection and 128 mg/L following additional cycles. In contrast, following 11 induction/selection cycles, the MIC of levofloxacin for mutants derived from strain 1314 (*emm* type 6-1) only reached 8 mg/L and a maximum MIC of 64 mg/L was reached at cycle 14 (Table 2 and Figure 1).

Point mutations in the QRDRs of gyrA/B and parC/E

No mutations were detected in the QRDRs of *gyrB* and *parE* in all mutants and parent strains; however, point mutations were found in *gyrA* and *parC* subunits. Individual mutations resulting in an amino acid substitution in the QRDRs of *gyrA* and *parC* corresponding to increased MICs for mutants are presented in Table 2.

As the MICs increased in mutants, except for strain 6 (*emm* type 94), residue 79 was changed from the initial residue to alanine, valine, tyrosine or phenylalanine dependent upon a particular parent-mutant set. The substitution of valine at position 79 of *parC* has not been observed from reported point mutations of clinical isolates of *S. pyogenes*. Strains 79 (*emm* type 4-1) and 6 (*emm* type 94) also developed a change in mutants from aspartic acid to glycine at position 83, which was not found in mutants derived from other strains. The remaining strains had no point mutation at this residue.

Point mutations were found in the QRDR of *gyrA* in mutants from each strain regardless of *emm* type and the point mutation only occurred in the serine residue at position 81, with two types of amino acid substitutions, either to Tyr-81 (in 8 of 12 strains) or Phe-81 (4/12). However, the occurrence of a point mutation at this residue did not consistently correlate to an increase in MICs. For example, it took as low as a 4-fold increase in MIC in strain 6 to reveal a substitution from Ser-81 to Phe-81, while for strain 13, the same residue replacement occurred after the MIC was increased more than 256-fold.

Discussion

Although *in vitro* generation of fluoroquinolone-resistant mutants in *S. pyogenes* has been studied previously,[10,11] our study has improvements over those studies for the purpose of further understanding the mechanism of fluoroquinolone resistance in *S. pyogenes*. Boos *et al.*[10] induced *S. pyogenes* to become fluoroquinolone-resistant *in vitro* by exposure to fluoroquinolone drugs, but that study did not investigate the changes in the QRDRs of the DNA gyrase and topoisomerase IV genes and it did not determine the *emm* types of the parent strains studied. In addition, the highest MIC increase in their generated resistant mutants was only 9-fold. We show an increase in MICs up to 512-fold, suggesting that the appropriate combination of stepwise induction and selection cycles are an efficient way to produce resistant mutants *in vitro*. The availability of mutants with a wide MIC distribution reveals more variety of point mutations associated with an increase in resistance level. Schmitz *et al.*[11]

used ciprofloxacin, gatifloxacin, moxifloxacin and BMS-284756 (garenoxacin) to generate fluoroquinolone-resistant mutants and demonstrated that, despite the choice of fluoroquinolone used, the potential for induction of resistant mutants in *S. pyogenes* is comparable. In the current study, we used levofloxacin to generate fluoroquinolone-resistant laboratory mutants from 12 parent strains of different *emm* types. Instead of testing with different fluoroquinolone agents, we enhanced the efficiency by repeating the induction/selection process until significantly high-level resistant mutants were selected. For each set of parent-mutants, an array of mutants was produced with a stepwise increase in MICs of levofloxacin corresponding to point mutation information in the QRDRs of both *gyrA* and *parC*. This allows a systematic analysis of mutations through the study of the relationship between the changes of residues involved in the point mutations and increasing MICs.

In conjunction with previous studies, the findings from the current study provide the following conclusions. (i) Induction coupled with selection in a laboratory setting is a reliable method for yielding stable fluoroquinolone-resistant *S. pyogenes* mutants. (ii) Despite the fact that many mutants with a wide range of MICs were produced from a dozen parent strains with different baseline MICs, the number of residues affected and variety of amino acid substitutions found in *gyrA* and *parC* are limited (Table 2), which are comparable to those demonstrated in reported clinical resistant isolates.[1–4,6,13–15] (iii) The concentration of a fluoroquinolone agent used and number of induction/selection cycles required to yield high MIC mutants vary with *emm* types/strains. (iv) Of the representative *emm* type strains, the majority of the non-*emm* 6 types were comparable in their ability to yield highly resistant mutants, suggesting that resistance to fluoroquinolones can develop independent of *emm* type.

Previous studies indicated that >90% of clinical isolates that are either resistant or have reduced susceptibility to fluoroquinolones are *emm* type 6.[4,13] However, our *emm* type 6 isolate took 14 induction/selection cycles to produce resistant mutants with a maximum MIC of 64 mg/L (Table 2 and Figure 1), while 10 non-*emm* 6 types took fewer cycles (from 1 to 7 cycles) to reach the same level of resistance. We challenged six other *emm* 6 strains collected from different geographic regions in Japan to the same induction/selection experiments and found that the generation of highly resistant mutants in these *emm* 6 strains was also relatively difficult (data not shown).

The disparity of ease of yielding high-level fluoroquinolone-resistant mutants between *emm* 6 and other types was further analysed by comparison of amino acid substitutions. The *emm* 6 parent strain used in this study had an MIC of 2 mg/L of levofloxacin and ciprofloxacin and had alanine substituted for serine-79 in *parC* before induction. Orscheln *et al.*[4] raised the concern that the intrinsic reduction in susceptibility to ciprofloxacin and the amino acid polymorphism at this residue of the *parC* gene in *emm* type 6 *S. pyogenes* might set the stage for the emergence of high-level resistance if a single point mutation were to occur in the *gyrA* gene. Our data seem to indicate that this may not be so. The mutation that leads to the substitution of tyrosine for serine-81 in *gyrA* took five steps to appear in the *emm* type 6 strain, but it took only one or two steps for a substitution of this residue with either tyrosine or phenylalanine in seven other *emm* type strains.

Point mutations in *gyrA* and *parC* appear to explain the primary mechanism of loss of susceptibility to fluoroquinolones in *S. pyogenes*. Stepwise acquisition of mutations in *S. pneumoniae* has been known for sometime.[8,16,17] It has been suggested that *S. pyogenes* mutations are also acquired in a stepwise fashion.[4,18] This study demonstrated stepwise acquisition of mutations in *S. pyogenes* through repeated *in vitro* exposure to increasing concentrations of a fluoroquinolone followed by a selection process. Based on comparison of our data and reports of point mutations in fluoroquinolone-resistant clinical isolates of *S. pyogenes*, amino acid substitutions associated with fluoroquinolone resistance in *S. pyogenes* seem far less complicated than that in *S. pneumoniae*.[19–22] A limitation of our study was that we studied a single isolate of each emm type for all our 12 different types. Because isolates from different *emm* types demonstrate similar phenotypic and genotypic changes we would expect different isolates of the same *emm* type to respond similarly. We are now conducting experiments using multiple isolates of *emm* type 6 to determine whether different strains of the same *emm* type respond in the same way. Precise correlation between residue replacements and increased MIC in mutants may not be solely explained by the point mutations, because it is still difficult to explain why mutant strains with the same point mutations demonstrated various levels of MIC of levofloxacin. Therefore, exploration of other possible mechanisms, such as an activated efflux pump specific for fluoroquinolone drugs, among the resistant mutants may be necessary.

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Transparency declarations

No interest has influenced the conclusions drawn from the work.

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Efficacy of a novel oral carbapenem, tebipenem pivoxil (TBM-PI), against experimental otitis media caused by penicillin resistant *Streptococcus pneumoniae* in chinchilla

Muneki Hotomi[a,b], Masaki Suzumoto[a,b], Koju Itahashi[c], Jun Nagura[c], Takayoshi Fukushima[c], Jun Shimada[a], Dewan S. Billal[a,b], Kazuma Yamauchi[a,b], Keiji Fujihara[a,b], Noboru Yamanaka[a,b,*]

[a] *Infection and Immunity Research Center, Wakayama Medical University, Wakayama, Japan*
[b] *Department of Otolaryngology, Wakayama Medical University, 811-Kimiidera, Wakayama-shi, Wakayama, 641-8509 Japan*
[c] *Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Yokohama, Japan*

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Abstract

An animal model of otitis media using chinchillas was developed to evaluate the efficacy of tebipenem pivoxil (TBM-PI) against experimental otitis media. Chinchillas inoculated via the transbullar approach with *Streptococcus pneumoniae* serogroup 6 were included in the efficacy study with TBM-PI, amoxicillin (AMX) or untreated as controls. TBM-PI resulted in survival rate of 83%, compared with 25% survival for AMX and 0% survival for controls ($p < 0.01$). Quantitative cultures in the middle ear effusions at day 5 of the TBM-PI group yielded $3.5 \pm 2.4 \log_{10}$ CFUs/ml. TBM-PI is a promising antibiotic for the treatment of acute otitis media.

Keywords: Tebipenem pivoxil; Experimental otitis media; Chinchilla; *Streptococcus pneumoniae*

1. Introduction

Acute otitis media (AOM) is one of the most common infectious diseases among children. By 3 years of age, more than 80% of children have had at least one episodes of AOM [1]. *Streptococcus pneumoniae* (*S. pneumoniae*) is a leading causative pathogen for AOM [2,3]. This pathogen causes >35% of the estimated 16.8 million cases of AOM in children younger than 7 years of age in the United States [4]. Clinical improvement of AOM is highly correlated with the eradication of pathogens from the middle ear [5]. Amoxicillin (AMX) has long been the drug of the first choice against AOM [6,7]. This antimicrobial agent eradicated the pathogen from the middle ear in greater than 90% cases before 1990. However, penicillin resistant *S. pneumoniae* (PRSP) with reduced susceptibility to both cephalosporins and other antimicrobial agents have been observed in recent years [8]. As the prevalence of PRSP has increased, microbiological and clinical failures have been reported in the treatment of pediatric AOM [9,10]. Unlike the mechanism of antimicrobial resistance in *Haemophilus influenzae* and *Moraxella catarrhalis* in which plasmid-mediated β-lactamase production was associated, in *S. pneumoniae* involves structural modification of penicillin binding proteins [11–13]. Thus, the development of alternative agents that have broad coverage with respect to the various strains of *S. pneumoniae* including PRSP are strongly required if the prevalence of the antimicrobial resistant pathogen continues to be increased.

Carbapenems are well recognized to have broad and strong antibacterial activities [14]. Four parenteral derivatives such as imipenem, panipenem, meropenem, and biapenem are clinically introduced in Japan [15–17]. However, oral carbapenem has not been marketed as yet worldwide.

* Corresponding author at: Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, 811-Kimiidera, Wakayama-shi, Wakayama, 641-8509 Japan. Tel.: +81 73 441 0651; fax: +81 73 446 3856.
E-mail address: ynobi@wakayama-med.ac.jp (N. Yamanaka).

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Tebipenem pivoxil (TBM-PI) is a novel oral carbapenem [18,19]. This compound is an ester-type prodrug of an active parent compound, tebipenem. In vitro studies have demonstrated that Tabipenem (TBM) has superior antibacterial activities against PRSP. In this study, we evaluate the clinical efficacies of TBM-PI against experimental otitis media caused by PRSP in the chinchilla.

2. Material and methods

2.1. S. pneumoniae strains

S. pneumoniae capsular group 6 strain SP00221, capsular group 19 strain SP00341, and serotype 14 strain SP00396 were used in this study. All strains were resistant to penicillin G (PCG) but were susceptible to TBM and AMX (Table 1).

2.2. Animals

Chinchillas (*Chinchilla lanigera*), male, 350–552 g (CLEA Japan Inc., Tokyo, Japan) were used in this experiment. They were maintained at 21–25 °C, 50–70% of moisture, 7:00–19:00 h in light. Food and water were given ad libidum.

2.3. Antibiotics

Tebipenem pivoxil (TBM-PI), TBM (Meiji Seika Kaisha, Tokyo, Japan) and AMX (Kyowa Hakko Inc., Tokyo, Japan) were used for the efficacy experiment. Both antibiotics were dissolved into 0.5% (w/v) carboxymethyl cellulose sodium salt buffer (Nacalai Tesque, Kyoto, Japan) at 25 mg/ml of the final concentrations. TBM-PI concentration was expressed as TBM equivalent.

2.4. Experimental infection

An experiment was performed for experimental infection. Prior to infections, both ears of chinchillas were confirmed having normal healthy middle ear status using otomicroscopy. The mid-log phase of *S. pneumoniae* strains grown in Todd-Hewitt broth (Becton Dickinson Microbiology Systems, NJ, USA) with 0.5% yeast extracts (Difco Laboratories, NJ, USA) were diluted appropriately to a concentration of 100–1000 CFUs/ml in sterilized saline. Ten to 100 CFUs of pneumococcal strains in 100 μl saline were inoculated directly into the right middle ears of the chinchilla via the transbullar approach by a 25-g needle and tuberculin syringe under intra-peritoneal anesthesia with pentobarbital sodium.

Table 1
S. pneumoniae strains used in this experiment

Strain	Serogroup	MIC (μg/ml)		
		PCG	AMX	TBM
SP00221	6	4	2	0.06
SP00396	14	2	1	0.06
SP00341	19	2	1	0.06

TBM-PI: tebipenem pivoxil, AMX: amoxicillin.

The chinchillas were monitored for 9 days after the inoculation with *S. pneumoniae*. Otomicroscopic examinations were performed on days 2, 5–6, and 9 after the inoculation. Middle ear washes with 0.2 ml of sterilized saline were collected for MEEs through a 3 mm hole in the superior bulla on days 2, 5–6, and 9. The middle ear washes were diluted up to total 2 ml with 1.8 ml of sterilized saline containing 0.05% agar. The 100 μl of serial 1:10 dilutions of middle ear washes were plated on the brain heart infusion agar (BHIA) with 5% defibrinized horse blood and were incubated at 35 °C for 16–19.5 h under the 5–6% CO_2 atmosphere. The lowest limit of detecting CFUs was 100 CFUs/ml in middle ear washings. The CFUs data were expressed as average $\log_{10}$ CFUs ± the standard deviation.

2.5. Efficacy experiment

Three experiments were performed for efficacy of antimicrobial treatment. After examinations of both ears confirming normal healthy status, the right ear of chinchillas were inoculated via transbullar approach with approximately 40–60 CFUs of *S. pneumoniae* capsular group 6 strain SP00221 in 100 μl of saline. After confirming the development of AOM by otomicroscopy 24 h after the inoculation, the chinchillas were randomly assigned into one of three groups for treatments with AMX, TBM-PI, or without antibiotics (controls). Both TBM-PI and AMX were administrated at 25 mg/kg/dose twice in a day (b.i.d.) by orogastro feeding tube at 9:00 a.m. and 7:00 p.m. for 5 days. The 0.5% (w/v) carboxymethyl cellulose sodium salt solution was used as control.

The chinchillas were observed for 9 days after the inoculation with *S. pneumoniae* for death. Otomicroscopic examinations of tympanic membranes and bacterial examinations of middle ear washes were done similar as described above.

2.6. Determination of antimicrobial concentration in serum and middle ear

Concentrations of TBM and AMX in sera and middle ears were determined by bioassay using *Staphylococcus aureus* ATCC6538 and *Micrococcus luteus* ATCC9341. Briefly, blood for sera were collected subsequently at 15, 30, 60, 120, 240, and 360 min after the administration of either 25 mg/kg TBM-PI or 25 mg/kg AMX. For evaluating concentrations of the antibiotics in the middle ear, the chinchilla were infected with 40 CFUs of *S. pneumoniae* SP00221 via the right middle ear cavity prior to collecting middle ear samples. Either antibiotic were administrated to the chinchilla by orogastric feeding tube. The chinchillas were sacrificed at 30 min after administration of TBM-PI or at 90 min after administration of AMX and middle ear mucosa were collected from both sides. The homogenized middle ear mucosa was further used as samples for determining concentration of the antibiotics.

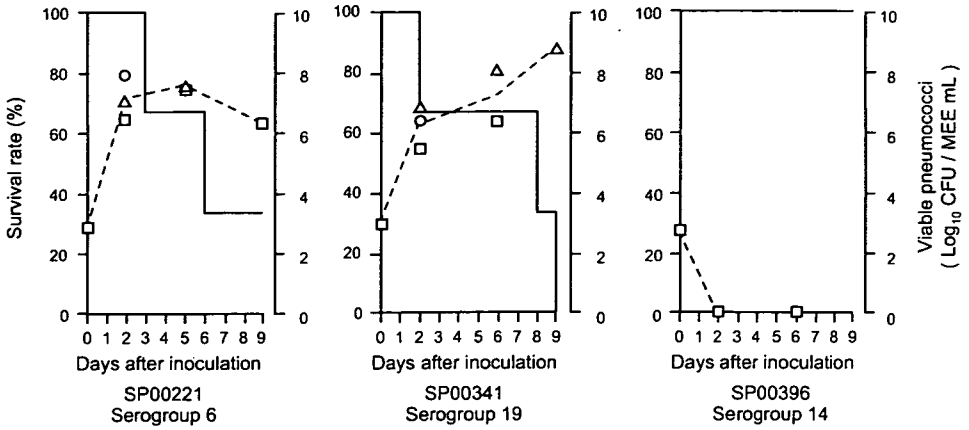

Fig. 1. Development of experimental otitis media by three different serogroups of pneumococci. Solid line: survival rate, dotted line: viable pneumococci in middle ear effusions. Three chinchillas were used per group.

2.7. Statistical analysis

Statistical comparisons of numbers of *S. pneumonia* in the middle ear washings were carried out on log-transformed data by the Student's *t*-test and/or the Mann–Whitney *U*-test. Statistical comparisons of the survival of the chinchilla were also carried out by the Kaplan–Meier method and/or the Log-rank test. A *p*-value of <0.05 was considered statistically significant. All statistical analysis was carried out with SAS8.2.

3. Results

3.1. Experimental pneumococcal AOM models

First appropriate capsular serogroups and doses of bacteria were evaluated to develop experimental otitis media in chinchillas. *S. pneumoniae* capsular group 6 strain SP00221 and group 19 SP00341 colonized in the tympanic bulla of chinchillas and developed AOM. The density of these strains in the middle ears was increased up to 10^6–10^8 CFUs/ml by days 3–9 after inoculation (Fig. 1). Both strains were highly virulent and 66.6–100% of chinchillas inoculated with either strain of *S. pneumoniae* died from days 2–9 after inoculation. However, the *S. pneumoniae* strain of serotype 14 did not colonize in the bulla of the chinchillas.

The appropriate doses of bacteria needed to develop experimental otitis media in chinchillas were also evaluated. One of three doses of *S. pneumoniae* capsular group 6 SP00221 was inoculated into the tympanic bulla of chinchillas. All doses of inoculation of the strain could colonize in the bulla, and the numbers of pneumococci increased at 10^6–10^8 CFUs/ml on day 3 after inoculation. The mortality rate was not different between chinchillas inoculated with three different doses of *S. pneumoniae* (Fig. 2). All chinchillas developed acute inflammation in the middle ear including retention of middle ear fluids, bulging and redness of tympanic membrane, swelling of the external auditory canals (Fig. 3).

3.2. Pharmacokinetics of TBM-PI and AMX

The respective C_{max} values of TBM were 7.2 μg/ml and showed markedly improved rather than that of AMX as 6.1 μg/ml. The T_{max}, $t_{1/2}$, $AUC_{0\sim\infty}$, CL/F values of TBM

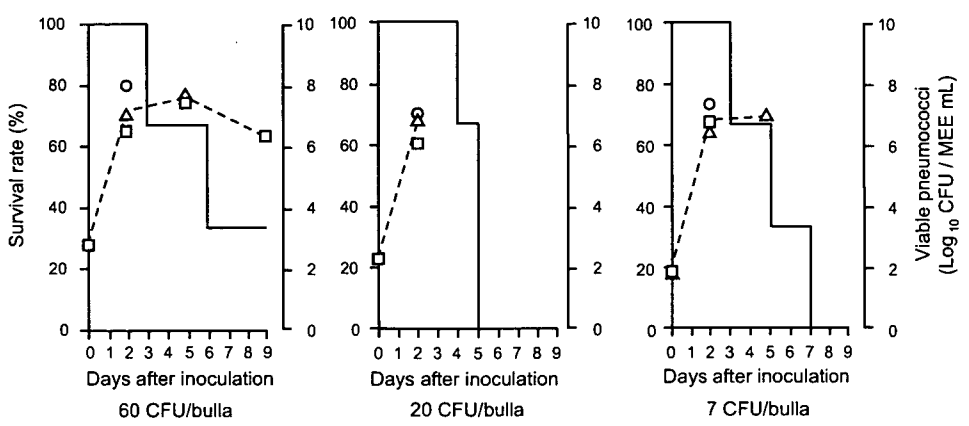

Fig. 2. Development of experimental otitis media with serogroup 6 according to inoculated doses. Solid line: survival rate, dotted line: viable pneumococci in middle ear effusions. Three chinchillas were used per group.

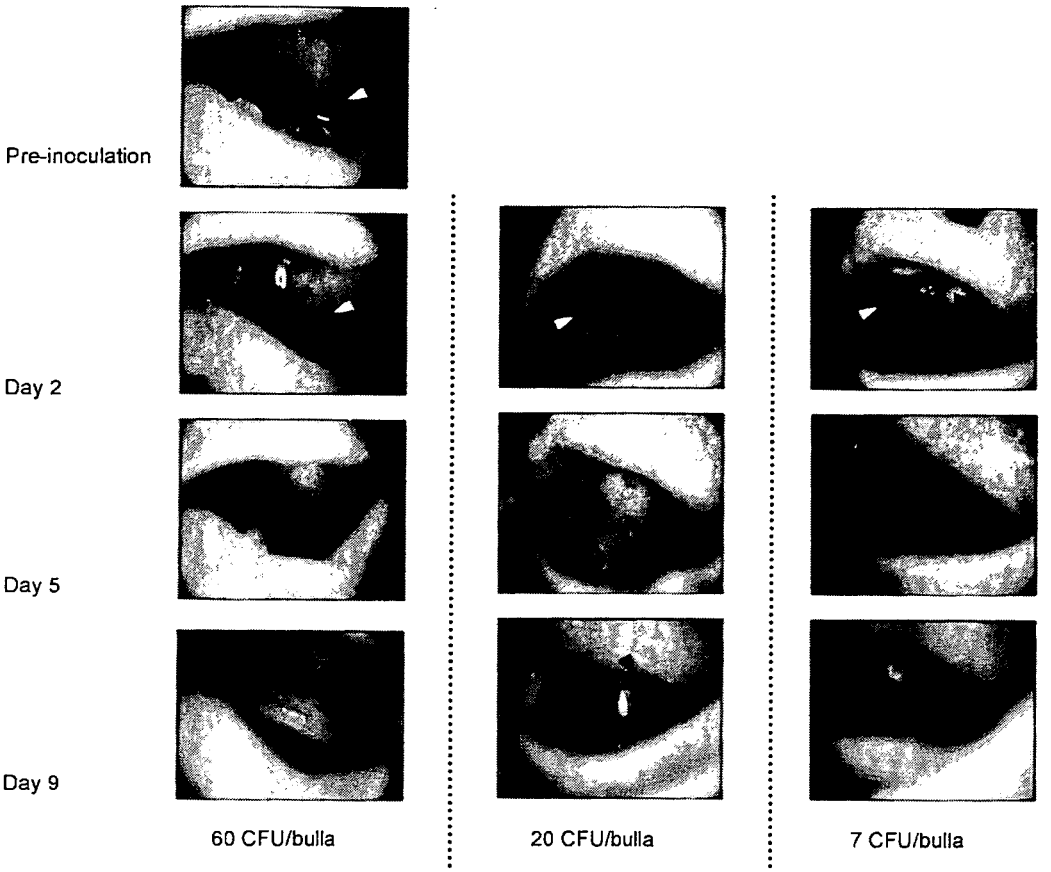

Fig. 3. Tympanic membrane findings of experimental otitis media. Open triangle: erythema of tympanic membrane, closed triangle: middle ear effusions, (*) bulging, arrow: pus.

and AMX were 0.67 h, 0.43 h, 8.2 μg h/ml, 3129 ml/h/kg, and 1.3 h, 2.3 h, 18.8 μg h/ml, 1384 ml/h/kg, respectively (Table 2). The concentrations of TBM in the infected middle ears and non-infected middle ears were 0.74 and 0.49 μg/g, respectively (Fig. 4). The concentrations of AMX in the infected middle ears and non-infected middle ears were 0.39 and 0.14 μg/g, respectively (Fig. 4).

3.3. Efficacy experiment

The outcomes of treatments were evaluated by mortality, bacteria culture, and improvement of otitis media. The mortality rates of chinchillas treated with TBM-PI were 16.7% (2 out of 12 chinchillas). On the other hand, in chinchillas treated with AMX mortality rate was 75% (9

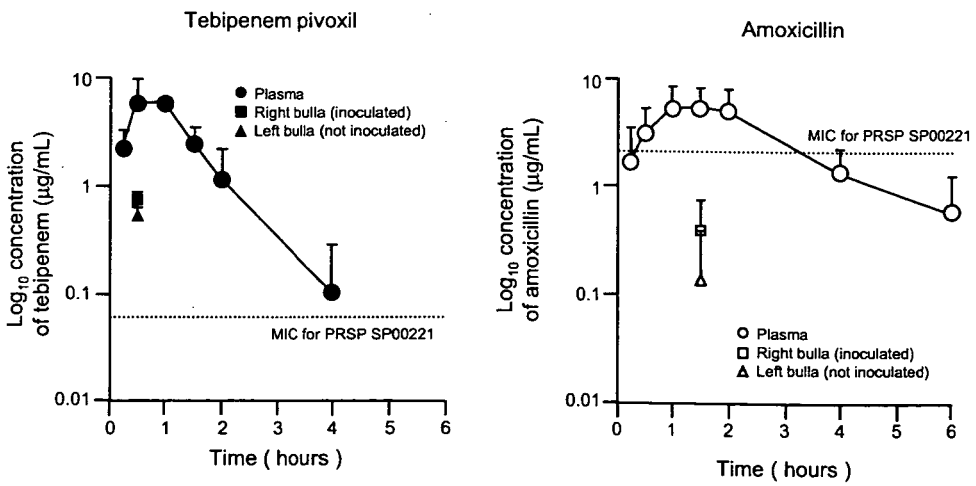

Fig. 4. Pharmacokinetic of TBM-PI and AMX. Twelve chinchillas were used per group.

Table 2
Pharmacokinetic of TBM-PI and AMX

Parameter	TBM	AMX
C_{max} (μg/ml)	7.22 ± 2.40	6.06 ± 3.14
T_{max} (h)	0.67 ± 0.29	1.33 ± 0.29
$t_{1/2}$ (h)	0.43 ± 0.23	2.33 ± 2.75
$AUC_{0-\infty}$ (μg h/ml)	8.22 ± 1.63	18.82 ± 4.31
CL/F (ml/h/kg)	3128.5 ± 685.6	1383.5 ± 362.9

C_{max}: maximum serum concentration, T_{max}: maximum concentration time, $t_{1/2}$: half time, $AUC_{0-\infty}$: area under the curve, CL/F: apparent total body clearance, TBM: tebipenem, AMP: ampicillin.

out of 12 chinchillas). All chinchillas in untreated control group died within day 9 after transbullar infections. There were statistically significant differences in mortality among treatment groups and between treatment with TBM-PI and AMP ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 5).

Quantitative cultures in middle ear effusions at day 2 of TBM-PI yielded $3.84 \pm 2.00 \log_{10}$ CFUs/ml which was significantly compared to AMX yielded $6.75 \pm 0.82 \log_{10}$ CFUs/ml and control yielded $6.60 \pm 0.77 \log_{10}$ CFUs/ml ($p < 0.001$). Quantitative cultures in middle ear effusions at day 5 of TBM-PI yielded $3.53 \pm 2.39 \log_{10}$ CFUs/ml which was significantly compared to AMX yielded $6.04 \pm 1.31 \log_{10}$ CFUs/ml and control yielded $6.88 \pm 0.78 \log_{10}$ CFUs/ml ($p < 0.05$). Quantitative cultures in middle ear effusions at day 9 of TBM-PI yielded $4.66 \pm 2.25 \log_{10}$ CFUs/ml which was not significantly different from AMX that yielded $6.51 \pm 0.33 \log_{10}$ CFUs/ml (Fig. 6.). Four of 12 on day 2, 5 of 10 on day 5, and 3 out of 10 chinchillas treated with TBM-PI did not have detectable *S. pneumoniae* in the middle ear washes.

Clinical cure was defined based on the results of otomicroscopic examinations. Existence of middle ear effusions, redness and bulging of tympanic membrane, purulent ear discharge were evaluated under the otomicroscope. The clinical cure was designated as markedly improved, slightly improved, no improvement, and progressive disease. TBM-PI treatment showed marked improvement of experimental otitis media (Fig. 7).

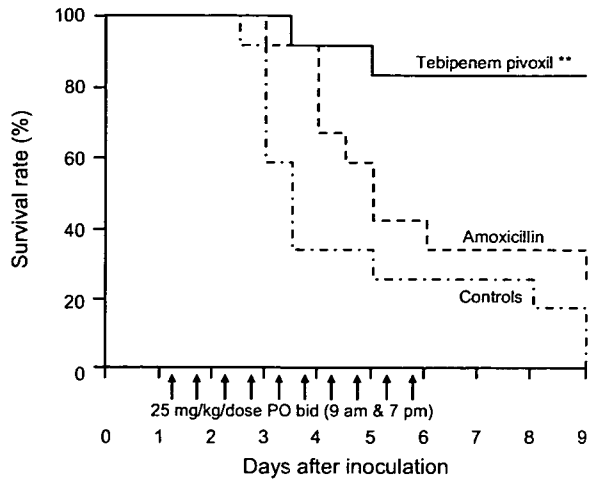

Fig. 5. Clinical course of chinchilla infected with *S. pneumoniae*. Twelve chinchillas were used per group.

4. Discussion

Because of *S. pneumoniae* as the most frequent causative pathogen responsible for AOM, antimicrobial therapies against AOM have been targeted against this microorganism [2–4]. Amoxicillin is recommended as the drug of the first choice for AOM [5–7]. However, there has been an alarming increase in *S. pneumoniae* resistance to antibiotics worldwide [8]. Penicillin resistant *S. pneumoniae* represented a greater proportion of treatment failures in AOM [9,10]. To control the intractable AOM caused by PRSP, a variety of options including increasing the dose of AMX at the initial therapies or including alternative antibiotics for primary treatment failures are suggested in the antimicrobial treatment of this disease [20–22]. The development of experimental animal models of AOM caused by PRSP that mimics the human condition are useful for evaluating the clinical efficacy of various antimicrobial treatment options. The chinchilla models of AOM caused by β-lactamase producing *H. influenzae* have been successfully used to evaluate the efficacy of new

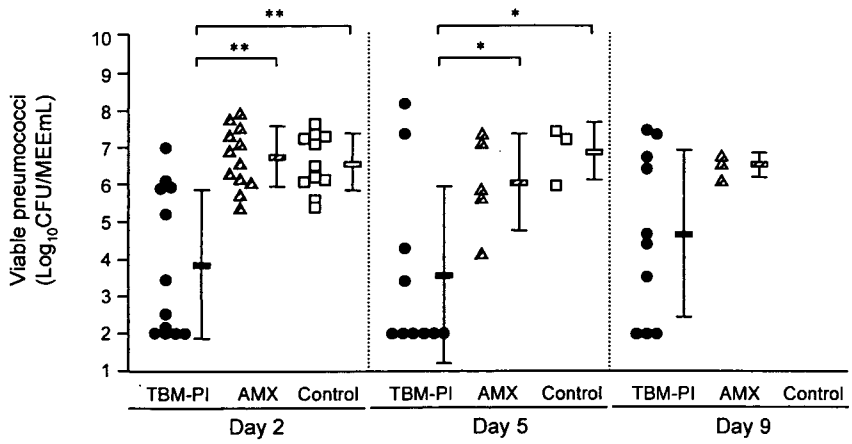

Fig. 6. CFUs of *S. pneumoniae* identified in middle eat fluids. Bar: mean ± S.D., dot: CUFs of *S. pneumoniae* identified in the middle ear. Twelve chinchillas were used per group.

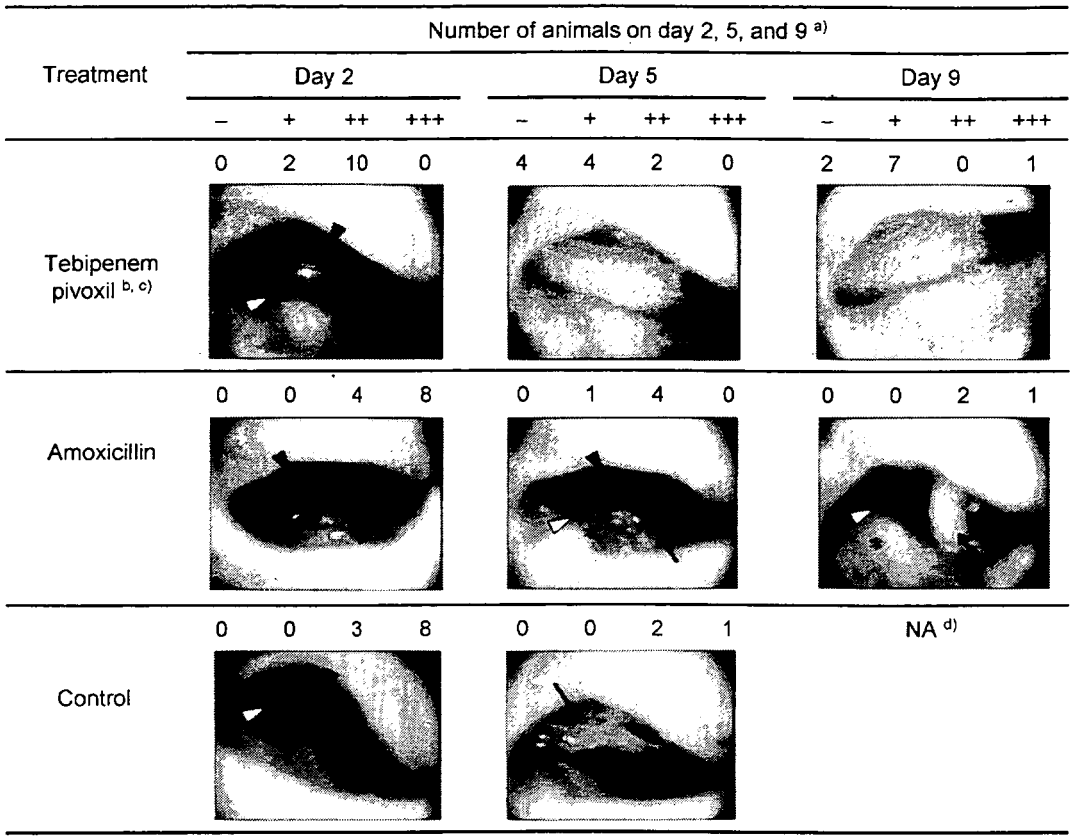

Fig. 7. The changes in tympanic membrane findings after treatments with antibiotics. (a) (–) Marked improvement, (+) slight improvement, (++) no improvement, (+++) progressive disease. (b) $p < 0.001$ compared with other groups on day 2 (Mann–Whitney U-test). (c) $p < 0.05$ compared with other groups on days 5 and 9 (Mann–Whitney U-test). (d) NA: data not available.

antimicrobial agents in the treatment of that disease [23–25]. The models have proven valuable for assessing the potential efficacy of new antimicrobial agents to eradicate middle ear infection due to *S. pneumoniae* [26,27].

In the current study, *S. pneumoniae* challenge with strains of serogroups 6 and 19 resulted in acute infection and development of AOM in the chinchilla. All of the chinchillas had obvious MEEs during the observation periods. These serogroups of *S. pneumoniae* showed varied mortality rates between 20 and 100%. Prior to death the behavioral signs exhibited by the chinchillas included head tilt, gait disturbance, labored breathing and lethargy and the death of the chinchilla had attributed due to disseminate infection causing labrynthitis, meningitis, and sepsis. On the other hands, the strain of *S. pneumoniae* serogroup 14 tested failed to infect in the middle ear and was eliminated from the middle ear cavity 2 days after the inoculation. Alper et al. also suggested that the differences in mortalities of experimental otitis media using chinchillas varied among the different *S. pneumoniae* serogroups [5]. The virulence of *S. pneumoniae* strains are different depending on the capsular serotypes in the chinchilla models. The high rates of mortality secondary to pneumococcal infections of the middle ear suggested the conditions of AOM in chinchillas differed from those of human and represented a limitation of this model when evaluating the pathogenesis of AOM. However, the models clearly showed the benefit when evaluating the efficacies of antimicrobial agents. However, the models clearly showed the benefit when evaluating the efficacies of antimicrobial agents on the experimental otitis media rather than mice and rats. Investigators can monitor the status of middle ear cavity via tympanic membrane and evaluate subsequent numbers of pathogens in the tympanic bulla.

Tebipenem pivoxil is the most recently developed oral carbapenem susceptible to PRSP (MIC = 0.06 μg/ml) and is a promising antimicrobial agent against PRSP [18,19]. Prior to evaluating the efficacy of TBM-PI in treating the AOM caused by PRSP, the pharmacokinetics of TBM-PI was evaluated in the chinchillas with acute middle ear infections in comparison with those of AMX. The single dose of oral administration of TBM-PI at 25 mg/kg to the chinchilla resulted in good serum levels and appreciable drug levels in middle ear mucus. Then, TBM was cleared from sera faster than AMX. Hikida et al. suggested a high correlation between the in vivo efficacy of TBM-PI and the maximum concentration of drug in plasma/MIC or area under the concentration time curve/MIC [19]. The in vivo efficacy data in the current study showed that both TBM-PI and high dose AMX were equally effective in the clinical course of AOM. Treatments with TBM-PI significantly reduced the numbers of *S. pneumoniae* in the middle

ear, while the numbers of *S. pneumoniae* in the middle ear of the chinchilla treated with high dose of AMX increased on day 9 again. These results strongly suggested that TBM-PI is more effective against AOM caused by PRSP.

In summary, the current study showed the chinchilla offers a reasonable animal model for evaluating clinical efficacies of antimicrobial agents against AOM caused by PRSP. The limited disadvantage of this experimental AOM model is the relatively high mortality rate demonstrated secondary to the pneumococcal infections in the middle ear. Without that limitation, this experimental AOM model using the chinchillas is attractive for evaluating the clinical efficacy of antibiotics. Tebipenem pivoxil is a promising antibiotic for the treatment of AOM caused by PRSP.

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Vaccine adverse events reported in post-marketing study of the Kitasato Institute from 1994 to 2004

Tetsuo Nakayama [a,*], Kazumasa Onoda [b]

[a] *Laboratory of Viral Infection I, Kitasato Institutes for Life Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641, Japan*
[b] *Division of Post Marketing Research, Research Center for Biologicals, Kitasato Institutes for Biologicals, Arai 6-111, Kitamoto City 364-0026, Saitama Prefecture, Japan*

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Abstract

General physicians, pediatricians and parents realize that serious adverse events occur with an extremely rare incidence, but have no information on the incidences of vaccine-associated adverse events. A proper understanding of vaccine adverse events would be helpful in promoting an immunization strategy. Causal association can rarely be determined in adverse events through laboratory examinations. We examined the cases reported in the post-marketing surveillance of the Kitasato Institute, categorizing them into two groups: allergic reactions and severe systemic illnesses. Anaphylactic patients with gelatin allergy after immunization with live measles, rubella and mumps monovalent vaccines have been reported since 1993, but the number of reported cases with anaphylaxis dramatically decreased after 1999 when gelatin was removed from all brands of DPT. The incidence of anaphylactic reaction was estimated to be 0.63 per million for Japanese encephalitis virus (JEV) vaccine, 0.95 for DPT and 0.68 for Influenza vaccine, but the causative component has not yet been specified. Among 67.2 million immunization practices, 6 cases with encephalitis or encephalopathy, 7 with acute disseminated encephalomyelitis (ADEM), 10 with Guillain–Barré syndrome and 12 with idiopathic thrombocytopenic purpura (ITP) were reported. The wild-type measles virus genome was detected in a patient with encephalitis and in two of four bone marrow aspirates obtained from ITP after measles vaccination. Enterovirus infection was identified in two patients after mumps vaccination (one each with encephalitis and ADEM), one patient with encephalitis after immunization with JEV vaccine, and one with aseptic meningitis after immunization with influenza vaccine. The total estimated incidence of serious neurological illness after vaccination was 0.1–0.2 per million immunization practices. We found that enterovirus or wild-type measles virus infection was coincidentally associated with vaccination in several cases suspected of being vaccine adverse events.

Keywords: Vaccine adverse events; Anaphylaxis; Gelatin allergy; Enterovirus infection

1. Introduction

The number of patients with vaccine-preventable diseases has decreased since the introduction of effective vaccines, and many kinds of vaccines are used in developed countries [1]. But, in Japan, acellular pertussis vaccine combined with tetanus and diphtheria toxoids (DPT), inactivated Japanese encephalitis vaccine, live polio vaccine, further attenuated measles vaccine and rubella vaccines are recommended for routine immunization. Mumps and varicella vaccines are voluntary and the immunization strategy is therefore stagnant. One of the reasons for this is the issue of vaccine safety, and some parents and physicians worry unnecessarily about vaccine adverse events. This has been an obstacle to increasing vaccine coverage and we should therefore clarify this matter to catch up with the worldwide standards [1].

There is no vaccine that is absolutely safe, and subsequently, increased vaccine usage inevitably results in an increased number of adverse medical events associated with

* Corresponding author. Tel.: +81 3 5791 6269; fax: +81 3 5791 6130.
E-mail address: tetsuo-n@lisci.kitasato-u.ac.jp (T. Nakayama).

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vaccination. A key issue is to achieve a better understanding of the balanced concept of potential risk, vaccine efficacy, and the incidence of vaccine adverse events. The incidence of adverse events was evaluated at the time of field trials before the licensure of vaccine. It covered only mild well-known reactions, including systemic reactions, such as febrile illness, eruption and local reactions. General physicians, pediatricians, and parents realize that serious adverse events occur but have no information on the incidence of vaccine-associated serious adverse events. A proper understanding will depend upon the post-marketing surveillance (PMS) research. Indistinct information and rumors about serious adverse events influence the popularity of vaccines.

The vaccine adverse event reporting system (VAERS) was developed in the US to increase the capacity to evaluate vaccine safety [2–4]. Approximately 6 million members (2% of the US population) participated and became a valuable resource for information on vaccine safety. Causal association between the vaccine and adverse events can rarely be determined through specific examinations, and false assumptions regarding causality are likely to occur without careful investigation of etiology and pathogenesis [5].

In this study, we analyzed the cases reported in the PMS of the Kitasato Institute and they were classified into two categories: allergic reactions and systemic serious illnesses. We performed virological examinations in the patients who had experienced serious adverse events after vaccination. Virus isolation and genomic diagnosis were performed to detect the corresponding virus species. We also performed the examination to detect the enterovirus genome in samples obtained from sterile sites, such as cerebrospinal fluids, and sera in addition to the virological diagnostic procedures related to the vaccine virus species.

2. Patients and methods

2.1. Data collection and definition

In Japan, physicians who experience cases with serious vaccine-associated events after vaccination should report these to the regional government through the regional Public Health Center. The case is then registered by the Ministry of Health, Welfare and Labor, through legitimate channel, as shown in Fig. 1. Clinical samples are obtained and specific diagnostic examinations are principally carried out at the Regional Institutes for Public Health. Also, the case is reported to the respective vaccine manufacture and should be registered through the private manufacturer's channel. The accuracy of the case identification is confirmed through both channels. In this study, clinical samples obtained by physicians were transferred to our laboratory and clinical data was also obtained from the physicians (Fig. 1).

We summarized the data reported in the PMS Research Unit of the Kitasato Institutes for Biologicals from April 1994 to December 2004. Reported cases were classified into two groups: allergic reactions and severe systemic illnesses, as shown in Table 1. In the allergic reactions observed within 48 h after vaccination, we classified them into four categories: anaphylaxis, urticaria, eruption, and local reaction. Details of the clinical case definition and co-relation with the presence of IgE antibodies against gelatin were reported previously [6,7]. Briefly, anaphylaxis is defined as the case where the patient demonstrated urticaria or angioedema with respiratory symptoms, cardiovascular shock, or both. The second group consists of the case with only urticaria as their reactions. The third group consists of those with generalized eruption different from nature of urticaria. The fourth, local reactions are defined as redness and swelling greater than 5 cm in diameter. The systemic serious illnesses reported are also shown in Table 1. Criterion of encephalitis and/or encephalopathy is acute onset of fever with neurological symptoms such as seizure, decreased level of consciousness, and positive findings with electroencephalography (EEG) or computerized tomography (CT). Aseptic meningitis is defined the occurrence of a febrile illness with meningeal irritation (headache, vomiting, or stiff neck) with pleocytosis in the CSF (>15/mm^3). Acute disseminated encephalomyelitis (ADEM) is diagnosed by several neurological signs (decreased level of consciousness, visual disturbance, motor

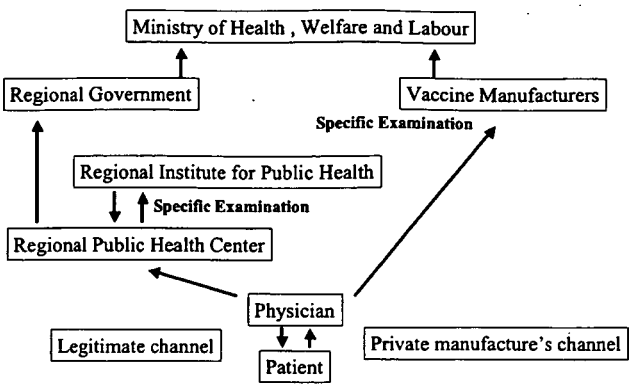

Fig. 1. Reporting system for serious vaccine-associated adverse events after vaccination in Japan.

Table 1
Vaccine adverse events

Allergic reactions
Anaphylaxis
Urticarial eruption
Generalized eruption
Severe local reaction
Severe systemic illness
Encephalitis/encephalopathy
Aseptic meningitis
Acute disseminated encephalomyelitis (ADEM)
Guillain–Barré syndrome
Acute cerebellar ataxia
Acute hemiplegia
Idiopathic thrombocytopenic purpura (ITP)
Allergic purpura

neuron disorders, etc.), and by the presence of demyelinating changes in magnetic resonance imaging (MRI) [8]. Guillain–Barré syndrome is diagnosed by clinical symptoms, such as progressive muscle weakness and loss or decreased level of deep tendon reflex [9]. Idiopathic thrombocytopenic purpura (ITP) is diagnosed by a tendency to bleed along with a decreased number of thrombocyte count (<100,000/mm^3).

We produce three kinds of live attenuated measles, rubella and mumps vaccines, and three kinds of inactivated JEV, DPT and Influenza virus vaccines. Live vaccines are packaged in a single dose but inactivated vaccines before 2003 contain two doses per package. We calculated the shipping doses of vaccine as the estimated number of immunization practices.

2.2. Virus isolation and detection of the viral genome

We obtained clinical samples of nasopharyngeal secretion (NPS), cerebrospinal fluid (CSF), peripheral venous blood mononuclear cells (PBMC) or bone marrow aspirates from the patients suspected of having vaccine adverse events. The samples were transferred within 48 h from individual hospitals. B95a cells for measles virus, Vero cells for mumps virus and RK13 cells for rubella virus were used for virus isolation [10–12].

Total RNA was extracted from 200 μl of clinical samples and genomic RNA was transcribed by AMV reverse transcriptase at 50 C for 1 h using virus-specific primers for measles virus, rubella virus, mumps virus, and enterovirus. The measles virus genome was amplified in the nucleocapsid (N) and hemagglutinin (H) regions by RT-PCR, as previously reported [13]. The phospho (P) protein and hemagglutinin-neuraminidase (HN) protein regions of the mumps virus were amplified [14,15], and NS4 and E1 regions of rubella virus were used for RT-PCR amplification [16]. We designed a nested RT-PCR to detect the 5′ non-coding region of enterovirus genome, which is considered to be a relatively conserved region among different serotypes. We reported the detection of enterovirus genome from sera, CSF and NPS, despite being negative for virus isolation [17,18].

2.3. Differentiation between vaccine and wild strains

The measles AIK-C vaccine strain was differentiated from the wild-type strains using restriction fragment length polymorphism (RFLP) of the PCR products of the H gene, which was digested by *Ava* II, *Bst* P I, and *Hpa* II [19]. The differentiation of mumps Hoshino vaccine strain was performed using RFLP of the HN gene digested by *Alw* 44 I and *Sca* I, as previously reported [15]. As for the rubella Takahashi strain, PCR products were excised from the gel and the nucleotide sequence was determined with the dye terminator method, using ABI 377 A.

3. Results

3.1. Allergic reactions

Among vaccine adverse events, anaphylactic reaction is rare event, but is sometime serious. From 1993, allergic reactions were increasingly reported after vaccination with live measles, mumps and rubella monovalent vaccines, and are summarized in Fig. 2. After 1999, when the trace amount of gelatin was removed from all brands of DPT, the incidence of gelatin allergy decreased, along with the modification of the gelatin material as a stabilizer [20]. No anaphylactic reaction

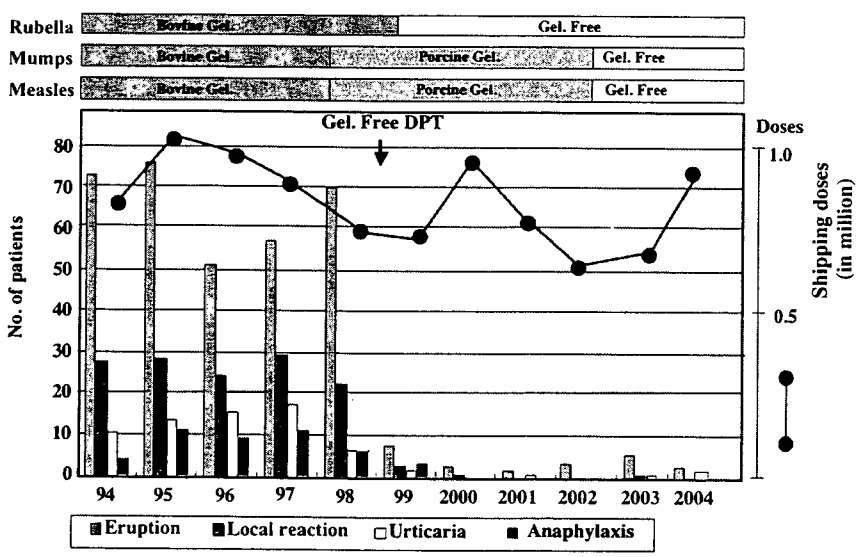

Fig. 2. Annual number of reported cases with allergic reactions, anaphylaxis, urticaria, local reaction and generalized eruption, after vaccination with live measles, mumps and rubella vaccines. The 0.2% bovine gelatin was used in all live vaccines. Gelatin was removed from rubella vaccine in 1999. Porcine gelatin was used in mumps and measles vaccines from 1998 to 2002 and removed from them in 2002. Gelatin was removed from all brands of DTaP in 1999. The dot represents the annual shipping doses of three live vaccines in million.

Table 2
Incidence of allergic reaction after vaccination with inactivated JEV, DPT, and Influenza vaccines, 1994–2004

Allergic reaction	JEV			DPT		Influenza			
	P-M	P-P	G.T. (−)	Gel. (−)	G.T. (−)	P-M	P-P	Gel. (−)	G.T. (−)
Anaphylaxis	2	3	1	9	1	0	4	19	3
Urticaria	3	6	1	6	0	0	2	28	1
Eruption	7	1	1	91	11	5	65	196	9
Local reaction	3	4	1	70	19	4	95	131	14
Shipping (million)	4.78	3.22	1.45	9.68	0.88	0.58	2.12	32.86	2.46
	JEV			DPT		Influenza			
	P-M	P-P	G.T. (−)	Gel. (−)	G.T. (−)	P-M	P-P	Gel. (−)	G.T. (−)
Incidence of anaphylaxis (cases/1 million doses)									
Thimerosal (+)	0.63			0.93		1.48		0.58	
Thimerosal (−)			0.69		1.148				1.22

P-M, porcine gelatin (Merck) 0.02%; Gcl. (−), gelatin free; P-P, porcine gelatin (Prionex) 0.02%; G.T. (−), gelatin, thimerosal free; JEV, Japanese encephalitis virus vaccine.

was reported after vaccination of live vaccines in PMS after 1999 and now, gelatin allergy is settled.

But some anaphylactic reactions were reported after the administration of inactivated vaccines and the results of PMS study for allergic reactions are shown in Table 2. The components of gelatin as a stabilizer and thimerosal as preservatives were modified during the study period of Japanese encephalitis virus vaccine (JEV), DPT and Influenza vaccines. Gelatin has not been used in Kitasato DPT, but the incidence of anaphylaxis is calculated as 0.93 per million immunization practices (nine cases/9.68 million doses). That of DPT free of thimerosal was 1.14 per million doses (one case/0.88 million doses) after 2003. A total incidence of anaphylaxis was 0.95 per million doses of DPT (10 cases/10.56 million doses). We investigated the serum IgE against vaccine components, but we could not identify the causal relationship of the anaphylactic reactions. The incidence of anaphylactic reaction is 0.63 per million for JEV (6 cases/9.45 million doses), and 0.68 for Influenza vaccine (26 cases/38.02 million doses). Most inactivated vaccines have been free of gelatin and thimerosal since 2003, but the incidence of anaphylaxis seemed to be similar, irrespective of the removal of thimerosal.

3.2. Incidence of serious adverse events after vaccination

We summarized the data reported to the PMS Research Unit from April 1994 to December 2004. Table 3 shows the number of reported cases diagnosed as encephalitis and encephalopathy, aseptic meningitis, ADEM, acute cerebellar ataxia, Guillain–Barré syndrome, acute hemiplegia, ITP, and allergic purpura. Shipping doses of measles, rubella, mumps, DPT, JEV, and influenza vaccine are also shown in Table 3.

Among 3.64 million doses of measles vaccine, encephalitis and/or encephalopathy were reported in two patients. One patient with encephalitis had a high fever, measles like eruption, convulsion and loss of consciousness 9 days after vaccination in 2002, and the H1 genotype of the measles virus was detected, which was the circulating genotype in 2002, different from the AIK-C vaccine strain [13]. Among five ITP cases, four bone marrow aspirates were examined and two measles wild-type D5 lineage genomes were detected, circulating strain from 1990 to 1997 [13]. An acute hemiplegia was reported in one case that was diagnosed with congenital intracranial arterial malformation, Moya-moya disease.

Table 3
Number of patients with severe adverse events after vaccination, 1994–2004

	Measles	Rubella	Mumps	DPT	JEV	Influenza
Encephalitis	2[a]	1	1[b]	1	1[b]	0
Aseptic meningitis	0	0	134	0	0	1[b]
ADEM	0	1	1[b]	0	2	3
Acute cerebellar ataxia	0	0	1	0	0	1
Guillain–Barré syndrome	0	1	0	0	0	9
Acute hemiplegia	1	0	0	0	0	0
ITF	5[a]	4	1	0	0	2
Allergic purpura	0	1	1	0	0	0
Toxic shock syndrome	1	0	0	0	0	0
Shipping doses (million)	3.64	4.00	1.53	10.56	9.45	38.02

JEV, Japanese encephalitis virus vaccine.
[a] Wild-type measles.
[b] Enterovirus infection.

One case each of encephalitis, ADEM and Guillain–Barré syndrome and four cases of ITP were reported among 4.00 million doses of rubella vaccine.

Among 1.53 million doses of mumps vaccine, one case each of encephalitis, ADEM, acute cerebellar ataxia, and ITP and 134 cases of aseptic meningitis were reported. Through virological examinations, the enterovirus genome was detected in the CSF despite being negative for the detection of the mumps virus genome in each patient with encephalitis and ADEM. One hundred and thirty-four patients with aseptic meningitis were reported and we obtained 55 CSF samples from the patients with aseptic meningitis. Mumps virus genome was detected in 40 of them and 35 were identified as the vaccine strain and five as the wild-type.

One patient with encephalitis/encephalopathy was reported out of 10.56 million doses of DPT.

One patient with encephalopathy and two with ADEM were reported among 9.45 million doses of JEV vaccine. The patient was diagnosed as encephalitis caused by enterovirus type 71 infection after a virological examination performed by the Regional Institute for Public Health.

One patient with aseptic meningitis, three with ADEM, nine with Guillain–Barré syndrome, and two with ITP were reported among 38.02 million doses of influenza vaccine. The enterovirus genome was detected in the CSF obtained from the patient with aseptic meningitis.

4. Discussion

The reliability of the surveillance of vaccine adverse events depends on how accurately the surveillance system picks up the cases. The limitation of this study is the self-reporting system by physicians or parents. We realized that the number of mild cases might be underestimated, but the cases with serious illness requiring medical treatment in hospitalization would most likely be registered without missing, because the medical costs for vaccine adverse events are completely compensated under the decision in the advisory committees organized in the regional government or the Ministry of Health, Welfare and Labour by the immunization remedy law, and the cases are verified through legitimate and manufacturer's channels.

Gelatin allergy was first reported after MMR vaccination in the USA [21] and we reported that the anaphylactic reaction was caused by gelatin allergy, with a high prevalence of IgE antibodies against gelatin [6,7]. We speculated that the repetitious administration of DPT containing gelatin enhanced the sensitization against gelatin. After 1999, when even trace amounts of gelatin were removed from all brands of DPT, the incidence of gelatin allergy decreased, along with the modification of gelatin material [20]. No anaphylactic reaction has been reported after immunization with live vaccines in PMS since 1999. In the US, trace amounts of hydrolyzed gelatin are used in DPT and there is 14,500 ug of gelatin in live vaccines. The VAERS study group examined 22 subjects with anaphylactic reaction and anti-gelatin IgE was demonstrated in 6(27%). The incidence of anaphylactic reaction was 1.8 per million doses at the time, 1991–1997, and did not increase after the introduction of DTaP containing gelatin [22]. A different incidence of gelatin allergy between Japan and the US was thought to depend on the quality of gelatin used and genetic predisposition [23]. In the case of inactivated vaccines, the incidence of anaphylactic reaction was 0.63 per million for JEV, 0.95 for DPT, and 0.68 for influenza vaccine. Most inactivated vaccines, DPT, JEV, and influenza vaccines have been free of gelatin and thimerosal since 2003, but there was no significant difference in the incidence of anaphylaxis among vaccines with or without gelatin or thimerosal. We could not identify the substances responsible for the allergic reactions, and some patients were reported to have gelatin allergy [24].

Further attenuated vaccines, although extremely less pathogenic, have similar characteristics to their respective wild-types. The wild-type strains have neuro-tropic characteristics by nature [25–27]. Adverse reactions after vaccination were inevitable, and the incidence of encephalitis or encephalopathy was reported as 0.3 cases per 1 million doses of measles vaccine [25]. Measles inclusion body encephalitis (MIBE) and ADEM cases were reported after MMR vaccination, but the causal relationship remains unidentified. One report suggested that the measles virus genome obtained from MIBE after MMR vaccination had a similar sequence to the vaccine strain [28]. Cases of encephalitis after the administration of mumps or rubella vaccines are supposed to be extremely rare events after vaccination.

In a total of 67.2 million immunization practices, 6 cases with encephalitis, 135 aseptic meningitis, 7 ADEM, 10 Guillain–Barré syndrome, 1 hemiplegia, 12 ITP, 2 allergic purpura, and 1 toxic shock syndrome were reported. Two patients with encephalitis were reported after vaccination with measles and the wild-type measles genome was detected from the NPS obtained from one patient. In another case of encephalitis reported after measles immunization, the patient developed febrile reactions with neurological symptoms 3 h after vaccination, and we supposed that it seemed to be a coincidental infection with other pathogens because the events attributed to vaccination occurred between days 6 and 14, at a peak of day 10 after MMR vaccination [29]. MMR vaccine containing the Urabe strain was withdrawn from the market in 1993 because of an unexpectedly high incidence of vaccine-associated aseptic meningitis [30]. The results in this study, using the Hoshino strain, estimated the incidence of aseptic meningitis to be one case in 10,000 vaccine recipients. Among 40 cases where the mumps genome was detected, the vaccine strain was detected in 35, but five were identified as wild strains.

A long-term discussion has been carried out about the causal relationship between vaccines and miscellaneous demyelinating neurological diseases, such as ADEM, Guillain–Barré syndrome, and multiple sclerosis [31,32].

The patho-physiological mechanisms are not fully understood, but possible mechanisms have been proposed; molecular mimicry between vaccine antigen and myelin protein, immunological stimulation by exogenous materials in vaccine components and immunological toxicity of vaccine components. The total estimated incidence of ADEM was 0.10 per million immunization practices and 0.15 per million immunization practices for Guillain–Barré syndrome. Behringwerke reported that the estimated incidence of central nervous system demyelinating illness was 3.5 cases per 10 million within a month of vaccination in 10 years of PMS results [33], similar to our results. The incidence of ITP was 0.18 per million immunization practices.

Enterovirus infection was identified in two patients after mumps vaccination (one each with encephalitis and ADEM), one patient with encephalitis after immunization with JEV vaccine and one with aseptic meningitis after immunization with influenza vaccine. Enterovirus consists of 61 serotypes: coxsackie virus type A (23 serotypes), coxsackie virus type B (6 serotypes), echovirus (28 serotypes), and enetrovirus types 68–71 (4 serotypes) [34]. It causes a variety of illnesses, ranging from minor respiratory illness to generalized serious illness, with a short incubation period. In addition, it causes aseptic meningitis, encephalitis, myositis and acute cardiac diseases. Aseptic meningitis is the most common clinical illness caused by enterovirus infection. In Taiwan, epidemics of enterovirus 71 were associated with a high incidence of aseptic meningitis and severe cases with encephalitis had a relatively high case fatality rate in different geographic areas, ranging from 7.7% to 31% [35]. In the present study, one case with encephalitis was diagnosed with enterovirus 71 infection.

In conclusion, several serious vaccine-adverse events have been reported in the PMS study, although the incidence was extremely low, at less than 0.2 per million doses. The coincidental enterovirus infection was identified in some vaccine-adverse events through specific virological laboratory examinations. Thus, we believe that vaccines are safe and that vaccine-adverse events should be continuously monitored with specific laboratory based surveillance to prevent misunderstandings of the causality.

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Simple differentiation method of mumps Hoshino vaccine strain from wild strains by reverse transcription loop-mediated isothermal amplification (RT-LAMP)☆

Naoko Yoshida[a], Motoko Fujino[a], Yoshinori Ota[b], Tsugunori Notomi[b], Tetsuo Nakayama[a,*]

[a] *Kitasato Institute for Life Sciences, Laboratory of Viral Infection I, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641, Japan*
[b] *Eiken Chemical Co. Ltd., Kita-ku, Tokyo 114-0002, Japan*

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Abstract

Mumps virus is still circulating and annual mumps outbreaks occur with fluctuating magnitudes in Japan. Aseptic meningitis has been reported after vaccination and it would be of importance to determine whether this was related to the vaccination. The objective of this study was to develop a sensitive, specific and rapid diagnostic method for the differentiation of the Hoshino vaccine strain from circulating wild types. We developed a reverse transcription loop-mediated isothermal amplification (RT-LAMP) method of the hemagglutinin neuraminidase (HN) region for the detection of mumps virus genome from clinical samples. The typical ladder pattern disappeared after the LAMP products of the Hoshino vaccine strain were digested with *Sca*I, but those of wild types were not cut by *Sca*I. We obtained 19 cerebro spinal fluids (CSF) from the patients with aseptic meningitis and 17 salivary swab samples from the patients with acute parotitis after mumps vaccination, in which one case was complicated with orchitis. Mumps virus genome was detected in 18 CSF samples and in all NPS by RT-LAMP. The Hoshino vaccine strain was identified in 16 out of 18 CSF RT-LAMP positives and in 11 out of 17 NPS samples and the remaining samples were identified as wild types. RT-LAMP followed by *Sca*I digestion is a sensitive, simple and rapid differential method and useful for laboratory surveillance for vaccine-adverse events.

Keywords: Mumps virus; Vaccine-associated aseptic meningitis; Reverse transcription loop-mediated isothermal amplification (RT-LAMP); Vaccine-associated parotitis; Rapid diagnosis

1. Introduction

Mumps virus is an enveloped, single-stranded negative-sense RNA virus that belongs to a member of the genus Rubulavirus of the family Paramyxoviridae. Mumps virus genome structure is 3′N-P-M-F-SH-HN-L-5′, encoding the following proteins: the nucleocapsid (N), phospho (P), membrane (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN) and large (L) proteins [1]. The mumps virus has two envelope proteins of F and HN, and these two proteins have critical functions [2]. The HN protein binds the cell surface sialic acid receptor and the subsequent conformational change of the HN protein triggers the F–HN protein interaction. Thus, F and HN proteins play an important roll for virus–cell fusion at the initial step of the infection. Mumps virus is still circulating throughout Japan, the UK and Europe and causes an acute communicable disease in childhood [3–5]. Mumps virus is transmitted through oropharyngeal secretions and the primary virus infection site is the upper respiratory epithelial cells. Pri-

☆ Experimental guidelines; the study design was approved by ethical committee of the Kitasato Institute for Life Sciences. Informed consent was obtained from a guardian of the patient.

* Corresponding author. Tel.: +81 3 5791 6269; fax: +81 3 5791 6130.
E-mail address: tetsuo-n@lisci.kitasato-u.ac.jp (T. Nakayama).

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