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Effective and safe immunizations with live-attenuated vaccines for children after living donor liver transplantation

Masayoshi Shinjoh[a,*], Isao Miyairi[a], Ken Hoshino[b], Takao Takahashi[a], Tetsuo Nakayama[c]

[a] Department of Pediatrics, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[b] Department of Pediatric Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[c] Laboratory of Viral Infection, Kitasato Institutes for Life Sciences, 5-9-1 Shirokane Minato-ku, Tokyo 108-8642, Japan

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ABSTRACT

Immunizations using live-attenuated vaccines are not recommended for post-liver transplant children due to its theoretical risks. However, they will encounter vaccine-preventable viral diseases upon returning to real-life situations. We performed a total of 70 immunizations with four individual live-attenuated vaccines to 18 pediatric post-living donor liver transplant (LDLT) recipients who fulfilled a clinical criteria including humoral and cell-mediated immunity. The seroconversion rates at the first dose for measles (strain AIK-C), rubella (strain TO-336), varicella (strain Oka), and mumps (strains Hoshino) were 100% (15/15), 100% (15/15), 82% (9/11), and 82% (9/11), respectively. During observed period (–5 years 11 months), a few cases with waning immunity (antibodies were once produced but the levels fell over time) were seen except after rubella immunization. Clinical diseases after seroconversion or definite serious adverse effects due to immunization were not observed. Immunizations using selected live-attenuated vaccines were safe and effective for post-LDLT children who were not severely immunosuppressed.

1. Introduction

Immunizations using live-attenuated vaccines are often difficult to perform in children undergoing orthotopic liver transplantation (OLT). Before OLT, the opportunity for vaccination is usually limited because children may have unstable courses of disease. Also, the vaccine immunity does not always persist after OLT [1,2], partly because of long-term immunosuppressant treatment. On the other hand, when immunizations are performed after OLT, the seroconversion rate can be lower, and theoretical risks of serious adverse effects and rejection due to live vaccine viruses exist. In practical way, therefore, all family members especially children were requested to be immunized to prevent transmission of vaccine-preventable agents.

However, these children will be exposed to vaccine-preventable diseases upon returning to real-life situations. For example, the patients who do not have antibodies against these viruses may be advised to refrain from attending schools or kindergartens for several weeks during epidemics, especially highly contagious measles and varicella because immunocompromised patients are at high risk of these diseases [3]. Measles epidemics are not rare in Japan and reported to be an exporting country [4]. Also, varicella epidemics are common due to the low vaccination rate of 35% [5], versus the 96.5% coverage among children enrolled in kindergarten in U.S. [6]. Although post-OLT patients may not be at high risk of rubella and mumps [3], they should be protected from these diseases similarly to normal children. Thus, it is reasonable to consider immunization with these live-attenuated vaccines for post-OLT patients who are at minimal risk of having adverse effects.

A few studies examining the seroconversion rates for immunization of measles, rubella, varicella and mumps after OLT have been published [2,7–9]. We achieved safe and effective immunizations using live-attenuated vaccines on 18 patients with post-living donor liver transplantation (LDLT) since 2002, using criteria which include evaluation of their humoral and cell-mediated immunity.

2. Methods

Pediatric post-LDLT recipients who are followed-up in the Pediatric Surgery Clinic are referred to the Pediatric Infectious Disease Clinic at the discretion of the surgeon to be considered for vaccination. The pediatrician reviews the patient's history and determines whether the child meets the criteria shown below for immunization by using a "candidate checklist". An informed consent is required. Generally, blood was collected immediately prior to tak-

* Corresponding author. Tel.: +81 3 3353 1211; fax: +81 3 5379 1978.
E-mail address: m-shinjo@z2.keio.jp (M. Shinjoh).

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ing immunosuppressants in order to measure the serum trough concentration of the drugs.

We investigated the response to each vaccine, duration of seropositivity as well as the rates of primary vaccine failure and waning immunity, clinical disease, booster responses and adverse effects after each immunization.

2.1. Criteria for administering live-attenuated vaccine in patients after LDLT

We established clinical criteria in order to maximize the safety and efficacy of immunizations with live-attenuated vaccines such as measles, rubella varicella and mumps after LDLT, as follows.

(1) Negative or borderline antibody titers to one or more of the following viruses: measles, rubella, varicella or mumps.
(2) More than 2 years after LDLT.
(3) Liver enzyme levels, such as AST, ALT and total bilirubin, are stable, and no signs of graft rejection within the last 6 months.
(4) No use of gamma-globulin or systemic steroids (>0.2 mg/kg/day) within the last 6 months.
(5) Children who were not severely immunosuppressed considering the following clinical laboratory examinations;
   - Lymphocyte counts: >1500/μl for children younger than 6 years old and >1000/μl for children 6 years old and older,
   - CD4 counts: >700/μl for children younger than 6 years old and >500/μl for children 6 years old and older, CD4/8: >approximately 1.0
   - Normal lymphocyte function: lymphocyte proliferation in response to phytohemagglutinin, PHA (Lymphocyte-blast transformation test using PHA): normal or high (normal range; 20,500–56,800 counts per minute)
   - Immunoglobulin G: >500 mg/dl.
(6) Low serum trough concentration of immunosuppressants; Tacrolimus and Cyclosporine are <5 and <100 ng/ml, respectively. (No criteria were established for other immunosuppressants such as mizoribine and mycophenolate mofetil and enrollment was based on an individual basis).
(7) All participants (the parents, surgeon and pediatrician) approve the immunization and have obtained a written informed consent. The parents wish their child to be immunized.

2.2. Measurement of titers

The antibody titers against the four viruses were evaluated in the post-LDLT children prior to immunization. Then titers were measured 1–2 months after each immunization, and twice a year thereafter. Standard laboratory cutoffs were used to determine seropositivity.

The titers are measured using a hemagglutinin inhibition (HI) test, IgG (Enzyme immunoassay, EIA) and/or neutralizing antibody (NT) for measles, HI and/or IgG (EIA) for rubella, and IgG (EIA) for varicella and mumps.

HI test for measles and rubella was considered negative if titers were less than 1:8. NT for measles was considered negative for a titer of less than 1:4. Measles, rubella, varicella and mumps IgG measured by EIA, were reported as positive (EIA index of 2.0 or more), borderline (1.0 to less than 2.0), and negative (less than 1.0).

HI and NT only have "positive" or "negative" titers. However, IgG (EIA) has "positive", "borderline" and "negative" titers. Negative antibody titers for NT even with "positive" or "borderline" IgG (EIA) titer are regarded as "negative" for measles. We used the kits produced by Dade Behring Marburg GmbH for IgG (EIA).

2.3. Immunization and vaccine strains

Children were generally immunized at his or her second visit to the Pediatric Infectious Disease Clinic. Each patient was immunized with one vaccine strain at a time, in order to monitor the adverse effect for each strain, and because MMR (Measles–Mumps–Rubella) vaccine has not been approved in Japan. The vaccine strains that have been used to date were AIK-C for measles, TO-336 for rubella, Oka for varicella, and Torii or Hoshino for mumps. Since the Torii strain for mumps was not able to raise the antibody titers sufficiently, we later replaced the Torii strain with the Hoshino strain. Patients with negative antibody titers and who fulfill the criteria were then immunized with each vaccine subcutaneously (0.5 ml) at an interval greater than 4 weeks or more. Patients who have had a history of severe allergy to food or medications were considered at risk for allergic reactions from vaccine components. These patients received an intradermal skin test using one hundredth of the vaccine dose (0.05 ml of 1:10 diluted vaccine) and were observed for 15 min for skin reactions. Erythematous skin reactions greater than 10 mm or control reactions to 0.05 ml of saline were considered positive and excluded from immunization. Adverse effects were interviewed at the next visit.

2.4. Primary vaccine failure, waning immunity and re-immunizations

Primary vaccine failure means that antibodies were not produced within 2 months (HI < 1:8, NT < 1:4 or IgG (EIA) < 2.0) when first immunized, and waning immunity means that antibodies were initially produced but the levels fell over time (HI < 1:8, NT < 1:4 or IgG (EIA) < 1.0). We simply judged waning immunity by loss of antibodies, not by loss of cellular immunity (even for varicella) as shown in Weinberg et al. [9]. Re-immunizations were performed when one of the following criteria had been met (1) primary vaccine failure, (2) waning immunity, (3) antibodies titers fell down to low positive (NT = 4) or to "borderline" titers, and the family wishes the child to be re-immunized. The maximum dose was 3 times for each strain.

2.5. Booster responses

We defined booster responses as significant elevation of viral titers after >3 months post immunization. Significant elevation for measles and rubella is defined as greater than 4 fold elevation of HI or NT. For varicella and mumps, since the coefficient of variation (CV, standard deviation/average of the data) of IgG (EIA) for varicella and mumps was calculated as 4.5–9.6% in our institute (data not shown), we defined booster responses as elevation of IgG (EIA) points of more than 40% (>4× maximum CV).

3. Results

A total of 70 immunizations were performed on 18 post-LDLT patients who fulfilled the criteria, regardless of their prior immunization status. Clinical profiles of the immunized patients are shown in Table 1. The median age at the time of LDLT and the first vaccine after transplantation was 10-month-old (range 5–135) and 55-month-old (range 34–225), respectively. The median time between the LDLT and the first immunization was 43 months (ranged 27–133). The ratio of females to males was 12 to 6. The underlying liver diseases were biliary atresia in 17 cases and fulminant hepatitis in one case (Patient 3). All but one patient (Patient 2) was immunized during the immunosuppressant treatment. Patient 5 did not receive the full measles vaccine dose at first time because of a positive intradermal skin test to the vaccine.

Table 1
Clinical profiles of the immunized patients after LDLT.

No	Sex	Liver disease	Age at LDLT		Age at first vaccine post-LDLT		Interval between LDLT and first vaccine		Primary immuno-suppressive agent	Immunized live-attenuated vaccine	
			Y	M	Y	M	Y	M		pre-LDLT	post-LDLT
1	Female	BA	1	10	4	2	2	4	Tacrolimus	Me	Me, R, V, Mu
2*	Female	BA	1	1	4	4	3	2	Tacrolimus*	ND	Me, R, Mu
3	Male	Hepatitis	1	5	3	9	2	3	Tacrolimus	ND	Me, R, V, Mu
4	Female	BA	2	7	5	2	2	7	Tacrolimus	Me	R, V, Mu
5**	Male	BA	0	5	2	10	2	5	Tacrolimus	ND	Me, R, V
6	Female	BA	0	8	3	1	2	4	Cyclosporine	ND	Me, R, V
7	Female	BA	0	10	4	5	3	6	Cyclosporine	Me, V	Me, R, V, Mu
8	Female	BA	0	7	4	4	3	8	Cyclosporine	ND	Me, R, V, Mu
9	Male	BA	0	10	5	0	4	1	Tacrolimus	Me, V	Me, Mu
10	Male	BA	11	3	17	10	6	7	Tacrolimus	Me, R, Mu	Me, R, Mu
11	Male	BA	0	6	8	2	7	7	Tacrolimus + Mizoribine	ND	Me, R
12	Female	BA	0	11	4	3	3	4	Cyclosporine	ND	Me, R, V, Mu
13	Male	BA	0	8	3	4	2	8	Cyclosporine	ND	Me, R, V, Mu
14	Female	BA	1	6	5	5	3	11	Cyclosporine	Me, V	Me, R, V, Mu
15	Female	BA	0	8	11	10	11	1	Tacrolimus + Mizoribine	Me, V	R, Mu
16	Female	BA	8	3	18	9	10	6	Tacrolimus	Me, R, V, Mu	Mu
17***	Female	BA	2	4	8	9	6	4	Cyclosporine+ Mizoribine+ PSL	Me, R, V	Me, V
18	Female	BA	0	8	10	4	9	8	Tacrolimus	ND	Me, R

LDLT, living donor liver transplantation; BA, biliary atresia; Me, measles; R, rubella; V, varicella; Mu, mumps; ND, not done.
* No immunosuppressants were taken at the time of immunization.
** Received one hundredth of the measles vaccine dose (0.05 ml of 1:10 diluted vaccine) at first time.
*** Immunosuppressants including low dose (2 mg/day) of PSL is used but repeated immunity tests revealed normal.

Table 2
Seroconversion rates for each vaccine.

Vaccine	Strain	*N* total vaccinees	% Seroconverted at first time/total vaccinees	% Seroconverted/total dose*
Measles	AIK-C	15	100 (15/15)	100 (19/19)
Rubella	TO-336	15	100 (15/15)	100 (15/15)
Varicella	Oka	11	82 (9/11)	87 (13/15)
Mumps	Torii	3	0 (0/3)	43 (3/7)
Mumps	Hoshino	11	82 (9/11)	86 (12/14)

* Some patients received two or more doses except for rubella as shown in Table 3 and Fig. 1.

The cell-mediated immunity was checked twice for some of the patients when the serum concentration of the Cyclosporine-A was low (trough) or high (several hours after intake). Patient 8 had test results indicative of normal cell-mediated immunity regardless of whether serum concentration of the Cyclosporine-A was as low as 38 ng/ml (trough) or as high as 241 ng/ml; lymphocyte counts were 2030 and 1880/μl, CD4 positive cells were 669 and 658/μl, and lymphocyte proliferation in response to PHA was 53,685 and 63,000 counts, respectively. Similarly, for Patient 17, lymphocyte counts were 2425 and 1890/μl, CD4 positive cells were 1091 and 832/μl, and lymphocyte proliferation in response to PHA was 60,800 and 44,600 counts, when the serum concentration of Cyclosporine-A was 93 and 149 ng/ml, respectively.

The overall seroconversion rates for each vaccine are shown in Table 2. The rates for measles (strain AIK-C) and rubella (strain TO-336) were 100%, varicella (strain Oka), and mumps (strains Hoshino) were greater than 80%. Since the seroconversion rate for mumps (strain Torii) was rather low, we then used the Hoshino strain.

The occurrences of primary vaccine failure and waning immunity are shown in Table 3, and the duration of seropositivity for each patient is shown in Fig. 1. Primary failure did not occur for measles and rubella. Waning immunity did not occur for rubella. Waning immunity for measles, varicella and mumps occurred 9–38 months after immunization (Fig. 1).

Patient 5, who had received only one hundredth of the measles vaccine dose had persistent titers for more than 2.5 years but was re-immunized since the level of the NT titer fell down to low positive and the family wished to be re-immunized (Fig. 1).

Clinical diseases after seroconversion were not observed. One patient (Patient 5) who did not seroconvert (0.0 to 0.0 for IgG (EIA)) after varicella immunization, developed varicella 18 months later, but only 10 vesicles were observed during 5 day course of oral acyclovir treatment and he required no admission. The IgG titer increased from 0.0 to 54.0 by IgG (EIA).

A booster response to a particular virus was observed 16 times (Table 4); once for measles in Patient 3, twice for rubella in Patients 3 and 10, 6 times for varicella in Patients 1, 3 and 6, 7 times for mumps in Patients 4, 7, 9, 10 and 13. For example, Patient 6 experienced 3 varicella epidemics at kindergarten after immunization and had elevation of varicella IgG (EIA) titer each time without any clinical symptoms.

No serious adverse effects or rejections were seen due to immunization. In addition, none of the immunized patients experienced re-transplantation. One patient (Patient 9) had transient parotid swelling 3 weeks after mumps immunization. Patient 12 was admitted for several days because of a fever without a focus 2 weeks

Table 3
Occurrence of primary vaccine failure and waning immunity.

Vaccine	Strain	Total vaccinees	Primary vaccine failure	Waning immunity
Measles	AIK-C	15	0	2
Rubella	TO-336	15	0	0
Varicella	Oka	11	2	2
Mumps	Torii	3	4*	2
Mumps	Hoshino	11	2	2

* Patient 3 experienced primary vaccine failure twice.

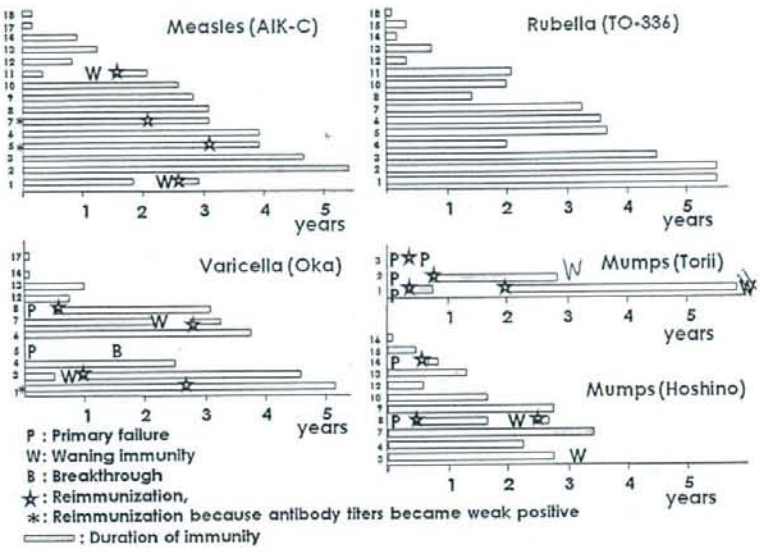

Fig. 1. Duration of immunity after immunization for measles, rubella, varicella and mumps.

Table 4
Occurrence of booster responses.

Vaccine	Strain	Patient number	Methods	Antibody titers (years and/or months after immunization)		Comments
				Before booster response	After booster response	
Measles	AIK-C	3	NT	<4 (4 years 3 months)	8 (4 years 9 months)	NT was 8 at 3 years 1 month after immunization
Rubella	TO-336	3	HI	256 (3 years 6 months)	1024 (3 years 11 months)	
		10	HI	64 (5 months)	256 (10 months)	HI was 256 at 1 month after immunization
Varicella	Oka	1	IgG (EIA)	1.2 (6 months)	5.2 (7 month)	IgG (EIA) was 20.0 at 1 month after immunization
		3	IgG (EIA)	6.7 (1 year 8 months)	11.0 (2 years 1 month)	
		3	IgG (EIA)	3.0 (3 years 3 months)	4.7 (3 years 9 months)	
		6	IgG (EIA)	1.3 (5 months)	10 (7 months)	IgG (EIA) was 3.6 at 2 months after immunization
		6	IgG (EIA)	1.9 (1 year 7 months)	9.3 (1 year 9 months)	
		6	IgG (EIA)	1.6 (2 years 7 months)	3.4 (2 years 11 months)	
Mumps	Hoshino	4	IgG (EIA)	9.2 (5 months)	69.0 (8 months)	
		7	IgG (EIA)	1.7 (4 months)	2.9 (5 months)	IgG (EIA) was 4.6 at 1 month after immunization
		7	IgG (EIA)	1.7 (6 months)	3.5 (9 months)	
		7	IgG (EIA)	2.4 (1 year 9 months)	4.2 (2 years 4 months)	
		9	IgG (EIA)	2.7 (1 year 2 months)	5.0 (1 year 10 months)	
		10	IgG (EIA)	6.3 (6 months)	36.0 (8 months)	IgG (EIA) was 9.4 at 2 months after immunization
		13	IgG (EIA)	28.0 (4 months)	54.0 (7 months)	

after measles immunization. The etiology of the fever was unclear but the patient defervesed (diagnosis for the patient was not made because no cultures were collected before antibiotic use by the attending doctor). The patient could continue other immunizations without any adverse effects thereafter.

4. Discussion

In our study, high seroconversion rates after immunizations in selected post-LDLT patents were observed for the measles, rubella, varicella and Hoshino strain of mumps. Previous reports have demonstrated various rates of seroconversion [2,7–9]. First, Rand et al. [7] immunized 18 children, aged 6–26-month-old at OLT and 15–73-month-old at immunization with measles vaccine between 1.5 and 65 months after OLT. The seroconversion rate was 41% (7/17), but only 29% (4/14) remained seropositive 6 months after immunization. Second, Kano et al. [2] immunized 13 children, aged 9 months to 17 years old at OLT, with four live-attenuated vaccines more than a year after OLT. The seroconversion rates were 85% (11/13) for measles, 100% (2/2) for rubella, 71% (5/7) for varicella, and 100% (6/6) for mumps. Third, Khan et al. [8] immunized 42 children, aged 0–73-month-old at OLT and 12–218-month-old at immunization with MMR or varicella vaccine 4–201 months after OLT. The seroconversion rates were 73% (19/26) for measles, 64.5% (20/31) for varicella. Fourth, Weinberg et al. [9] immunized 15 children, aged 13–76 months old at immunization with varicella vaccine more than 257–2045 days after OLT. The seroconversion rate was 87% (13/15). In our study, a larger number of children were analyzed for rubella and mumps vaccine than in these reports. The seroconversion rates, especially for measles, in our study were also generally higher than previous reports. This may be because of the more stringent criteria we established which included the immunological studies of the recipient. However, waning immunity was observed in all vaccine types except for rubella TO-336 strain during the observed period. Therefore, periodical measurement of each antibody is required. The seroconversion rates were

similar to those for healthy population except mumps Torii. It is not clear why the seroconversion rate for the Torii strain of mumps was particularly low, since the expected rate of seroconversion for recipients of the Torii strain had been reported to be greater than 90% for the healthy population [10].

The 16 episodes of booster responses in the absence of any clinical symptoms were likely due to subclinical infections. Subclinical infections after seroconversion are expected to lead to sustained protective immunity. Children who had seroconverted had no clinical disease and often had booster responses demonstrating the effectiveness of the vaccines. Although seroconversion had not been seen after varicella immunization in Patient 5, it is likely that he had partial immunity (including cellular immunity) given the mild clinical presentation of varicella, which occurred 1.5 years after immunization.

No definite adverse effects were seen in our study. The relationship between the measles immunization in Patient 12 and the fever was unclear. It is common to experience transient fever 7–10 days post immunization in healthy children, although infection secondary to transient immunosuppression by the measles vaccine might have occurred. Transient parotid swelling after mumps immunization have been seen in general population at the rate of 3.5% for this strain [11], thus this phenomena seen in one patient (Patient 9) in our study may not be due to immunosuppression. Rand et al. [7] reported no serious adverse effects had been seen except for one episode of acute rejection that had occurred 3 weeks after immunization in a patient who had OLT. However, the authors stated that the rejection and immunization were not related because the patients did not show any symptoms of measles. Weinberg et al. [9] reported that four children developed fever and four developed rashes at non-injection site (three were treated with oral acyclovir) as adverse events but all were transient. Also, Kano et al. [2] and Khan et al. [8] reported that no serious adverse effects or rejections had been seen. Therefore, immunization with live-attenuated vaccine appears to be safe among the selected post-OLT patients.

Although the trough serum concentrations of the immunosuppressants in our study were low, there may be concerns that cell-mediated immunity may be suppressed at the time of peak serum concentrations. However, we have confirmed that cell-mediated immunity (lymphocyte counts, CD4 counts, and lymphocyte proliferation in response to PHA) was within the criteria even at the high concentration of Cyclosporine in Patients 8 and 17. Furthermore, these patients did not have clinical history suggestive of immunodeficiency.

In our study, we included the humoral and cell-mediated immune status in the criteria for post-transplant immunization. Past studies do not necessarily support the need for immunological studies from a safety standpoint [2,7–9]. However, our study suggests that documenting immunological status within the criteria may account for a better seroconversion rate. Children who do not meet our criteria and have compromised immunity are at high risk for viral infection where management strategies are limited. The appropriateness of live vaccinations in this population needs to be made on an individual basis by weighing the risk of each vaccine with the risk of acquiring the infection in the community.

To sustain good antibody titers against these pathogens, special attention must be paid to the selection of the strain, timing and the number of immunizations. Also, more clinical data should be collected to evaluate the effectiveness and safety of immunizations with live vaccines for children after OLT.

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Histidine at position 1042 of the p150 region of a KRT live attenuated rubella vaccine strain is responsible for the temperature sensitivity

Masafumi Sakata[a], Katsuhiro Komase[b], Tetsuo Nakayama[a,*]

[a] Laboratory of Viral Infection I, Kitasato Institute for Life Sciences, 5-9-1 Shirokane, Minato-ku, 108-8641 Tokyo, Japan
[b] Department of Virology III, National Institute of Infectious Diseases, Tokyo, Japan

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ABSTRACT

The Japanese live attenuated KRT rubella vaccine strain has a temperature sensitivity (*ts*) phenotype. The objective of this study is to identify the region responsible for this phenotype. Genomic sequences of the KRT strain and the wild-type strain (RVi/Matsue.JPN/68) with the non-*ts* phenotype were investigated and reverse genetic systems (RG) for these strains were developed. The *ts* phenotype of KRT varied drastically on replacement of the p150 gene (encoding a methyltransferase and a nonstructural protease). Analysis of four chimeric viruses showed the region responsible for the *ts* phenotype to be located between Bsm I and Nhe I sites (genome position 2803–3243). There were two amino acid differences at positions 1007 and 1042. Mutations were introduced into the KRT cDNA clone, designated G1007D, H1042Y and G1007D-H1042Y. H1042Y and G1007D-H1042Y grew well at a restrictive temperature with a 100-fold higher titer than G1007D and the KRT strain, but a 10-fold lower titer than RVi/Matsue.JPN/68. Since the growth of H1042Y was not completely the same as that of the wild-type strain at the restrictive temperature, we also assessed whether other genomic regions have an additive effect with H1042Y on the *ts* phenotype. H1042Y-RViM SP having structural proteins of RVi/Matsue.JPN/68 grew better than H1042Y, similar to RVi/Matsue.JPN/68. Thus, we concluded that one mutation, of the histidine at position 1042 of p150, was essential for the *ts* phenotype of the KRT strain, and structural proteins of KRT had an additive effect with H1042Y on the *ts* phenotype.

1. Introduction

Rubella virus (RV) is the sole member of the genus *Rubivirus* in the family *Togaviridae*. RV is an enveloped, single-stranded, positive-sense RNA virus with an approximately 10-kb genome, having a cap structure at the 5′ end of the genome and polyA tail at the 3′ end. The genome contains three untranslated regions (UTRs) and two open reading frames (ORFs). One UTR is located at the 5′ end, one at the 3′ end, and one between the ORFs. The UTR between the two ORFs is the junction-UTR (J-UTR). The 5′ end ORF encodes two nonstructural proteins (NSPs) named p150 and p90, while the 3′ end ORF encodes three structural proteins (SPs); capsid protein and the two envelope proteins, E1 and E2. NSPs are translated from genomic RNA and act as a viral genome replication complex. The full-length negative-sense RNA (cRNA) is replicated from genomic RNA. cRNA acts as a template for replicating progeny viral RNA and sub-genomic RNA (SG RNA). Three SPs, capsid, E2, and E1, are translated from SG RNA to form virion structures. Nucleocapsids are comprised of the genomic RNA and capsid protein, and constitute progeny virions surrounded by a lipid viral membrane embedded with E1 and E2 [1,2].

RV infection is one of many transmissible diseases in infants and children. Most patients with RV demonstrate mild symptoms, maculopapular rash, lymphadenopathy, low-grade fever, conjunctivitis, sore throat and arthralgia, and recover in several days without any complications or sequelae. However, infection in unimmunized women during the early stages of pregnancy, especially within the first trimester, cause fetal death or congenital rubella syndrome (CRS). CRS is characterized by multiple malformations: deafness, cataracts, cardiac disease and neurological abnormalities [3–5]. For the prevention of CRS, live attenuated vaccines have been used in vaccination programs in many countries. Recently, in the United States and several European countries, the indigenous circulation of rubella virus has been disrupted and CRS has been eliminated [3]. The prevention of CRS and rubella epidemics mainly depends on efficacious vaccination programs. In Japan, a nationwide outbreak of rubella has not occurred since 1993, but some cases of CRS in sporadic regional outbreaks have been reported. Rubella infection still remains an important issue in Japan and the accelerated control of RV infection is anticipated for the elimination of CRS.

* Corresponding author.
E-mail address: [illegible] (T. Nakayama).

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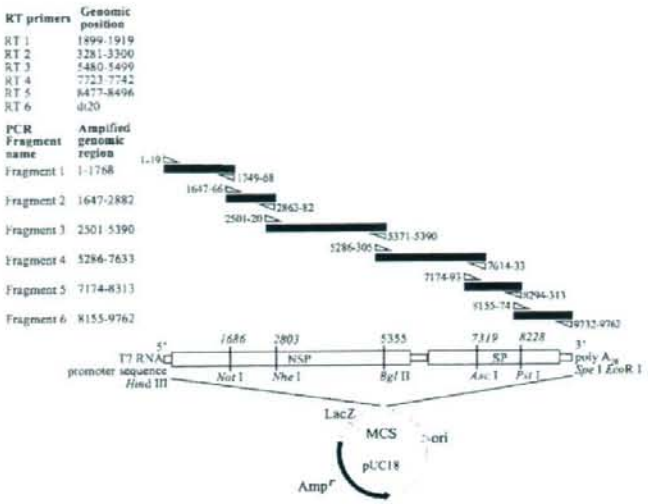

Fig. 1. Construction of the infectious cDNA clones from KRT and RVi/Matsue.JPN/68. Gray arrows indicate the RT primers and direction of cDNA synthesis, and numbers are genomic positions of RT primers. The six RT products were amplified by PCR with primer sets. Open arrowheads indicate the positions of primers. Six PCR fragments were cloned at Eco RI and Hind III in the MCS of pUC18. The full-length cDNA clones were constructed by combining each cDNA clone, using the restriction enzyme sites described in the panel.

Four live attenuated rubella vaccine strains have been used in Japan. The attenuation process differed for each strain with serial passages of the wild-type rubella viruses in different primary cells and cell lines at 35 °C or less [6]. Ohtawara et al. [7] also reported that all Japanese rubella vaccine strains exhibit unique characteristics of temperature sensitivity (*ts*) in cultured cells. Although the wild-type strains show approximately 10-fold lower infective titers at a restrictive temperature of 39 °C than at a permissive temperature of 35 or 37 °C, the vaccine strains with *ts* phenotype demonstrate growth at 39 °C that is 1/1000 that at 35 or 37 °C.

In this study, we determined the complete genomic sequences of both the KRT live attenuated rubella vaccine and the wild-type RVi/Matsue.JPN/68 strain circulating at the same time and in the same region as the progenitor wild-type of KRT. Reverse genetic systems (RG) for the two strains were developed [8–13], and a series of recombinant chimeric viruses and point-mutated viruses were generated from KRT and RVi/Matsue.JPN/68 and point-mutated viruses were generated to investigate the region responsible for the *ts* phenotype. Through infection experiments in cultured cells, the p150 gene, especially the histidine at position 1042, was determined to be responsible for the *ts* phenotype of the KRT strain. This is the first report to identify the region responsible for the *ts* phenotype of a live attenuated rubella vaccine at the molecular level.

2. Materials and methods

2.1. Cells and viruses

Vero and RK13 cells were maintained in Eagle's minimum essential medium (MEM) (Sigma–Aldrich, MO, USA) supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). RVs of the KRT and RVi/Matsue.JPN/68 strains propagated in RK13 and Vero cells were used in this study. The KRT vaccine strain was supplied by the Kitasato Institute Research Center Biologicals and the wild-type RVi/Matsue.JPN/68 strain was kindly provided by Y. Umino, NIID Japan.

2.2. Preparation of viral RNA, amplification of viral cDNA and nucleotide sequencing

Monolayers of RK13 cells grown in 6-well plates were infected with RVi/Matsue.JPN/68, and the culture medium was harvested on day 5 post-infection. Viral RNA was extracted from the culture medium of RVi/Matsue.JPN/68 and bulk material of the KRT vaccine with MagExtractor -Viral RNA- (TOYOBO, Osaka, Japan) following the instruction manual, and used for first-strand cDNA synthesis. The primers used for reverse transcription (RT) and the polymerase chain reaction (PCR) are shown in Fig. 1. First-strand cDNA synthesis was carried out at 50 °C for 1 h in a 10-μl reaction mixture containing viral RNA, 5 pmol of reverse primer based on the RV genome or poly deoxythymidine (poly dT) primer, 100 U of SuperScript III reverse transcriptase (Invitrogen, CA), 40 U of RNasin Plus Ribonuclease Inhibitor (Promega, Madison, WI) 5.0 mM DTT, 125 μM dNTP Mix (125 μM each of dATP, dGTP, dCTP and dTTP), 50 mM Tris–HCl, 75 mM KCl, and 300 μM $MgCl_2$. First-strand cDNA, PCR, and sequencing primers were designed by referring to the sequence reports of TO-336 wt (Genbank accession number AB047330), TO-336vac (AB047329) and RA27/3 vaccine (L78917). For sequencing the 5′ and 3′ end of the genome, 5′ and 3′-Full RACE core kits (TakaRa, Shiga, Japan) were used with 5′-TCACTGACCTGCATCT-3′ (genome position [gp] 219–234) and poly dT. The first-strand cDNA was amplified by nested or semi-nested PCR in six overlapping fragments. The amplification was performed in a 50-μl reaction mixture, containing cDNA, 25 pmol of the primer set, 5% dimethyl sulfoxide (DMSO) and *TaKaRa La Taq* polymerase (TaKaRa) with the PCR mixture provided by the manufacturer. The reaction was carried out under thermal cycling conditions for 3 min at 95 °C followed by 30 cycles of 20 s at 95 °C, 30 s at 62 °C, and 90 s at 68 °C with some modifications. PCR products were excised and used for sequencing with a DYEnamic ET Terminator Cycle sequencing Kit (GE Healthcare Bio-Science, NJ), and analyzed with a 377 XL DNA sequencer (Applied Biosystems, CA). M13RV and M13m4 sequencing primers were used to determine the nucleotide sequence of the

Table 1
Comparison of nucleotide (nt) and amino acid (a.a.) sequences between KRT (vac) and RVi/Matsue.JPN/68 (wt).

	5′UTR[a]	NSP[b]		J-UTR[a]	SP[c]			3′UTR[a]
		p150	p90		C	E2	E1	
nt	40	3903	2442	123	900	846	1443	62
vac/wt[d] (%)	2 (5.0)	69 (1.77)	65 (2.66)	5 (4.17)	27 (3.0)	27 (3.19)	36 (2.49)	1 (1.61)
a.a.		1301	814		300	282	481	
vac/wt[d]		15	1		4	8	5	
(%)		(1.15)	(0.12)		(1.33)	(2.84)	(1.04)	

[a] UTR was an untranslated region.
[b] NSP was a nonstructrual precursor polyprotein encoding two nonstructural proteins, p150 and p90.
[c] SP was a structural precursor polyprotein encoding three proteins, capsid, E2, and E1.
[d] The number of nucleotides or amino acid residues differed between the KRT vaccine (vac) and wild RVi/Matsue.JPN/68 (wt) strains.

5′ and 3′ ends of the genome cloned into the multi-cloning site (MCS) of pUC18 that were constructed with the 5′ and 3′-Full RACE core kits.

2.3. Construction of infectious cDNA clones of KRT and RVi/Matsue.JPN/68

Based on the entire genome sequence of the KRT and RVi/Matsue.JPN/68 strains, viral cDNA was synthesized with six primers and cDNAs were amplified with KOD plus DNA polymerase (TOYOBO). The amplified regions and primer sets are summarized in Fig. 1. For cloning of the cDNAs into the MCS of pUC18, Hind III (*italic: aagctt*) and Eco RI (*italic: gaattc*) restriction enzyme sequences were linked to the 5′ end of the forward and reverse primers, respectively. These fragments were digested with Hind III and Eco RI and cloned into the MCS of pUC18. All fragments inserted into the plasmid were confirmed by using appropriate restriction enzymes and sequenced. Individual clones were digested with restriction enzymes as shown in Fig. 1 and ligated with each other to construct the full-length cDNA clones of KRT and RVi/Matsue.JPN/68. The T7 RNA promoter sequence was introduced at the 5′ end and a polyA tail at the 3′ end of the full-length cDNA, and sub-cloning of cDNAs covering the 5′ and 3′ ends of the viral genome was carried out. The T7 RNA promoter sequence (double underline) was introduced after amplification, using as a forward primer 5′-tgt*caagctt*taatacgactcactataggCAATGGGAGCTATCGGACC-3′ for KRT and 5′-tgt*caagctt*taatacgactcactataggCAATGGAAGCTATCGGACC-3′ for RVi/Matsue.JPN/68 (gp 1–19) with the reverse primer 5′-agtc*gaattc*ACTCGGCAGCAGACCAGCCG-3′ (gp 2863–2882). A polyA tail was also introduced after the amplification, using as a forward primer, 5′-agta*agctt*GTCTCTTGATCACGCCCTCG-3′ (gp 8110–8129) and reverse primer, 5′-atta*gaattcactagt*T_{19}CTATGCAGCAACAG-3′ (gp 9649–9762), which includes restriction sites for Eco RI and Spe I (*italic: actagt*) downstream of the poly dT_{19} tract [11]. Modified 5′ and 3′ ends were confirmed by sequencing and reintroduced into the full-length cDNA with appropriate restriction enzymes. The infectious cDNA clones constructed from KRT and RVi/Matsue.JPN/68 were named pKRT and pRViM, respectively.

2.4. Construction of cDNA clones of recombinant chimeric viruses and point mutation viruses

Recombinant cDNA clones based on pKRT and pRViM were generated by using appropriate restriction enzymes. Along with the genome structure of RV, we generated eight recombinant infectious cDNA clones designated pKRT-rec1–8. The recombinant construct pKRT-rec1 was generated by replacing the region from Hind III to Nco I (gp 5′ end to 39) containing the 5′ UTR sequence, respectively. The recombinant rec2 and rec3 constructs which cover the entire ORF of the NSP genes (p150 and p90) were generated by replacing the region between the two Nco I sites (gp 39–4023) containing most of the p150 gene and the region from Bsm I to Not I (gp 3243–6623) containing most of the p90 gene, respectively. The recombinant rec4 construct was generated by replacing the region between the two PspX I sites (gp 6338–6557) containing the J-UTR sequence. As for the ORF of the SP genes, the recombinant rec5, rec6, and rec7 constructs were generated by replacing the region from Xmn I to Asc I (gp 6514–7319), Asc I to Pst I (gp 7319–8232), and Pst I to AsiS I (gp 8232–9457), for C, E2, and E1, respectively. The recombinant rec8 construct was generated by replacing the region from AsiS I to Eco RI (gp 9457–3′ end) containing the 3′ UTR sequence. To construct the recombinant cDNA clones in the p150 region, we generated the four recombinant constructs pKRT-p150 rec1, 2, 3, and 4 by using the restriction enzyme sites Mfe I (gp 126),

Table 2
Differences in amino acid residues of KRT and RVi/Matsue.JPN/68.

Coding region	Amino acid position	Strain	
		KRT	RVi/Matsue.JPN/68
p150	295	A	T
	407	S	G
	466	L	F
	483	A	T
	674	V	I
	717	L	S
	739	P	H
	740	S	L
	751	V	A
	777	E	G
	790	V	A
	795	G	D
	961	V	A
	1007	G	D
	1042	H	Y
p90	195	I	T
Capsid	11	G	A
	34	P	S
	72	K	R
	226	H	T
E2	6	V	A
	7	H	D
	14	P	L
	104	S	P
	105	L	F
	122	S	A
	234	P	S
	235	P	S
E1	5	A	T
	177	D	N
	203	M	L
	333	T	A
	398	R	A

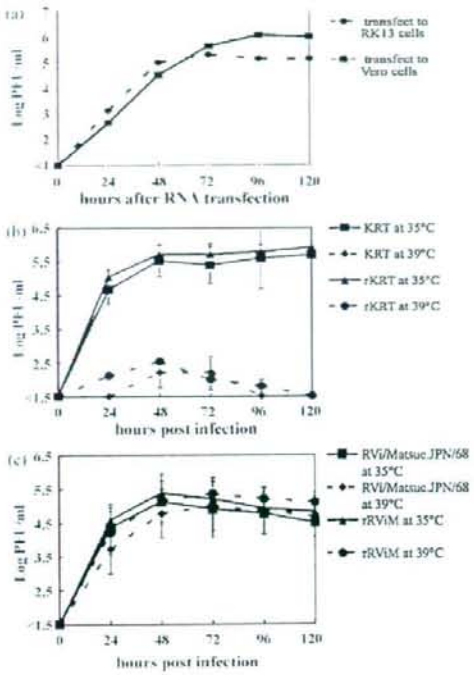

Fig. 2. Recovery of the clone virus from an infectious cDNA clone of pKRT and the growth properties of the clone and original virus. (a) Recovery of infectious clone. Vero and RK13 cells were transfected with viral genomic RNA synthesized from pKRT. The culture medium was harvested and the infective titer was determined by the plaque assay. The average titer for two independent experiments is shown. (b) Growth kinetics of KRT and rKRT at 35 and 39 °C. RK13 cells were infected at a MOI of 0.01. The culture medium was harvested and the infective titer was measured by the plaque assay. The results show the average for three independent experiments and the error bar indicates ±standard deviation (SD). (c) Growth kinetics of RVi/Matsue.JPN/68 and rRViM at 35 and 39 °C.

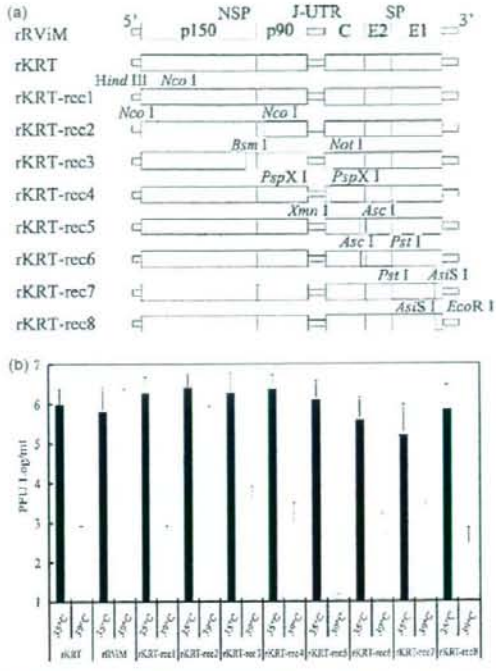

Fig. 3. Construction of a series of recombinant viruses based on rKRT and growth at 35 and 39 °C. (a) Construction of rKRT-rec1–8 based on the rKRT. The genomic structure of RV is indicated in the panel. The broad boxes demonstrate ORFs containing the NSP (p150 and p90) at the 5′ end and the SP (capsid, E2, and E1) at the 3′ end. Narrow boxes between two ORFs indicate untranslated regions (5′ UTR, J-UTR, and 3′ UTR). A series of viruses, rKRT-rec1–8, based on rKRT backbone were constructed by replacing the fragments of rKRT with those of rRViM after digestion with appropriate restriction enzymes. Fragments derived from rRViM are shown as gray bars and from the rKRT backbone, as open bars. (b) Infectivity of respective recombinant viruses at 35 and 39 °C. RK13 cells were infected at a MOI of 0.01. The culture medium was harvested at 96 hpi, and the infective titer was measured. The black columns indicate the infective titers at 35 °C and the gray columns, those at 39 °C. The average infective titers in three independent experiments are shown and the error bar indicates ±SD.

Nde I (gp 1872), Nhe I (gp 2803), Bsm I (gp 3243), and Eco RV (gp 4213), shown in Fig. 5.

There were three nucleotide substitutions with two amino acid changes [G and D at amino acid (a.a.) position 1007, H and Y at 1042] in the Nhe I-Bsm I region of p150 between the KRT strain and wild-type RVi/Matsue.JPN/68 strain. Nucleotide mutations were introduced either independently or in combination into pKRT by PCR amplification with the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen), using as a forward primer, 5′-GCCGGCGaCCCGGGCCGACCGGCTCAGCG-3′ (gp 3053–3082), and as a reverse primer, 5′-GGTCGGCCCGGGtCGCCGGCGGGGCAAGAT-3′ (gp 3043–3072), for the mutation of G to A at gp 3060 (G to D at a.a. position 1007) and 5′-GGTGCGAACTCTGCCGGtAt-ACGCGCGTCA-3′ (gp 3147–3176) and 5′-CCGGCAGAGTTCGCACCC-CTGGCATCCGC-3′ (gp 3135–3163) for the mutations of C to T at gp 3164 and C to T at gp 3166 (H to Y at a.a. position 1042, nucleotide mutations indicated in lower case). The three point-mutated viruses were designated G1007D, H1042Y and G1007D-H1042Y, respectively (Fig. 6). Two recombinant viruses H1042Y-RViM p90 and H1042Y-RViM SP were constructed, using the restriction enzyme sites Bsm I (gp 3243), Not I (gp 6623), Xmn I (gp 6514), and Eco RI (3′ end) as shown in Fig. 7.

2.5. Recovery of clone viruses from infectious cDNA clones of RV

The full-length viral genomic RNA was synthesized from the infectious cDNA clones with the mMESSAGE mMACHINE T7 kit (Applied Biosystems) following the instruction manual. Vero and RK13 cells were prepared at 8.0×10^5 cells/well in 6-well Plates 24 h before RNA transfection. After the cells were washed with 2.0 ml of OPTI-MEM, RNA transfection was carried out with a mixture of 12.5 μg of synthesized RNA and 15.0 μl of DMRIE-C (Invitrogen) in 1.0 ml of OPTI-MEM. After incubation at 35 °C for 4 h, the mixture was removed and replaced with 2.0 ml of MEM containing 5% FBS. For calculating the recovery kinetics of clone viruses, a 100-μl aliquot of culture fluid was harvested every 24 h until 120 h after transfection. The kinetics of infectious clone viruses was monitored with a plaque assay.

2.6. Analysis of temperature sensitivity

Monolayers of RK13 cells in 6-well plates were infected at a multiplicity of infection (MOI) of 0.01. After adsorption, each well was washed twice with 2.0 ml of PBS and replaced with 2.0 ml of MEM

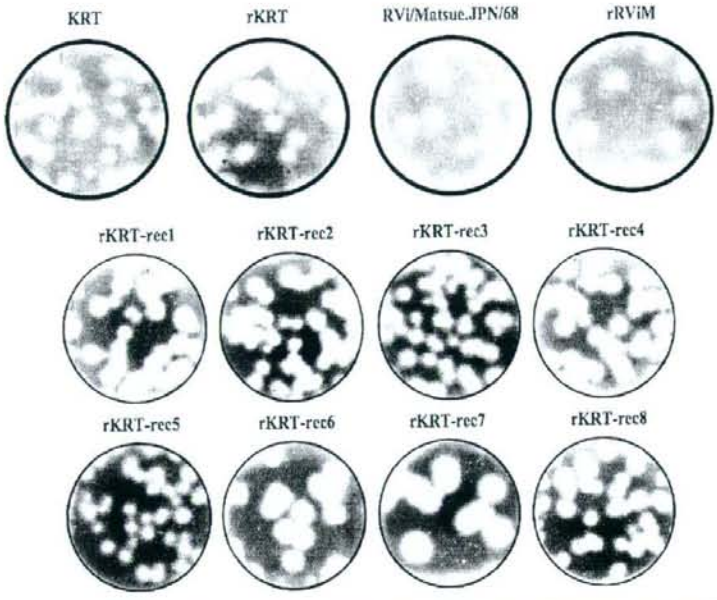

Fig. 4. The plaque morphology of rKRT, rRViM, the original KRT and RVi/Matsue.JPN/68, and the chimeric viruses. Plaques were visualized by fixation with a staining solution containing crystal violet.

containing 5% FBS and antibiotics. The plates were incubated at 35 or 39 °C in a 5% CO_2 incubator, and the culture medium was collected at 24, 48, 72, 96, or 120 h post-infection (hpi). The infective titer of the medium was determined by the plaque assay.

2.7. Viral titration by plaque assay

Monolayers of RK13 cells in 6-well plates were infected with 10-fold serial dilutions of samples. The inoculum was removed after 1 h of contact at room temperature and replaced with 3.0 ml of MEM containing 2% FBS, 40 μg/ml of DEAE dextran, 0.07% sodium bicarbonate, 0.7% agarose, penicillin 100 U/ml and streptomycin 100 U/ml. The plates were incubated at 35 °C in a 5% CO_2 incubator. On day 7 post-infection, plaques were visualized by staining with PBS containing 0.1% crystal violet and 4% formalin [9,14,15].

2.8. Nucleotide sequence accession numbers

The entire sequences of the KRT vaccine strain and wild-type RVi/Matsue.JPN/68 strain were submitted to the GenBank database with accession numbers AB222608 and AB222609, respectively.

3. Results

3.1. Identification of the full-length genome sequence of the KRT vaccine strain and wild-type RVi/Matsue.JPN/68 strain

The live attenuated rubella vaccine KRT was developed by passaging a wild-type rubella virus in rabbit cells. It has been reported that the progenitor wild-type virus of the KRT strain was isolated from a patient with rubella in Matsue city, Japan in 1968 [6]. Although the progenitor was not available, the RVi/Matsue.JPN/68 strain was isolated in the same city in the same year. Consequently, RVi/Matsue.JPN/68 was used a reference for the wild-type of the KRT strain in this study. KRT has a temperature sensitivity (*ts*) phenotype, while the wild-type strains have no temperature sensitivity (non-*ts*) phenotype. The *ts* phenotype of KRT means restricted viral replication at 39 °C [7]. Although little is known about the mechanism of *ts*, it is widely recognized that the phenotype relates to viral attenuation [7,16–19]. The genome of both the KRT and RVi/Matsue.JPN/68 strains was 9762 nt in length. Both genomes consisted of a 40-nt 5′ UTR, 6348-nt NSP, 123-nt J-UTR, 3189-nt SP, and 62-nt 3′ UTR, and were classified into the clade Ia. At the nucleotide level, the entire genomes of the two strains varied by 2.38% (232/9762 nt), while at the amino acid level, they varied by 1.04% (33/3179 a.a.). Tables 1 and 2 show the nucleotide and amino acid differences between the KRT and RVi/Matsue.JPN/68 viruses. Nucleotide differences in the E2 region were highest at 3.19% (27/846 nt) with 2.84% (8/282 a.a.) of amino acids differing. In each region 1.77–3.19% of nucleotides differed and 0.12–2.84% of amino acids differed.

3.2. Construction and characterization of infectious cDNA clones of KRT and RVi/Matsue.JPN/68

Infectious cDNA clones were constructed from the KRT and RVi/Matsue.JPN/68 viruses (Fig. 1). The amplified cDNA fragments were cloned into pUC18 and these clones were assembled into a full-length cDNA clone by using appropriate restriction enzymes. To synthesize the viral RNA, the T7 promoter sequence and a polyA tract were introduced into the full-length cDNA clone. The infectious cDNA clones of KRT and RVi/Matsue.JPN/68 were named pKRT and pRViM, respectively.

Clone viruses of rKRT and rRViM were obtained after transfection of RNA synthesized *in vitro* from the infectious cDNA clones of RVs. To monitor the kinetics of recovery after the transfection of RNA into Vero and RK13 cells, aliquots were harvested and viral titers were determined by plaque assay (Fig. 2a). Maximum infec-

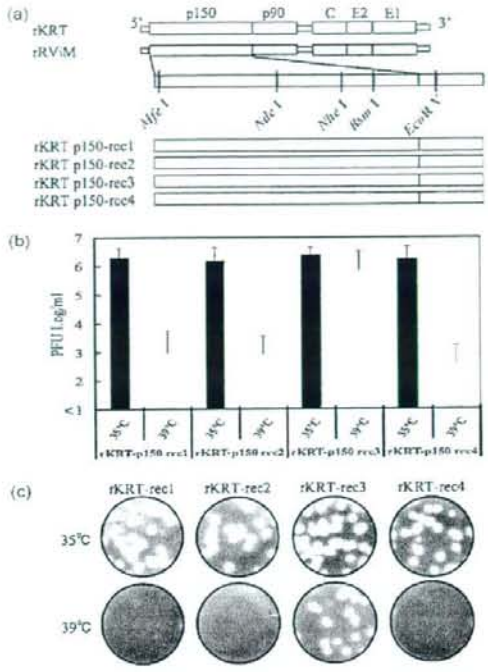

Fig. 5. Construction and characteristics of the recombinant viruses rKRT-p150 rec1–4. (a) Construction of a series of recombinant viruses in the p150 region based on rKRT and rRViM. Gray bars represent the regions derived from rRViM and open bars, those from rKRT. Four chimeric viruses were generated by introducing a part of the p150 region of rRViM into rKRT using appropriate restriction enzyme sites. (b) Infectivity of recombinant viruses. RK13 cells were infected with the rKRT p150 chimeric viruses. The black and gray columns show the infective titers at 35 °C and 39 °C, respectively. The average infective titer in three independent experiments is shown and the error bar indicates ±SD. (c) Plaque assay. Panels at 35 °C demonstrate the results of the plaque assay in 10^{-4} dilutions and panels at 39 °C, these in 10^{-3} dilutions.

tivity of the infectious clone virus (rKRT), 1.0×10^6 PFU/ml, was obtained in Vero cells 96 h after transfection, while the peak of infectivity in RK13 cells reached 2.0×10^5 PFU/ml 72 h after transfection. Thereafter, the clone viruses were recovered at 96 h after transfection in Vero cells. The viruses were propagated within two passages in Vero cells and used for further experiments.

It has been reported that KRT had the *ts* phenotype but the wild-type strain did not [7]. The growth of KRT at 39 °C decreased to 1/1000 of that at 35 °C. The infectivity of RVi/Matsue.JPN/68 at 39 °C was approximately 1/5 of that at 35 °C. The growth patterns of the recombinant viruses, rKRT and rRViM, were similar to those observed for the original viruses (Fig. 2b and c).

The clone viruses were efficiently recovered from the infectious cDNA clone of the KRT vaccine strain and of the wild-type RVi/Matsue.JPN/68 strain. The clones retained the characteristics of the original viruses *in vitro*.

3.3. Construction of chimeric viruses and properties of viral replication at 35 and 39 °C

In order to determine the genomic region responsible for the *ts* phenotype of the KRT vaccine strain, eight recombinant chimeric viruses were constructed based on rKRT, replacing parts of the KRT

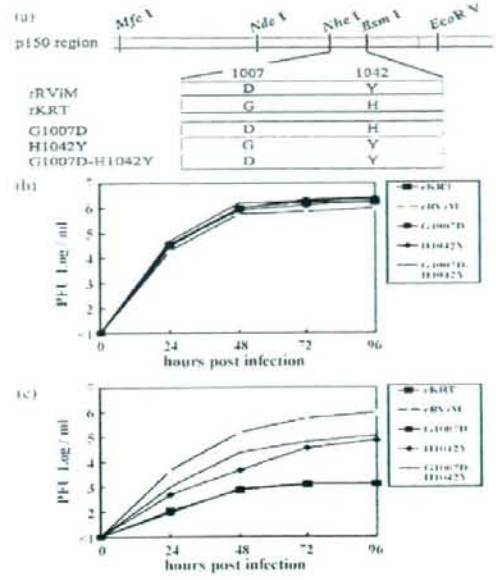

Fig. 6. Growth kinetics of point-mutated RVs at 35 and 39 °C. (a) Amino acid differences in the Nhe I-Bsm I region of p150 between KRT and RVi/Matsue.JPN/68 and construction of mutated viruses. Mutated viruses were generated by introducing the amino acid residues of rRViM into rKRT. Three constructs, G1007D, H1042Y, and G1007D-H1042Y, are illustrated. (b) Growth kinetics of the mutated viruses at 35 °C. The results show the average for three independent experiments and the error bar indicates the ±SD. (c) Growth kinetics of mutated viruses at 39 °C.

genome with the corresponding sequence of RVi/Matsue.JPN/68 (Fig. 3a). Suitable restriction enzymes were used in order to exchange the fragments of individual proteins and UTR regions between the KRT and RVi/Matsue.JPN/68 viruses. For example, rec2 was constructed by exchanging the amino acids in the p150 gene without exchanging those in the p90 gene.

To identify the region responsible for the *ts* phenotype, we examined the growth properties of the recombinant chimeric viruses (rKRT-rec1–8), rKRT, and rRViM, in RK13 cells at 35 and 39 °C. As shown in Fig. 3b, the rKRT-rec1–8 chimeric viruses, except rec2, demonstrated a reduction in growth at 39 °C and the difference in infective titer at 39 °C versus 35 °C varied from 1/100 to 1/10000, similar to that for rKRT. However, rKRT-rec2 grew well at 39 °C in comparison with the other recombinant viruses, its infectivity being 1/10 that at 35 °C. We also obtained complementary results with recombinant chimeric viruses based on rRViM (data not shown). Replacement of the p150 of rKRT with that of rRViM abrogated the *ts* phenotype and the introduction of the p150 of rKRT into rRViM reduced the growth at 39 °C with less efficiency. Thus, the *ts* phenotype of KRT depends on the p150 region.

The morphology of plaques was reported to differ among strains [5,10,12,13], and such a difference between KRT and RVi/Matsue.JPN/68 is shown in Fig. 4. KRT produced mainly small plaques with sharp and clear margins, while RVi/Matsue.JPN/68 generated mainly large plaques with vague and opaque margins. The clone viruses, rKRT and rRViM, exhibited the same plaque morphology as the original viruses. In addition, the plaques differed in size among the recombinant chimeric viruses. Among a series of rKRT-recs, rKRT-rec1, 2, 3, 4, 5, and 8 showed similar plaques, while rKRT-rec6 and rec7 generated apparently large plaques with clear and sharp margins. These results suggested that the envelope

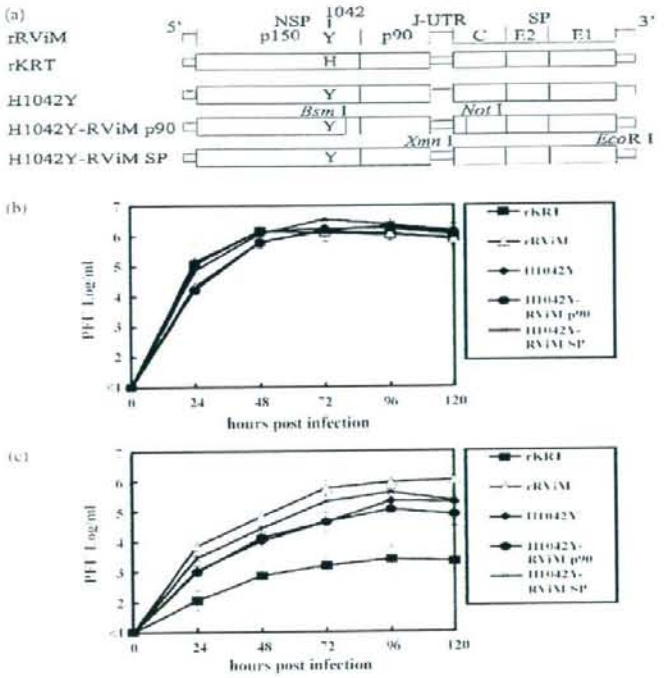

Fig. 7. Additive effect of other genomic regions on temperature sensitivity. (a) Construction of recombinant viruses (H1042Y-RViM p90 and H1042Y-RViM SP). The gray bars represent the fragments derived from rRViM and the white bars are those from rKRT. The recombinant viruses H1042Y-RViM p90 and H1042Y-RViM SP having the p90 and SP of rRViM together with the amino acid substitution H1042Y were generated, using the appropriate restriction enzyme sites (Bsm I, Not I, Xmn I, and Eco RI). (b) Growth kinetics of the recombinant viruses at 35 °C. The infective titer is shown as the average for three independent experiments and the error bar indicates ± SD. (c) Growth kinetics of recombinant viruses at 39 °C.

proteins, E1 and E2, of RVi/Matsue.JPN/68 were relevant to large plaques.

3.4. Identification of the amino acid residue in p150 responsible for the temperature sensitivity

The chimeric viruses altered in the p150 region (rKRT-rec2) varied in *ts* phenotype. Since there were 15 amino acid differences in the p150 region between the two strains, four recombinant viruses (rKRT p150-rec1–4) were generated based on rKRT, replacing the fragments in the p150 region between rKRT and rRViM (Fig. 5a). The growth of the rKRT p150-recs was investigated at 35 and 39 °C in RK13 cells and the results are shown in Fig. 5b and c. All rKRT p150-recs showed similar infectivity, over 10^6 PFU/ml at 35 °C, but the infective titers of p150-rec1, 2, and 4 were less than 10^3 PFU/ml at 39 °C. Only rKRT p150-rec3 demonstrated efficient growth, over 10^5 PFU/ml, at 39 °C. rKRT p150-rec3 consisted of the fragment between Nhe I and Bsm I in the p150 region of rRViM which seemed to be relevant to the *ts* phenotype of KRT. There were two amino acid differences in the Nhe I-Bsm I region of p150 between the two strains, at positions 1007 and 1042 (see Table 2). The amino acid at position 1007 of p150 was changed from aspartic acid (D) in RVi/Matsue.JPN/68 to glycine (G) in KRT. The other at position 1042 was changed from tyrosine (Y) in RVi/Matsue.JPN/68 to histidine (H) in KRT. In order to decide the amino acid residue(s) responsible for the *ts* phenotype, three mutated viruses, G1007D, H1042Y, and G1007D-H1042Y, containing a substitution at position 1007, 1042, or both of RVi/Matsue.JPN/68, were constructed (Fig. 6a). The growth kinetics at 35 and 39 °C are shown in Fig. 6b and c. G1007D, H1042Y, and G1007D-H1042Y grew well at 35 °C with similar titers, but G1007D showed poor growth similar to rKRT at 39 °C. Whereas, H1042Y and G1007D-H1042Y showed more efficient growth at 39 °C with 100-fold higher titers than rKRT. These results indicated that the histidine at position 1042 of p150 was responsible for the *ts* phenotype of the KRT.

3.5. Additive effect of other regions on the ts

The histidine at position 1042 of p150 was considered to be responsible for the *ts* phenotype of KRT, but the growth kinetics of H1042Y and G1007D-H1042Y at 39 °C did not completely match that of rRViM. The peak infective titers of those viruses at 39 °C were about 1/10 lower than those of rRViM (Fig. 6c). There remained the possibility that other gene(s) influenced the growth properties at restrictive temperatures in relation with the tyrosine at position 1042, thus we examined the additive effect of other gene(s) to allow better growth at 39 °C. We constructed two additional recombinant viruses having p90 or structural proteins (SP) including capsid, E2, and E1 of RVi/Matsue.JPN/68 together with the substitution H1042Y (Fig. 7a). There was no difference in growth among the recombinant viruses (H1042Y, H1042Y-RViM p90 and H1042Y-RViM SP) at 35 °C. The growth kinetics of H1042Y and H1042Y-RViM p90 showed a very similar pattern at 39 °C, while the growth of H1042Y-RViM SP was greater than that of H1042Y, the maximum titer (at 96 hpi) approaching that of rRViM. These results suggested that the p90 of RVi/Matsue.JPN/68 had no additive effect with the

tyrosine at position 1042 of p150 on the growth at a restrictive temperature, whereas the SP region of RVi/Matsue.JPN/68 did have an additive effect.

4. Discussion

For the prevention of CRS and rubella epidemics, live attenuated rubella vaccines have been used in vaccination programs in many countries. Although these vaccines have high efficacy and a low incidence of adverse reactions, their attenuation mechanism is not well understood. A few studies reported replication properties, plaque morphology and cell tropism of the RA27/3 and Cendehill vaccine strains, leading to some insight into the attenuation. RA27/3 has been used widely for vaccination programs and was reported to have unique characteristics *in vitro*, probably related to the attenuation, in comparison with the wild-type virus (Therien strain). RA27/3 formed smaller plaques with lower growth than Therien due to mutations in the 5′ UTR, the protease motif of p150, and the capsid region [8–10,22]. The Cendehill strain is known to cause acute arthritis at a very low incidence, a phenomenon that seems to correlate with a drastic reduction in growth in synovial cells compared with the wild-type virus [8,23]. Chantler et al. [20] also reported differences in plaque morphology and growth properties among rubella virus strains. All Japanese vaccines, RA27/3, and Cendehill, exhibit the *ts* phenotype, which is probably linked with immunogenic markers in rabbits and guinea pigs [7,16,20,24]. In some single positive strand RNA viruses, such as *Togaviridae* and *Flaviridae* family viruses: sindbis virus, semiliki forest virus, tick-borne encephalitis virus and dengue virus, the *ts* phenotype was shown to be related to attenuation in animal models [17,19,25,26]. Therefore, the *ts* phenotype of the KRT vaccine strain was considered a biological marker of attenuation.

In this study, the biological characteristics of the KRT vaccine were compared with those of RVi/Matsue.JPN/68, which was isolated in the same city and year as the KRT progenitor. The genomes of both strains consisted of 9762 nt and there were 232 nucleotide differences with 33 amino acid changes between the two. These differences were more frequent than those between the TO-336 vaccine and TO-336 progenitor wild-type strains: Kakizawa et al. [27] reported that the two genomic sequences differed at 21 nt with 10 amino acid changes. Although KRT and RVi/Matsue.JPN/68 belong to genotype Ia of rubella virus, consisting of the RA27/3, Cendehill, and TO-336 vaccine strains, and other wild-type strains, none of the mutations observed in KRT was common to those vaccine strains [8,27–32]. Thus, the region(s) responsible for the *ts* phenotype would differ among vaccine strains.

In order to investigate the genetic determinant(s) of the *ts* phenotype in KRT, we developed a RG system with KRT and RVi/Matsue.JPN/68. There were significant biological differences between KRT and RVi/Matsue.JPN/68. The clone virus, rKRT, and its original virus produced small plaques with sharp and clear margins, while rRViM and its original virus produced large plaques with vague and opaque margins. rKRT, rRViM, and their original viruses grew well at 35 °C without any changes in growth kinetics, but the growth kinetics differed significantly at 39 °C. The clones, rKRT and rRViM, showed the same biological characteristics as the original viruses.

A series of recombinant viruses (rKRT-rec1–8) based on rKRT and rRViM were generated, to investigate the contribution of individual regions of the genome to the *ts* phenotype of KRT. rKRT-rec2 (replacement of the p150 region) differed in phenotype from the original virus, however, the others showed similar growth properties to the parental viruses. We also found that some parts of the genome affected the morphology of plaques in the process of detecting the region responsible for the *ts* phenotype. The difference in the morphology of the plaques between the two strains was determined with crystal violet staining. Crystal violet stains living cells, and therefore plaque morphology, e.g. vague, opaque, clear and sharp may reflect apoptotic cell distribution, probably related to the difference in the regulation of viral replication and cytopathic effect between the two strains. Moreover, the introduction of E1 and E2 proteins of RVi/Matsue.JPN/68 changed the small plaques of rKRT to large ones. E1 and E2 having N-linked glycosylation sites (Asn-X-Ser/Thr) form heterodimers and compose the viral particle. After the formation of the heterodimer, N-linked glycosylation occurred during transport to the budding site. E1 and E2 play a key role in budding at the plasma membrane of infected cells, and the attachment to and entry of an uninfected cell occur through the fusion activity of these proteins [33,34]. It has also reported that the formation of the heterodimer and N-linked glycosylation influence infectivity and membrane fusion activity [35–41]. There were 13 amino acid differences in the E1 and E2 proteins, one of which was in the predicted N-linked glycosylation site at amino acid position of the E1 region (Table 2). This difference causes the absence of one predicted N-linked glycosylation site in E1 of KRT. As a result, two predicted N-linked glycosylation sites exist in the E1 region of KRT and three sites in that of RVi/Matsue.JPN/68, and a difference in the molecular weight of E1 between the two strains was observed by Western blotting (data not shown). The recombination of E1 and E2 (rKRT-rec6 and rec7) obviously altered plaque size. Thus, the large plaques of RVi/Matsue.JPN/68 might suggest an efficient expansion of infection *in vitro*. The different numbers of predicted N-linked glycosylation sites and amino acid changes may be involved in the membrane fusion activity and conformational change of those proteins leading to an influence on viral spread from cell to cell. Further investigation is required to clarify whether the characteristic morphology of plaques is correlated with the attenuation process.

There are 15 amino acid differences in the p150 region between the KRT and RVi/Matsue.JPN/68 strains. Four recombinant viruses (rKRT p150-rec1–4) and mutated viruses (G1007D, H1042Y, and G1007D-H1042Y) demonstrated that the histidine at position 1042 was critical for the *ts* phenotype of KRT. Although the growth of the H1042Y mutant was 100-fold greater than that of rKRT at 39 °C, the peak infective titer was 1/10 that of rRViM. Therefore, the additive effect of the other region(s) on growth at 39 °C was investigated, together with H1042Y. Although the p90 region of RVi/Matsue.JPN/68 had no additive effect on the *ts* phenotype, SP (structural proteins: capsid, E2, and E1) exhibited an additive effect on replication at 39 °C. The reduction in peak titer of the H1042Y mutant may be due to the differences in genetic background between KRT and RVi/Matsue.JPN/68.

In this study, we did not evaluate the effect of this histidine on p150 and the defective viral life cycle. The p150 region encodes a methyltransferase motif having a role in the capping of viral RNA and a cysteine protease domain that cleaves a precursor p200 polyprotein to p150 and p90 proteins as functional units. Balistreri et al. [42] reported that variation in the *ts* phenotype of Semliki forest virus having mutations in the protease domain indicated a great reduction of protease activity compared to the wild-type virus at a restricted temperature. The change in the *ts* phenotype of Sindbis virus with mutations in the protease domain reduced subgenomic RNA synthesis [43–46]. These reports provided new insights into the mechanism of the *ts* of KRT. Because the histidine at position 1042 is located in the protease domain, it may cause defects in NSP processing or viral RNA replication; synthesis of negative strand RNA (cRNA), subgenomic RNA, and progenitor genomic RNA.

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ORIGINAL ARTICLE

Kenji Gotoh · Liang Qin · Kiwao Watanabe
Dang Duc Anh · Phan Le Thanh Huong
Nguyen Thi Hien Anh · Nguyen Dinh Lien Cat
Luong Linh Ha · Le Thi Thuy Ai · Nguyen Manh Tien
Truong Tan Minh · Kazunori Oishi · Hiroshi Watanabe

Prevalence of *Haemophilus influenzae* with resistant genes isolated from young children with acute lower respiratory tract infections in Nha Trang, Vietnam

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Abstract Our study was undertaken to investigate the characteristics of *Haemophilus influenzae* in young children with acute lower respiratory tract infections in Nha Trang, Vietnam. The study population consisted of 116 children less than 5 years of age admitted to Khanh Hoa General Hospital due to acute lower respiratory tract infections between July 2004 and April 2005. Organisms could be detected from nasopharyngeal swabs (NP) in 72 (62.1%) of the 116 children. *Haemophilus influenzae* was the most common organism, and 39 strains were isolated from 39 children aged 2 to 60 months (mean age, 16 months). We examined 37 of these 39 *H. influenzae* strains. The serotypes of the 37 isolates were all nontypeable, and 22 strains (59.5%) were β-lactamase producing. Polymerase chain reaction (PCR) analysis to identify resistance genes revealed that 17 strains had the TEM-1-type β-lactamase gene alone, 6 strains had the *ftsI* gene with the same substitution as that in g low-β-lactamase-negative ampicillin-resistant (g low-BLNAR) strains, and 6 strains had both the TEM-1-type β-lactamase gene and the *ftsI* gene with the same substitution as that in g β-lactamase-producing amoxicillin clavulanic acid-resistant (g BLPACR I) strains, although no BLNAR strains were found. Molecular typing by pulsed-field gel electrophoresis (PFGE) showed that the 6 g low-BLNAR strains had five PFGE patterns and the 6 g BLPACR I strains had four PFGE patterns. Our results indicate that BLNAR strains are still not prevalent, but that g low-BLNAR and g BLPACR I strains are potentially spreading in Nha Trang, Vietnam.

Key words *Haemophilus influenzae* · Drug resistance · Pulsed-field gel electrophoresis (PFGE) · Child

K. Gotoh · L. Qin · H. Watanabe (✉)
Division of Infectious Diseases, Department of Infectious Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan
Tel. +81-942-31-7549; Fax +81-942-31-7697
e-mail: hwata@med.kurume-u.ac.jp

K. Watanabe
Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

D.D. Anh · P.L.T. Huong · N.T.H. Anh
National Institute of Hygene and Epidemiology, Hanoi, Vietnam

N.D.L. Cat · L.L. Ha · L.T.T. Ai · N.M. Tien
Khanh Hoa General Hospital, Nha Trang, Vietnam

T.T. Minh
Khanh Hoa Provincial Health Service, Nha Trang, Vietnam

K. Oishi
Department of Special Pathogens, International Research Center for Infectious Diseases, Research Institute for Medical Diseases, Osaka University, Osaka, Japan

Introduction

Haemophilus influenzae is a Gram-negative, rod-shaped bacterium that colonizes the human nasopharynx. Nontypeable *H. influenzae* (NTHi) can cause a variety of infections, including otitis media, sinusitis, conjunctivitis, bronchitis, and pneumonia.[1,2] Children are frequent carriers of *H. influenzae* in the nasopharynx, and the rate of carriage is high in infancy.[3–5] The colonization may subsequently lead to the development of infectious diseases caused by *H. influenzae*.[3,6] It has also recently been reported that β-lactamase-negative ampicillin-resistant (BLNAR) strains have increased in some countries,[7,8] although the global prevalence still remains low.[9,10] In Vietnam, one previous study demonstrated that BLNAR strains were not prevalent in Hanoi, in the northern region.[11] However, the resistance status of *H. influenzae* in the country is still unclear. The aim of our study was to investigate the characteristics of *H. influenzae* among young children in Nha Trang, in the southern region of Vietnam.

Methods

All studies described herein were approved by the Human Ethics Review Boards of Nagasaki University and Khanh Hoa General Hospital, and signed consent was obtained from each subject or their parent or guardian.

Patients and bacterial strains

The population in this study consisted of 116 children admitted to the Khanh Hoa General Hospital, Nha Trang, Vietnam, due to acute lower respiratory tract infections, between July 2004 and April 2005. Khanh Hoa General Hospital is the core hospital for the province around Nha Trang. Forty-three patients were diagnosed with pneumonia and 73 patients were diagnosed with acute bronchitis. None of the patients had severe disease showing multilobular infiltrates on chest X-ray and none required a ventilator. In Vietnam, people can buy antibiotics at a pharmacy without a prescription. In the present study, approximately 10% of the patients had not received any therapy before admission to the hospital, approximately 30% of the patients had received antibiotic treatment at a private hospital before admission to our hospital, and approximately 60% of the patients had taken oral antibiotics by themselves and had subsequently been treated with antibiotics at a private hospital before admission to our hospital.

Nasopharyngeal swabs (NP) were collected from the 116 patients with acute lower respiratory tract infections, using two flexible swabs (Medical Wire and Equipment, Wiltshire, Corsham, England). A quantitative culture and Gram's staining of the nasopharyngeal secretions were performed simultaneously within 24 h. After weighing the NP on a microbalance, the volume of the sample was determined to be approximately 0.01 ml. This sample was diluted in brain heart infusion broth (BBL; Becton Dickinson, Cockeysville, MD, USA), and a tenfold dilution was then prepared in saline. A quantitative bacterial culture was carried out on trypticase soy agar (BBL; Becton Dickinson) containing 7% defibrinated rabbit blood and incubated in a 5% CO_2 incubator at 37°C overnight.

Thirty-nine *H. influenzae* strains were isolated from the NP of 39 children aged 2 to 60 months (mean age, 16 months), and 37 of the 39 strains were investigated in this study, because 2 strains did not grow from the frozen stock at −80°C. As control cases, we also investigated the NP of 111 children aged 1 to 60 months (mean age, 19 months) without lower respiratory tract infection admitted to the hospital for other conditions, such as acute enterocolitis and convulsions.

Serotypes and β-lactamase production

H. influenzae isolates were serotyped by slide agglutination with antisera purchased from Difco Laboratories (Detroit, MI, USA), and β-lactamase production was detected by a disc impregnated with nitrocefin (Becton Dickinson, Sparks, MD, USA).

Polymerase chain reaction (PCR)

PCR was carried out for *H. influenzae* isolates by using mixed primers including the following (all from Wakunaga Pharmaceutical, Hiroshima, Japan): P6 primers to amplify the *p6* gene, which encodes the P6 membrane protein specific for *H. influenzae*; TEM-1 primers to amplify a part of the bla_{TEM-1} gene; *pbp*3-1 primers to identify the same substitution as that shown by g low-BLNAR strains in the *ftsI* gene; and *pbp*3-2 primers to identify the same substitution as that shown by BLNAR strains in the *ftsI* gene.[12]

Antimicrobial susceptibility test

The minimal inhibitory concentration (MIC) was determined by the agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute.[13] The susceptibility of the 37 *H. influenzae* isolates to the following 14 antibiotics was tested: ampicillin (Meiji Seika Kaisha, Tokyo, Japan), amoxicillin (Meiji Seika Kaisha), amoxicillin-clavulanic acid (GlaxoSmithKline, Tokyo, Japan), cefaclor (Shionogi, Osaka, Japan), cefuroxime (GlaxoSmithKline), cefotaxime (Aventis Pharma, Tokyo, Japan), ceftriaxone (Chugai Pharmaceutical, Tokyo, Japan), imipenem (Banyu Pharmaceutical, Tokyo, Japan), erythromycin (Abbott Japan, Tokyo, Japan), clarithromycin (Taisho Toyama Pharmaceutical, Tokyo, Japan), azithromycin (Pfizer Japan, Tokyo, Japan), gentamicin (Schering-Plough, Osaka, Japan), chloramphenicol (Daiichi Sankyo, Tokyo, Japan), and sulfamethoxazole-trimethoprim (Shionogi).

Pulsed-field gel electrophoresis (PFGE) analysis

After digestion with *Sma*I (Takara Shuzo, Shiga, Japan), PFGE was performed on 21 of the 37 *H. influenzae* isolates from the NP, as described previously,[14] and the interpretation of PFGE patterns was based on the criteria described by Tenover et al.[15]

Results

Bacterial strains

Organisms could be detected from the NP in 72 (62.9%) of the 116 children who were admitted to the Khan Hoa General Hospital due to acute lower respiratory tract infections between July 2004 and April 2005. Table 1 shows the differences in the organisms isolated from NP in the children with lower respiratory tract infections and the control patients. The organism detection rate (62.9%) in the children with lower respiratory tract infections was higher than that (12.6%) in the control patients. *H. influenzae* and *Moraxella catarrhalis* were frequently detected in the children with lower respiratory tract infections. On the other hand, only *M. catarrhalis* was detected in the control patients.

Serotypes, β-lactamase production and PCR analysis

The serotypes of the 37 *H. influenzae* isolates were all nontypeable. Twenty- two strains were β-lactamase producing,

Table 1. Comparison of organisms isolated from nasopharynx in children with acute lower respiratory tract infections and control patients in Nha Trang, Vietnam

	Patients with acute lower respiratory tract infections (n = 116)	Control patients (n = 111)
Mean age, in months (range)	16 (2–60)	19 (1–60)
Pathogenic organism detection rate	62.1% (72/116)	12.6% (14/111)
Haemophilus influenzae	32	0
Moraxella catarrhalis	24	14
H. influenzae + *M. catarrhalis*	7	0
Streptococcus pneumoniae	6	0
Staphylococcus aureus	2	0
M. catarrhalis + *S. pneumoniae*	1	0

Table 2. Distribution of MICs of 14 antibiotics against 37 *H. influenzae* strains isolated from the nasopharyngeal swabs of young children in Nha Trang, Vietnam

No. of isolates for which MIC (μg/ml) was: Antibiotic	≤0.004	0.008	0.016	0.032	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128
Ampicillin							5	2	6	3	3	3	7	6	1	1
Amoxicillin								6	3	8	2	4	7	6		1
Amoxicillin-clavulanic acid								6	12	10	8	1				
Cefaclor										6	9	3	5	11	3	
Cefuroxime									13	7	9	4	4			
Cefotaxime			13	6	13	5										
Ceftriaxone	12	6	10	8										1		
Imipenem							5	4	2	8	5	11	2			
Erythromycin									5	15	13	4				
Clarithromycin										2	5	17	13			
Azithromycin								1	2	14	13	7				
Gentamicin								7	28	2						
Chloramphenicol								16	2		1	17	1			
Sulfamethoxazole-trimethoprim														1	36	

and the remaining strains were β-lactamase-negative. PCR analysis to identify the resistance genes indicated that 17 strains had the TEM-1-type β-lactamase gene alone, 6 strains had the *ftsI* gene with the same substitution as that in g low-BLNAR strains, 6 strains had both the TEM-1-type β-lactamase gene and the *ftsI* gene with the same substitution as g low-BLNAR (β-lactamase-producing amoxicillin clavulanic acid-resistant: g BLPACR I) strains, and 8 strains were β-lactamase-negative ampicillin-susceptible (BLNAS) strains without a resistant gene. No BLNAR strain was found.

Antimicrobial susceptibility test

Table 2 shows the range of MIC values for the 14 antibiotics tested against the 37 *H. influenzae* isolates from the NP. Using CLSI breakpoints,[13] most isolates were susceptible to ceftriaxone and cefotaxime. Thirteen strains (35.1%) were susceptible to ampicillin; 3 strains (8.1%) were intermediate-resistant and 21 strains (56.8%) were resistant to ampicillin. On the other hand, 36 (97.3%) strains were susceptible to amoxicillin-clavulanic acid.

Interpretation of PFGE

We investigated the molecular typing of 21 *H. influenzae* strains by PFGE; 4 strains with the TEM-1 type β-lactamase gene alone, 6 g BLPACR I strains, 6 g low-BLNAR strains, and 5 BLNAS strains. Molecular typing by PFGE showed that these 21 *H. influenzae* strains had 18 PFGE patterns. The 6 g BLPACR I strains had 4 PFGE patterns, the 6 g low-BLNAR strains had 5 PFGE patterns. The 4 TEM-1 strains and the 5 BLNAS strains had various patterns (Fig. 1).

Discussion

In general, the major bacterial pathogens of acute lower respiratory tract infections in young children are *Streptococcus pneumoniae* and *H. influenzae*. In our study, the detection rate of *S. pneumoniae* in patients with acute lower respiratory tract infection was approximately 6%. Because approximately 90% of the patients in our study had already taken antibiotics before admission, especially penicillin or a first-generation cephalosporin, this result may indicate that these antibiotics reduce acute lower respiratory tract infections caused by *S. pneumoniae*. Indeed, the *S. pneu-*

Fig. 1. Pulsed-field gel electrophoresis (PFGE) patterns of *Sma*I-digested DNA from 21 *Haemophilus influenzae* isolates obtained from nasopharyngeal swabs (NP) in 21 children with acute lower respiratory tract infections. Molecular typing by PFGE demonstrated that the 21 *H. influenzae* strains from the NP had 18 PFGE patterns (*A* to *R*) without any predominant pattern *M*. Molecular weight marker; *BLNAR*, β-Lactamase-negative ampicillin-resistant; *BLNAS*, β-lactamase-negative ampicillin-susceptible

moniae strains in our study were all penicillin-resistant. On the other hand, the rate of β-lactamase-producing *H. influenzae* strains was high, as was also shown in the previous study in Hanoi,[11] and we also found other resistant strains, such as g low-BLNAR and g BLPACR I, although no BLNAR strains were found. In the previous study in young children in Hanoi,[11] only one g BLPACR I strain had been detected from NP. However, we identified a greater number of resistant strains with different PFGE patterns in Nha Trang. In Vietnam, as stated above, people can buy antibiotics (especially penicillin and first-generation cephalosporins) at a pharmacy without a prescription, which could potentially explain the high detection rate of β-lactamase-producing *H. influenzae* strains and the further appearance of such resistant strains as g low-BLNAR and g BLPACR I. In Japan, on the other hand, people cannot buy antibiotics at a pharmacy without a prescription, but third-generation cephalosporins, rather than penicillin or a first generation cephalosporin, have often been used in hospitals, and this may have caused the spread of BLNAR strains in Japan.

Infants and children tend to acquire *H. influenzae* in their respiratory tracts, and *H. influenzae* often causes infectious diseases because of their low immunity.[14] Nontypeable *H. influenzae* causes respiratory tract infections including pneumonia, otitis media, and sinusitis. In Japan, the proportion of BLNAR strains is extremely high,[8] and young children often suffer severe infections such as pneumonia and recurrent otitis media due to BLNAR strains. A recent study in the United States has described a novel resistance mechanism of BLNAR strains with unusually high resistance to ampicillin.[16] It has been reported that children can acquire *H. influenzae* at day care centers[14] or in the home[17] by person-to-person transmission. However, our PFGE studies showed that resistant strains such as g low-BLNAR and g BLPACR I did not have dominant genetic patterns. These results may provide evidence to show that such resistant strains are not spreading horizontally among children in Nha Trang.

In conclusion, our results demonstrate that, in Nha Trang, Vietnam, BLNAR strains are still not prevalent, but g low-BLNAR and g BLPACR I strains are potentially spreading. Therefore, further monitoring for such resistant strains should be considered.

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