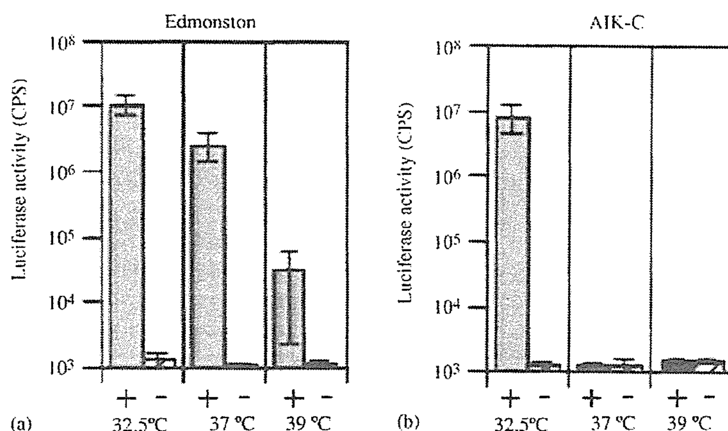

Fig. 1. The minigenome experiments supported by the Edmonston-N, P, and L protein expression plasmids or the AIK-C-N, P, and L protein expression plasmids at 32.5, 37, or 39 °C. HeLa cells prepared in 12-well plates were infected with vaccinia virus vTF7-3 at an MOI of 2–3 and then transfected with 0.5 μg of negative-sense minigenome RNA holding the luciferase gene and (a) the set of pCIN01 (0.5 μg), pCIP01 (0.25 μg), and pCIL01 (0.5 μg) plasmids derived from the Edmonston strain or (b) the set of pCIAN01 (0.5 μg), pCIAP01 (0.25 μg), and pCIAL01 (0.5 μg) plasmids derived from the AIK-C strain: (+), tranfected with minigenome RNA and the set of plasmids; (−), transfected with only minigenome RNA as a negative control. The transfected cells were maintained at 32.5, 37, or 39 °C in 5% CO_2 for 40 h and lysed to measure luciferase activity. The luciferase activity (counts per second, CPS) was counted by Top Count (Packard Instrument Company, Meriden, CT, USA). The experiments were carried out at 3 times and the mean values and ±1.0 S.D. were indicated.

3. Results

3.1. Minigenome replication supported by the set of N, P, and L protein-expression plasmids derived from Edmonston and AIK-C strains at various temperatures

The MV vaccine strain, AIK-C, exhibits the *ts* phenotype (shut off temperature at 39 °C), whereas the Edmonston strain, the parental strain of AIK-C, does not [16]. Recently, the *ts* propertiy of AIK-C was reported not to be attributable to either of the viral envelope proteins [23]. We assumed that viral RNA synthesis-related genes or proteins contribute to the *ts* phenotype of AIK-C and the minigenome system is helpful to analyze the phenotype. Our laboratory Edmonston strain which has been propagated in Vero cells does not show *ts* phenotype (shut off temperature at >40 °C). Thus, we attempted to identify the *ts*-related mutation in AIK-C strain using the minigenome system with the cDNAs derived from our Edmonston strain and from vaccine seed AIK-C strain. Nucleotide sequences of the leader, N gene, P gene, L gene and trailer regions were determined. The leader and trailer sequences of the Edmonston strain were identical to those of AIK-C strain [19]. This implies that these *cis*-element sequences for viral transcription/replication are not responsible for *ts* phenotype of AIK-C strain. Nucleotide differences that caused the amino acid substitutions between Edmonston and AIK-C strains were observe in 3 for the N gene, 5 for the P gene (including C and V genes) and 15 for the L gene.

When the negative-sense minigenome RNA containing the luciferase gene was transfected with the Edmonston-derived N, P and L proteins expression plasmids and incubated at the 32.5, 37, and 39 °C, high level of luciferase activity was observed at any temperature conditions, but considerable reduction at 39 °C (Fig. 1a). In contrast, when the AIK-C-derived N, P and L expression plasmids were used, the luciferase activity was detected only at 32.5 °C. No activity was detected even at 37 °C (Fig. 1b). These results suggested that AIK-C-derived protein(s) responsible for viral replication and/or transcription are involved in the *ts* phenotype. In addition, the temperature point for *ts* was shifted to lower temperature in the minigenome system, suggesting that the system may be more sensitive to temperature as compared with the infectious viruses. As the *ts* property seemed much highlighted at 37 °C rather than at 39 °C in the minigenome system (Fig. 1a and b), further study using minigenome system was performed at 32.5 and 37 °C.

3.2. Identification of the protein of strain AIK-C that contributes to the ts phenotype

To identify the protein that contributes to the *ts* phenotype of AIK-C, minigenome experiments were carried out with various combinations of the N, P, and L expression plasmids which are derived from either the Edmonston or the AIK-C strain (Fig. 2). All combinations of the plasmids supported high level of luciferase expression at 32.5 °C, suggesting that the minigenome RNA was replicated and transcribed. In contrast, when minigenome experiments were carried out at 37 °C using pCIAP01 plasmid, the AIK-C derived P expression plasmid, as expression partner, no luciferase activity was observed. The other combinations excluding the pCIAP01 plasmid could support the luciferase expression at 37 °C. These results indicate that the proteins derived from the AIK-C P gene functioned at 32.5 °C, but not at 37 °C in the minigenome system, suggesting the proteins expressed from P gene of AIK-C might contribute to the *ts* phenotype.

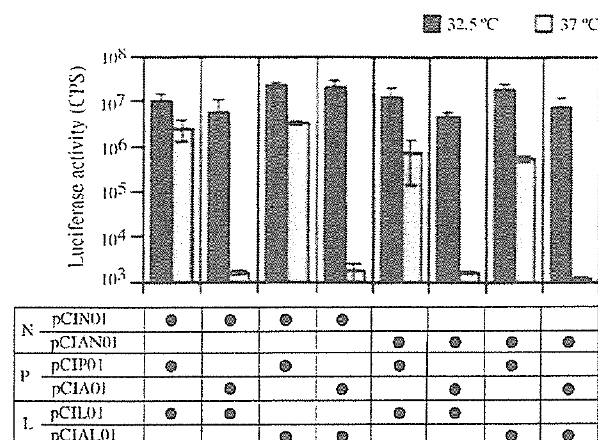

Fig. 2. Luciferase activity in the minigenome experiments supported by the various combinations of N, P, and L protein-expression plasmids at 32.5 or 37 °C. The vTF7-3 virus-infected HeLa cells were transfected with the minigenome RNA and various combinations of the pCIAN01 or pCIN01, pCIAP01 or pCIP01, and pCIAL01 or pCIL01 plasmids and incubated at 32.5 (dark gray bar) or 37 °C (light gray bar) for 40 h. After cell lysis, luciferase activity (CPS) was measured. Dots in the table under the figure indicate the plasmid employed in the minigenome experiments. The experiments were carried out at 3 times and the mean values and ±1.0 S.D. were indicated.

3.3. *Identification of the ts point(s) in the p gene*

The P gene of MV coded three proteins, P, C, and V proteins. Four nucleotide differences were found between the AIK-C and Edmonston P genes. Those nucleotide substitutions led to three amino acid substitutions in the P protein (aspartic acid to tyrosine at position 110; P110-Tyr, cysteine to tyrosine at 275; P275-Tyr and leucine to proline at 439; P439-Pro), one substitution in the V protein (aspartic acid to tyrosine at 110; V110-Tyr), and one substitution in the C protein (serine to tyrosine at 134; C134-Tyr) (Table 1). The V protein was not expressed from the P plasmid in the transfected cells due to a lack of RNA editing activity of the T7 RNA polymerase. Thus, the V110-Tyr did not attribute to lack of luciferase expression at 37 °C in the minigenome system. To identify *ts* point(s) in the P or C protein, 8 chimera P plasmids were constructed using appropriate restriction endonuclease sites (Fig. 3a and b), which were designed to express the chimera P or C proteins between the AIK-C and Edmonston strains. Following construction, the nucleotide sequences of the chimera P genes were entirely determined to verify the constructed P gene.

The minigenome experiments using the 8 chimera P plasmids were conducted at 32.5 and 37 °C. When the experiments were performed at 32.5 °C, all the chimeric P proteins induced the high level of luciferase activity, indicating that all chimeric plasmids expressed the functional P proteins (Fig. 4a and b). These chimeras, however, showed the significant differences when the experiments were held at 37 °C. A high level of luciferase expression was observed when Edmonston-P gene backbone plasmids (pCIP01, 03, 04 and 05) were used, but it was reduced dramatically when the pCIP02 was used, which had a proline residue instead of leucine at position 439 of the P protein (Fig. 4a). Conversely, the chimera plasmids originated from the AIK-C P gene except pCIAP02 failed the luciferase expression. The pCIAP02 which had a leucine instead of proline at position 439 of the P protein, restored the luciferase expression at 37 °C (Fig. 4d). Those results indicated that the proline residue at amino acid 439 of the P protein (P439-Pro) plays a key role in the *ts* phenotype of strain AIK-C.

3.4. *Replication of rescued recombinant AIK-C viruses containing various P proteins at various temperatures*

To confirm the results obtained from the minigenome experiments, the recombinant viruses having the various P genes were rescued using infectious cDNA clone system and tested their temperature-sensitivity. The Edmonston or chimeric P genes were excised at relevant endonuclease site from the pCIP01, pCIP02 and pCIAP02 plasmids and exchanged with the AIK-C P gene of the AIK-C genome cDNA (pMVAIK-C). These chimera genome cDNAs were designated pMVED-P (exchanged with pCIP01), pMVAIK/P439-Leu (pCIAP02) and pMVED/P439-Pro (pCIP02), respectively (Fig. 5). The full-genome cDNAs were transfected into MVAT7 pol-infected B95a cells together with pCIAN01,

Table 1
Nucleotide comparison of the P genes of the Edmonston and AIK-C strains and deduced amino acid substitutions in the P, V, and C proteins

protein	Nuclotide positon	virus Edmonston	virus AIK-C	Amino Acid position	virus Edmonston	virus AIK-C
	2134	G	T	110	D	Y
P	2229	C	A	141	L	L
	2630	G	A	275	C	Y
	3122	T	C	439	L	P
	2134	G	T	110	D	Y
V	2229	C	A	141	L	L
	2630	G	A	275	V	V
C	2134	G	T	102	V	V
	2229	C	A	134	S	Y

Nuclotide substitutions result in amino acid changes.

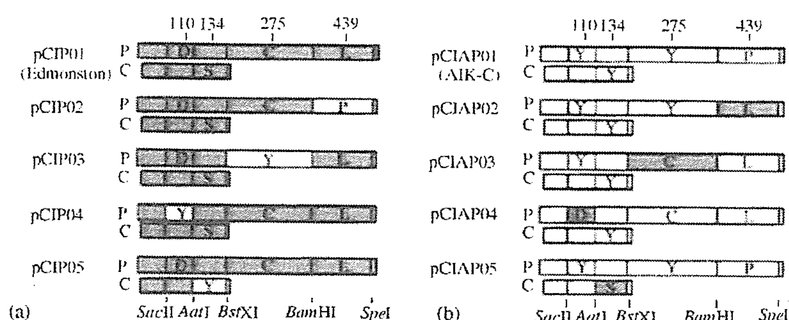

Fig. 3. Construction of chimera P genes. Eight chimera P gene plasmids were constructed. (a) pCIP02, 03, 04, and 05 were originated from pCIP01, the plasmid bearing the Edmonston P gene. In these plasmids, some regions of pCIP01 were replaced with the corresponding region of pCIAP01, the plasmid having the AIK-C P gene, using appropriate restriction endonuclease sites as shown in figure. The resultant plasmids expressed the Edmonston P protein bearing a mutation at amino acid 439 (pCIP02), 275 (pCIP03), and 110 (pCIP05) and the C protein with a mutation at position 134 (pCIP04). (b) pCIAP02, 03, 04, and 05 were originated from pCIAP01 and were changed in some regions to the corresponding region of pCIP01. The pCIAP02, 03 and 04 plasmids expressed the AIK-C P protein having a mutation at amino acid 439 (pCIAP02), 275 (pCIAP03), and 110 (pCIAP05) and the C protein with a mutation at position 134 (pCIAP04). Numbers indicate the amino acid position of the proteins.

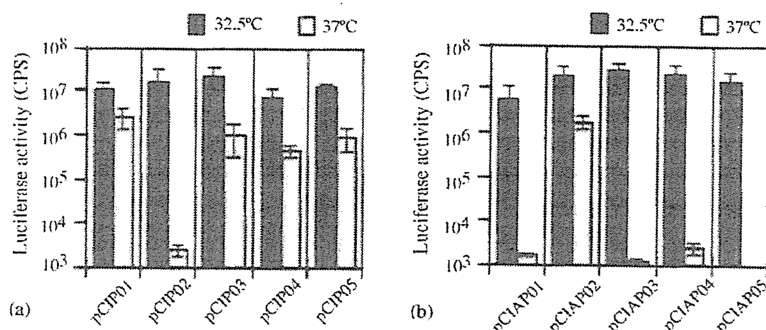

Fig. 4. Luciferase activity in the minigenome experiments using the chimera P plasmids. The vTF7-3 infected HeLa cells were transfected with minigenome RNA, pCIAN01, pCIAL01, and one of the chimera P plasmids. (c) pCIP01 and the derivative plasmids. (d) pCIAP01 and the derivative plasmids. The transfected cells were incubated at 32.5 (dark gray bar) or 37 °C (light gray bar) for 40 h and lysed to measure the luciferase activity. The luciferase activity (CPS) was counted by TopCount (Packard Instrument Company, Meriden, CT, USA). The experiments were carried out at 3 times and the mean values and ±1.0 S.D. were indicated.

pCIAP01 and pCIAL01 plasmids at 32.5 °C and the recombinant viruses were recovered. The P genes of the rescued viruses were amplified by RT-PCR and ascertained by direct sequencing. Each recombinant virus had the P gene corresponding to the transfected AIK-C genome cDNA. The recovered viruses were designated recAIK-C virus (derived from pMVAIK-C), recAIK/ED-P virus (pMVED-P), recAIK/P439-Leu virus (pMVAIK/P439-Leu), and recAIK/ED/P439-Pro virus (pMVED/P439-Pro), respectively.

To examine whether the P gene, especially P439-Pro, contributed to *ts* phenotype of AIK-C strain, the *ts* level of four recombinant AIK-C viruses were tested by plaque formation assay at 32.5, 36, 37, 38, 39 and 40 °C with Vero cells (Table 2). The recAIK-C virus showed the equal level of

Table 2
Efficiency of plaque formation in Vero cells at 32.5, 36, 37, 38, 39 and 40 °C

Virus (plasmid)	Amino acid at 439 position of the P protein	Virus titer ($\log_{10}$ PFU/ml) at an indicated temperature (°C)						Shut-off temperature (°C)
		32.5	36	37	38	39	40	
recAIK-C (pMVAIK-C)	Pro	5.6	5.4	5.3	4.7	<0.4	<0.4	39
recAIK/ED-P (pMVED-P)	Leu	6.7	6.7	6.6	6.4	6.2	5.4	>40
recAIK/P439-Leu (pMVAIK/P439-Leu)	Leu	6.6	6.5	6.3	6.2	5.8	<0.4	40
recAIK/ED/P439-Pro (pMVED/P439-Pro)	Pro	6.7	6.2	6.2	6.0	<0.4	<0.4	39
vacAIK-C	Pro	6.9	6.7	6.6	6.2	<0.4	<0.4	39
Edmonston	Leu	5.8	5.7	5.5	5.5	5.1	4.5	>40

Shut-off temperature is defined as the lowest temperature at which a 10^4-fold or greater reduction in titer observed.

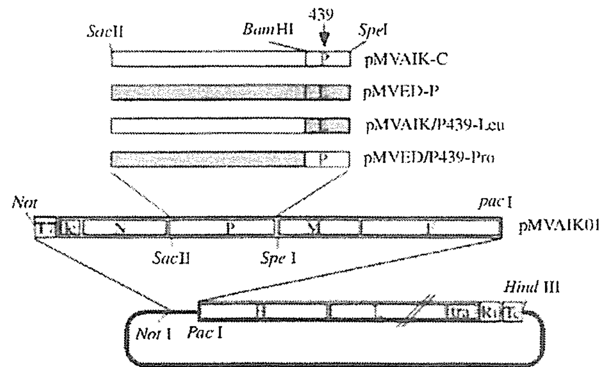

Fig. 5. Strategy for the construction of the recombinant AIK-C genome cDNAs that have various P genes. To rescue of the recombinant AIK-C viruses carrying various P proteins, three chimera cDNAs based on the AIK-C cDNA genome (pMVAIK-C) were constructed. To exchange the P gene of the full-genome cDNA, the *Not*I-*Pac*I fragment of pMVAIK-C was cloned into pUC193M-7 to give pMVAIK01. pMVAIK01 had unique restriction endonuclease sites, *Sac*II and *Spe*I sites, located at both ends of the P gene. The pCIP01 *Sac*II and *Spe*I fragment having the Edmonston P gene, pCIP02 *Sac*II and *Spe*I fragment having the Edmonston P gene containing a mutation that causes substitution of proline for leucine at position 439 of the P protein, and pCIAP02 *Sac*II and *Spe*I fragment having part of the AIK-C P gene with proline replaced by leucine at position 439 were inserted into the pMVAIK01 *Sac*II and *Spe*I site. The resultant plasmids were digested by *Not*I and *Pac*I restriction endonuclease and ligated into *Not*I-*Pac*I digested pMVAIK-C plasmid. These plasmid constructs were designated pMVED-P, pMVAIK/P439-Leu and pMVED/P439-Pro, respectively. Numbers indicate the amino acid position of the P protein: T7, T7 promoter; Ri, hepatitis delta virus ribozyme; Te, T7 terminator; le, leader sequence; tra, trailer sequence.

temperature-sensitivity with the vaccine AIK-C (vacAIK-C). In contrast, the recAIK/ED-P virus having Edmonston-P gene lost the *ts* phenotype, like Edmonston strain (shut off temperature at >40 °C), indicating that the P gene of AIK-C strain contribute to the *ts* phenotype. Further, recAIK/ED/P439-Pro (carrying Edmonston-P gene with P439-Pro) virus also exhibited the equal level of *ts* with recAIK-C and vacAIK-C (shut off temperature at 39 °C). These results showed that P439-Pro is crucial to the *ts* phenotype of strain AIK-C. In addition, recAIK/P439-Leu virus (carrying AIK-P gene with P439-Leu) showed the moderate level of *ts* phenotype (shut of temperature at 40 °C), suggesting that other amino acid substitutions found in the P gene of AIK-C strain might modulate the expression of the phenotype.

4. Discussion

Further attenuated live MV vaccines are widely used due to their high effectiveness and low incidence of adverse reactions and play a central role for prevention of measles infection in the world. Most of them, such as Edmonston–Zagreb, Moraten, Schwarz and AIK-C strains were attenuated by the classical and empirical approach from the Edmonston strain but genetic basis of the attenuation of those vaccines has not been well understood.

Strain AIK-C exhibits the *ts* property (shut off temperature at 39 °C). This property is unique to AIK-C strain among MV vaccines. There is little direct evidence that *ts* phenotype of AIK-C contributes for its attenuation, due to lack of an appropriate experimental animal model to evaluate MV pathogenesis. However, since the *ts* mutant is less vigorous in their growth at higher temperature than the parental virus, it is conceivable that this phenotype plays a part in viral attenuation. In fact, *ts*-mutant of some negative strand RNA viruses, such as Influenza A, human parainfluenza type 3 (HPIV 3) and respiratory syncytial virus (RSV) were evaluated as attenuated viruses in animal experimental model [24–27]. Thus, we considered that the *ts*-related mutation of AIK-C might involve attenuation of AIK-C strain. This study is the first report to identify the *ts* related-mutation of currently available MV vaccines at molecular level using reverse genetics.

Although the AIK-C stain propagated well at 37 °C, the minigenome system conducted with the AIK-C protein expression plasmids did not work at 37 °C. The reason of the discrepancy was not analyzed well at present. The minigenome system was considered to represent measles virus replication and transcription process, but not identical to the ordinary processes, because, at least, T7 RNA polymerase-expressing vaccinia virus was co-existed in order to express the N, P and L proteins in the system. Furthermore, due to high transcriptase activity of T7 RNA polymerase, amount and ratio of N, P and L proteins could be different from measles virus replication. It is mere speculation that these differences might make the minigenome system more sensitive to temperature than ordinary virus replication.

We identified the P439-Pro as the *ts* point of AIK-C strain. Recently, the nucleotide sequences of five Edmonston-derived vaccine strains including AIK-C, Moraten, Schwarz, Rubeovax and Edmonston–Zagreb were reported and compared with that of low-passage, wild-type Edmonston virus to analyze the genetic bases of attenuation of MV vaccine strains [28,29]. Some common amino acid changes among all vaccines were considered to play important roles for viral attenuation. Additional vaccine specific substitutions were considered to determine the property or attenuated level of individual strains. The P439-Pro was reported to be a unique substitution to AIK-C strain among other MVs, suggesting the molecular basis of peculiar phenotype to AIK-C strain.

The mechanism how P439-Pro contributes to the phenotype has not been analyzed. The P protein is considered to be multifunctional protein. It acts for viral RNA synthesis as a unit of RNA polymerase together with L protein as well as viral RNA encapsidation in company with N protein [30]. Thus, the P protein appears to play a central part of viral RNA synthesis. On the other hand, the P protein also regulates the N protein localization in cell [31]. The C-terminal region (including the position 439) of the P protein is essential for the interaction of N protein together with the extreme amino-terminal residue of the P protein, P–P self-interaction and L protein interaction [32–34]. These interactions are considered to be important for viral RNA synthesis. Thus, this

region is significant for exhibition for P function. It is mere conjecture that the substitution from Leu to Pro at position 439 of P protein might reduce the ability to bind these proteins at higher temperatures, and consequently, impair viral genome replication and/or mRNA synthesis.

The recAIK/P439-Leu virus showed a moderate *ts* level (shut-off temperature at 40 °C) (Table 2). This suggested the possibility that the other *ts* related-amino acid substitutions might exist in the P gene of AIK-C, besides P439-Pro. Sequence comparison of the various MV P gene showed that the P275-Tyr, P439-Pro, and C134-Tyr substitutions were characteristic to AIK-C strain. Conversely, P/V110-Tyr was conserved in all MVs as well as other moribilliviruses including canine distemper virus (CDV) [35], rinderpest virus (RV) [36] and Phocid distemper virus (PDV) [37]. The P/V110-Asp seems to be a peculiar mutation of our laboratory Edmonston strain, suggesting that P/V110-Tyr might have no contribution to the *ts* phenotype. Recently, the roles of C protein have been scrutinized using the reverse genetics. The recombinant MV defective in C protein showed that the C protein was dispensable for growth in Vero cells [38], but essential in human peripheral blood cells [39], played some roles in MV pathogenesis in vivo [4,5], and inhibited the type I interferons response [6]. Some mutations in the C protein of MV increase the RNA synthesis [40] and may relate to cell tropism [41]. These results indicate that the C protein is involved in the process of viral RNA synthesis and with a representation of virus phenotype. Thus, it is possible that the amino acid substitution in the C protein of AIK-C may affect the viral character at higher temperature. Anyhow, recombinant viruses recovered from modified AIK-C genome cDNAs might be necessary to examine the contribution of other mutations found in AIK-C P gene to *ts* phenotype.

We elucidated the mutation related to the *ts* characteristic, and probably having some connections with attenuation of AIK-C vaccine strain in this study. Other *moribillivirueses*, such as CDV, PDV, and RV are formidable pathogens in animals like a MV in human. Among these viruses, the P439-Leu and the surrounding sequence are well conserved. This implies it would be possible to add a *ts* phenotype (and probably an attenuation phenotype) to those viruses by changing leucine to proline at that position in the P protein. Current reverse genetics technology of negative strand RNA virus will facilitate to introduce the mutation on the RNA genome. Recovering viruses will be candidates for live virus vaccine.

The nucleotide sequence data reported in this paper is available in DDBJ/EMBL/GenBank nucleotide sequence databases with accession no. AB046113 (AIK-C genome cDNA), AB046218 (Edmonston-P gene), AB052821 (Edmonston-N gene), and AB052820 (Edmonston-L gene).

Acknowledgments

We thank B. Moss, G. Sutter, and H. Hashimoto for providing us with vaccinia viruses and AIK-C cDNAs. We thank Y. Morikawa (Kitasato University) for review of the manuscript. This work was supported by Grants-in-Aid for Scientific Research B, All Kitasato Project Study (AKPS), and a Grant from the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Passage in Vero cells alters the characteristics of measles AIK-C vaccine strain

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Received 4 January 2005; received in revised form 20 June 2005; accepted 17 August 2005
Available online 7 September 2005

Abstract

Objective of this study is to evaluate the feasibility of measles vaccine production in Vero cell culture. We constructed the full-length cDNA, pIC-MVAIK-F278Leu (small plaque-type) and pIC-MVAIK-F278Phe (large plaque-type) from the AIK-C measles vaccine strain attenuated from the Edmonston wild-type. MVAIK-S/B2 was rescued from pIC-MVAIK-F278Leu after two passages in B95a cells and MVAIK-SL/B2V1 was obtained through large plaque cloning in Vero cells. MVAIK-SL/B2V8 was obtained after eight passages in Vero cells. It produced large plaques in Vero cells, grew well at 39 °C, and thus the characteristics of the AIK-C vaccine strain were lost. Thirteen amino acid changes were observed; one in the N, two in the P, one in the C, three in the F, one in the H, and five in the L protein regions. Twelve of these changes excluding one in the L gene were back mutated to the Edmonston strain. Change from Leu to Phe at position 278 of the F protein was an early event during adaptation to Vero cells and the P gene was back-mutated to the Edmonston wild-type. As for the control, MVAIK-L/B9 strain was obtained after passages in B95a cells from pIC-MVAIK-F278Phe (large plaque-type). It maintained the same temperature sensitivity as the AIK-C vaccine strain and only four amino acid changes, one in the N and three in the L protein region, were observed without any mutations in the P, C, M, F, and H genes. The passage of the measles vaccine AIK-C strain in Vero cells lost the characteristics of small plaque inducibility and temperature sensitivity (*ts*) phenotype.
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Keywords: Measles virus; Temperature sensitivity; Measles vaccine; Vero cells; Plaque assay; Reverse genetics

1. Introduction

Measles virus is a member of the genus Morbillivirus of the family Paramyxoviridae and order Mononegavirales and consists of 15,894 nucleotides, coding six structural proteins. The nucleoprotein (N), phosphoprotein (P), large protein (L) and genome RNA constitute the ribonucleoprotein complex (RNP). Two glycoproteins of hemagglutinin (H) and fusion (F) are present as envelope spikes and they work in the process of virus attachment and cell fusion. The M protein acts in virus assembly and maturation [1]. The establishment of a rescue system of the infectious virus from cDNA of measles virus was reported [2,3]. We developed the rescue system of infectious virus from cDNA of the AIK-C measles vaccine strain [4]. Recent developments in reverse genetics of Mononegavirales have contributed to better understanding of the mechanisms of virus replication, transcription, and pathogenesis [5].

The measles vaccine strain AIK-C was developed from the Edmonston strain through plaque cloning in sheep kidney cells and chick embryonic cells at 33 °C [6,7]. It shows optimal growth at 33 °C with small plaques but extremely poor or no growth at 39–40 °C, demonstrating temperature sensitivity (*ts*). We reported that Leu at position 278 of the F protein was responsible for small plaque formation in Vero

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0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.08.082

cells and Phe at 278 of the F protein of the Edmonston strain induced large plaques in Vero cells [4]. The AIK-C strain was a mixture of two virus genomes, having Leu or Phe at position 278 of the F protein gene although it did not influence the *ts* characteristics. The P protein of the AIK-C strain was responsible for the *ts* characteristics and Pro at position 439 of the P gene significantly contributed to the *ts* phenotype from the results of minigenome and reverse genetic assays [8]. Recently, wild measles virus strains were isolated in B95a cells and showed no CPE in Vero cells [9]. Measles virus isolated in B95a cells had full pathogenicity in cynomolgus monkeys and no pathogenicity after adaptation in Vero cells [10]. During the adaptation process, several amino acid changes occurred: two amino acid changes in the P and V regions, one in the C protein, three in the H region, and two in the L region, but the critical regions responsible for pathogenesis were not identified [10].

The AIK-C strain is one of the candidate vaccine strains for the Expanded Programme on Immunization (EPI) to overcome maternally conferred immunity, resulting in a high sero-conversion rate in young infants at 6 months of age [11–13]. A vaccine manufacturer intended to produce the AIK-C measles vaccine in large scale using Vero cells for intra-nasal usage but we have no information on the stability of the characteristics of the AIK-C through passage in Vero cells.

In this study, we investigated the genetic stability of infectious virus recovered from cDNA with Leu at position 278 of the F protein, which induced small sized plaques, and purified large plaques by plaque cloning in Vero cells. After several passages in Vero cells the original *ts* phenotype was lost. For comparison, infectious virus was rescued from cDNA having Phe at position 278 of the F protein and propagated in B95a cells. We compared the characteristics of infectious virus rescued from the full-length cDNA after different passage histories in Vero cells or B95a cells.

2. Materials and methods

2.1. Measles virus strains

The AIK-C seed strain was used for vaccine production. The AIK-C strain was attenuated from the wild Edmonston virus kindly provided by the late Prof. Enders [6,7].

2.2. Construction of full-length cDNA of AIK-C and recovery of the recombinant virus

We constructed two full-length cDNA plasmids of the AIK-C vaccine seed strain as previously reported [4,14]. Briefly, we constructed two parts; the first half consisted of AIK-C cDNA from the leader sequence to the *Pac* I site at nucleotide position 7238 of the AIK-C genome and the second half of the AIK-C cDNA consisted of the H and L regions from the 7238 *Pac* I site to the trailer sequence. To change the amino acid at position 278 from Leu to Phe, a fragment of the recombinant plasmid derived from the Edmonston strain was used. The construct was designated as pIC-MVAIK-F278Phe and the original AIK-C cDNA as pIC-MVAIK-F278Leu. For the recovery of the recombinant measles virus, B95a cells were infected with MVAT7 pol., nonreplicative vaccinia virus expressing T7 RNA polymerase kindly provided by Dr. G. Sutter, and then pAIK-N, pAIK-P, pAIK-L and pIC-MVAIK-F278Leu (small plaque-type) or pIC-MVAIK-F278Phe (large plaque-type)were transfected, as reported previously [4]. B95a cells were cultured at 32.5 °C in 5% CO_2 and through two blind passages in B95a cells, typical cytopathic effect (CPE) was observed. The strains rescued from the respective cDNA were designated as MVAIK-S/B2 from pIC-MVAIK-F278Leu and MVAIK-L/B2 from pIC-MVAIK-F278Phe.

MVAIK-S/B2 was infected into Vero cells and the wells were overlayed with 0.5% agar in minimum essential medium (MEM) supplemented with appropriate antibiotics and 5% fetal calf serum (FCS). After 7 days of culture at 33 °C, three large plaques were cloned and passaged in Vero cells. After eight passages in Vero cells at 33 °C, MVAIK-SL/B2V8 was obtained. MVAIK-L/B2 was further propagated in B95a cells and through seven additional passages at 33 °C, and MVAIK-L/B9 was obtained (as shown in Fig. 1).

2.3. Plaque and infectivity assays

Monolayers of Vero cells were inoculated with measles virus or virus strains rescued from full-length cDNA and were overlayed with 0.5% agar in Eagle's MEM supplemented with antibiotics and 5% FCS, as previously reported [14]. After 7 days of incubation in 5% CO_2 at 37 °C, the cells were fixed with 0.25% glutaraldehyde. Hyperimmune rabbit antibodies to measles virus were added to the plate, followed by, after vigorous washing, biotinylated goat antibodies to rabbit IgG. The plates were stained with an VECTASTAIN Elite ABC kit (VECTOR Labo., Burlingame, CA). To examine the growth of infectious virus, monolayers of Vero cells were infected with the AIK-C, Edmonston, MVAIK-L/B9 and MVAIK-SL/B2V8 (approximately m.o.i. = 0.01). The culture fluid and infected cells were obtained at days 1, 3, 5 and 7 of culture and the infectivity in B95a cells was expressed as TCID50/ml.

2.4. Cloning of the P, F, H gene, sequence analysis, and expression experiments

Coding regions of the N, P, M, F, H, and L proteins were divided into several fragments which were amplified from genomic RNA using positive sense primers at the RT step of RT-PCR [15] and sequenced by a dye terminator method using the ABI 377A sequencer (Applied Biosystems, Foster City, CA, USA). The F and H gene expression plasmids were constructed from cDNA synthesized from genomic RNA of MVAIK-SL/B2V8 and expression experiment was carried

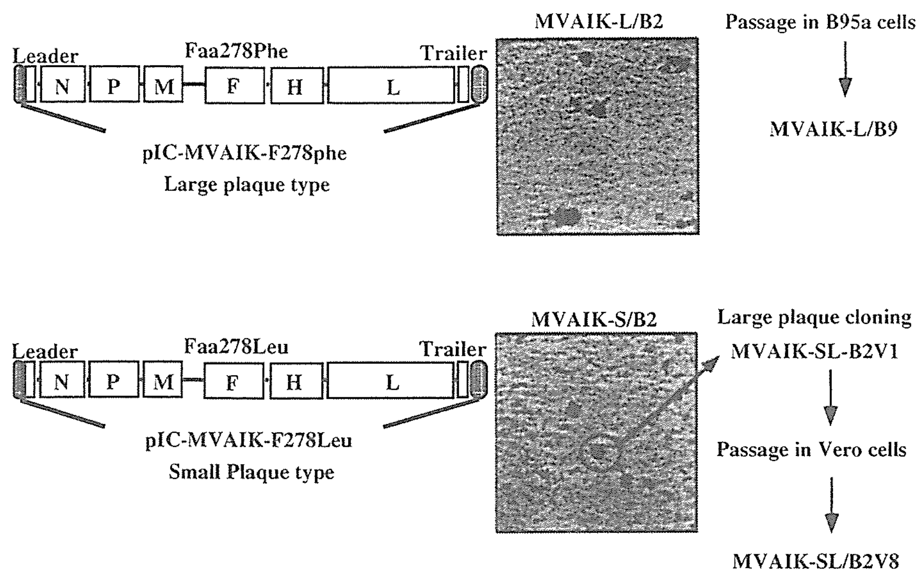

Fig. 1. Scheme of construction of recombinant viruses and passage history of rescued virus strains.

out under the control of T7 RNA polymerase [4,14]. PCR products of the P gene amplified from genomic RNA of MVAIK-SL/B2V8 and MVAIK-SL/B2V4 were cloned and sequenced.

3. Results

3.1. Construction of infectious clone and propagation of viruses

We constructed two infectious cDNAs having Leu (pIC-MVAIK-F278Leu) or Phe (pIC-MVAIK-F278Phe) at position 278 of the F protein gene. After two passages in B95a cells, infectious viruses were recovered and designated as MVAIK-S/B2 and MVAIK-L/B2, respectively (Fig. 1). MVAIK-L/B2 induced large plaques in Vero cells and MVAIK-L/B9 was propagated after seven additional passages in B95a cells. MVAIK-S/B2 induced mainly small or medium sized plaques in Vero cells and three large plaques were isolated and propagated independently in Vero cells (MVAIK-SL/B2V8).

3.2. Temperature sensitivity and plaque formation

The original AIK-C measles vaccine strain had *ts* and did not grow at 39–40 °C but grew well at 33 °C with small and medium size plaques in Vero cells. We reported that Leu at position 278 of the F protein was responsible for small plaques [4]. Plaque formation assay and virus growth analysis at different temperatures were carried out in Vero cells and the results are shown in Fig. 2. MVAIK-L/B9 induced large plaques in Vero cells. MVAIK-S/B2 induced mainly small plaques but some large plaques were demonstrated as shown in Fig. 1. MVAIK-SL/B2V1 was isolated through large plaque cloning in Vero cells and MVAIK-SL/B2V8 was propagated after additional eight passages in Vero cells. MVAIK-L/B9 and MVAIK-SL/B2V8 were inoculated in Vero cells at the m.o.i. of 0.01 and culture fluid was collected at days 1, 3, 5 and 7 of culture. Cells were freeze-thawed with 400 μl of MEM supplemented with 5% FCS and after sonication they were stocked at −70 °C. Infectivity was assayed in B95a cells and the results are shown in Fig. 2. MVAIK-L/B9 grew well at 33 °C but no virus grew at 39 °C in the culture fluid. Infectious particles were detected at 33 °C but none at 39 °C in the cell lysate. Large plaques were produced at 33 °C but not at 39 °C. MVAIK-SL/B2V8 grew well at 33 °C but no infectious virus was detected in the supernatant at 39 °C. Cell-associated infectious virus was recovered at 39 °C as well as 33 °C on day 3 of culture but reduced after day 5 of culture at 39 °C. MVAIK-SL/B2V8, which was propagated through eight passages in Vero cells, lost *ts* and produced large plaques in Vero cells at 39 °C as well as at 33 °C.

3.3. Nucleotide differences in MVAIK-L/B9 and MVAIK-SL/B2V8 strains in comparison with the original infectious clones (pIC-MVAIK-F278Leu and pIC-MVAIK-F278Phe)

Nucleotide sequences of six coding regions, N, P, M, F, H, and L genes, were investigated and the deduced amino acids that differed from the original cDNA of AIK-C are depicted in Table 1. MVAIK-L/B9 had five nucleotide changes with four amino acid differences; one at amino acid position 129 of the N protein (N129), and three in the L gene (L 371, L 542 and L 2032). MVAIK-SL/B2V8 had 16 nucleotide changes with 13 amino acid differences; one in the N protein, two in the P, one in C, three in the F, one in the H, and five in the L. Three nucleotide changes were observed in the P gene region with two amino acid changes at the P protein and with one

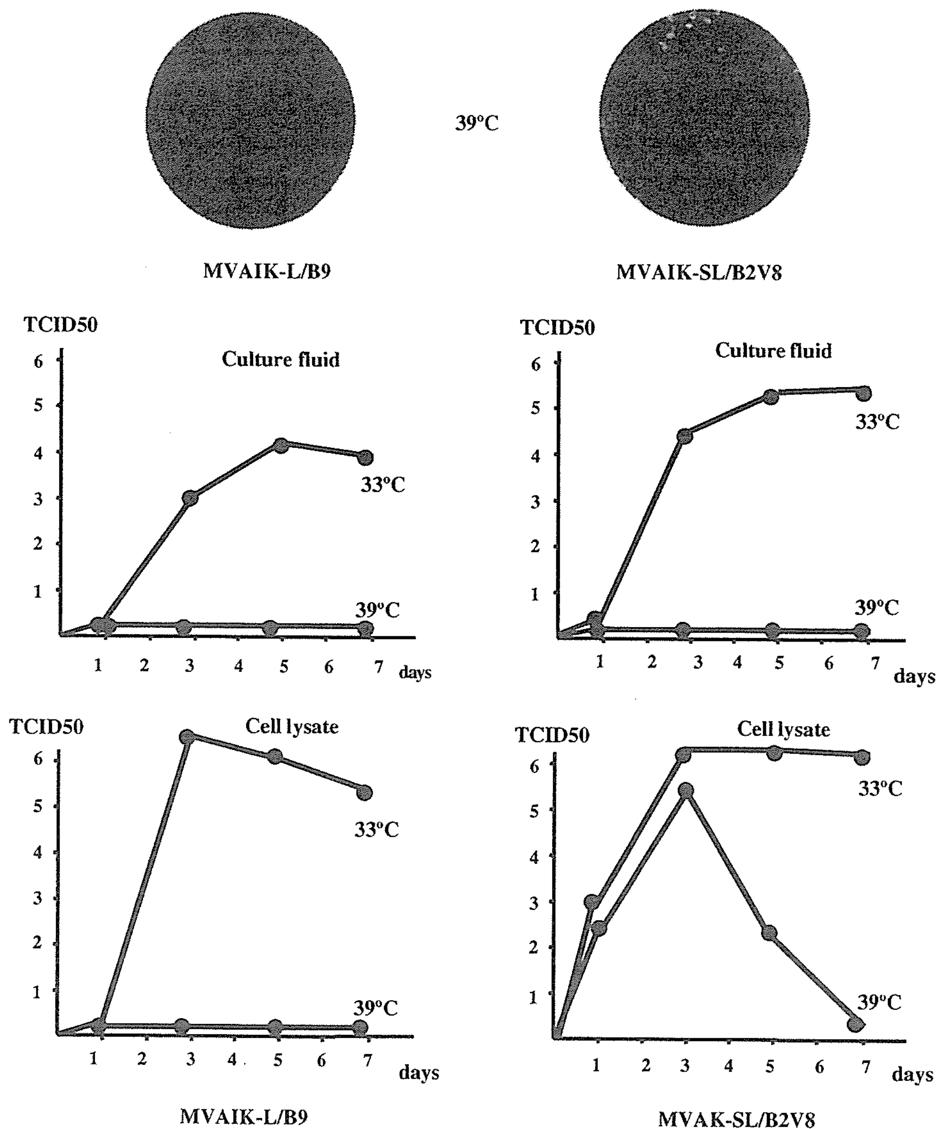

Fig. 2. Virus CPE and virus growth in Vero cells at different temperatures for MVAIK-L/B9 and MVAIK-SL/B2V8. Infectivity was examined in B95a cells.

of the C protein. Three amino acid substitutions, Lys N129 Gln (from Lys to Gln at the position 129 of the N protein), Ala L542 Thr and Gly L2032 Arg, were common in MVAIK-L/B9 and MVAIK-SL/B2V8. Amino acid changes except for the position 2032 of the L gene were all back-mutations to the Edmonston strain. MVAIK-SL/B2V8 had 13 amino acid changes that were the same as the Edmonston strain except for the position L2032. One silent mutation was observed in MVAIK-L/B9 and three in MVAIK-SL/B2V8.

MVAIK-SL/B2V8 had one amino acid change at the position 338 of the H protein region and three at the positions 278, 453, and 494 of the F protein region. The change at position 278 of the F protein region, which was critical for plaque size, was noted soon after large plaque cloning. MVAIK-SL/B2V2 had Phe at position 278 of the F region. We constructed the F and H expression plasmids from genomic RNA of MVAIK-SL/B2V8 and seven plasmids for F gene and five for H genes showed the same mutations as demonstrated in Table 1. These were expressed under the control of T7 RNA polymerase with no difference in the extent of cell fusion, which was demonstrated at 39 °C as well as at 33 °C (data not shown). Thus, the loss of *ts* was not related to the mutation in the F and H regions.

We cloned P gene region from genomic RNA of MVAIK-SL/B2V8 and all 11 clones showed the same mutations as shown in Table 1. Seven clones of the P gene from MVAIK-SL/B2V4 also showed the same mutations at the positions of C134, P275 and P439.

4. Discussion

The currently available further attenuated live measles vaccine strains, Moraten, Schwarz, Edmonston-Zagreb, and AIK-C, were developed from the Edmonston strain through extensive passages in different cells from susceptible primary

Table 1
Nucleotide differences and deduced amino acid changes of MVAIK-L/B9 and MVAIK-SL./B2V8 in comparison with the AIK-C vaccine strain and parental Edmonston strain

NT position (AA position)	Virus strain			
	AIK-C	Edmonston	MVAIK-L/B9	MVAIK-SL/B2V8
492 (N 129)	A (Lys)	C (Gln)	C (Gln)	C (Gln)
2229(C 134)	A (Tyr)	C (Ser)	*	C (Ser)
2630 (P 275)	A (Tyr)	G (Cys)	*	G (Cys)
3122 (P 439)	C (Pro)	T (Leu)	*	T (Leu)
6291 (F 278)	A (Leu)	C (Phe)	*	C (Phe)
6815 (F 453)	T (Leu)	C (Ser)	*	C (Ser)
6937 (F 494)	T (Cys)	A (Ser)	*	A (Ser)
8282 (H 338)	A (Thr)	C (Pro)	*	C (Pro)
10344 (L 371)	T (Trp)	A (Arg)	A (Arg)	*
10857 (L 542)	G (Ala)	A (Thr)	A (Thr)	A (Thr)
11158 (L 642)	G (Arg)	A (Gln)	*	A (Gln)
I1711 (L 826)	G (Ser)	A (Ser)	*	A (Ser)
12671 (L 1146)	A (Ala)	G (Ala)	G (Ala)	G (Ala)
14651 (L 1806)	A (Lys)	G (Lys)	*	G (Lys)
15039 (L 1936)	T (Tyr)	C (His)	*	C (His)
15327 (L 2032)	G (Gly)	G (Gly)	C (Arg)	C (Arg)
15622 (L 2130)	C (Thr)	T (Ile)	*	T (Ile)

(*) Represents no nucleotide change and underline shows the silent mutation.

cells used for virus isolation [16]. Differences in nucleotides of the Edmonston-derived vaccine strains were reported but the molecular basis of the attenuation has not been well defined as yet [17]. The AIK-C strain was developed through small plaque cloning in sheep kidney cells and chick embryonic cells and has two biological markers; small plaques in Vero cells and extremely low or no virus growth in culture at 39–40 °C. We reported that Leu at 278 position of the AIK-C virus F gene is related to the small plaques in the Vero cells and Phe at 278 of the F gene induces large plaques [4]. RNA virus has a nature of quasispecies and the AIK-C seed virus induced a mixture of small and medium sized plaques in Vero cells, having F278Leu (small plaque-type) and F278Phe (large plaque-type). The characteristics of temperature sensitivity depend upon the P protein, especially Pro at 439 of the P protein [8]. No infectious measles particle was detected in the culture medium of Vero cells at 39 °C culture infected with MVAIK-SL/B2V8 but infectious virus was obtained in cell lysate. It suggested that *ts* phenotype was lost during the passage in Vero cells (MVAIK-SL/B2V8). This was also found for the recombinant virus which the P protein of the AIK-C strain was replaced by that of the Edmonston strain [8]. Recombinant virus strains (MVAIK-SL/B2V8, MVAIK-Edm-P, and MVAIK-AK/Edm-P) produced infectious particles in the cell lysate but not in supernatants. We could not identify the reason why these strains have cell-associated characteristics at 39 °C culture.

As for the vaccine production, passages are limited to five times from the seed virus to prevent the accumulation of mutations. Most RNA viruses are considered to be a mixture of heterogeneous viruses because of their high incidence of mutation in the RNA genome in nature [18]. The seed of the AIK-C strain was established through plaque purification but may consist of heterogeneous viruses. In contrast, MVAIK strains rescued from the cloned cDNA are thought to be rather homogenous single genome species even after two passages in B95a cells. To investigate the genetic stability of recombinant measles virus rescued from the cloned cDNA, virus was passaged in Vero cells and B95a cells. In Vero cell passage, MVAIK-SL/B2V8 had a total of 13 amino acid differences; one in the N protein, two in the P, one in the C, three in the F, one in the H, and five in the L genes. The significance of the amino acid positions of 278 of the F protein and 439 of the P protein were elucidated in this report. In B95a cell passage, MVAIK-L/B9 had four amino acid differences; one in the N protein and three in the L gene. Four mutations observed for the MVAIK-L/B9 were common in the MVAIK-SL/B2V8 and all mutations were back mutations to the Edmonston strain except at position 2032 of the L protein region. Mutations have a fixed tendency of back-mutation to the parental wild-type Edmonston strain, not showing random mutation and the rate of mutation was higher through passages in Vero cells than in B95a cells.

The establishment of further attenuated live vaccines is thought as a selection of a mutant virus that can adapt to grow in different cell cultures from susceptible hosts. Vaccine seed might have a mixture of quasispecies of genomes and an extremely low proportion of minority genome was present as their past dominance. In biological adaptive systems, evolving viral quasispecies possessed a molecular memory in the form of minority components that populate their mutant spectra [18–20]. These were demonstrated in the selection of biological clones of foot-and mouth disease virus (FMDV) resistant to a monoclonal antibody [19]. Domingo et al. [18] reported that memory was a property of the quasispecies as a whole, and not the result of an inherent tendency of the dominant genomes to mutate to the memory genomes.

In this study, we started to generate a single genome construct from the vaccine seed strain having representative biological characteristics; introducing small plaques in Vero cells and *ts* phenotype. Infectious virus was recovered after two passages and was further propagated in Vero cells or B95a cells. After eight passages, it showed mutations in the fixed direction to the original Edmonston strain as a memory of a past attenuation process through passages at lower temperatures and different cells. Passage in B95a cells showed to induce less mutations and the genome soon after the rescue of the virus seems to be homogeneous. Large plaques were produced at the first passage in Vero cells and we selected a large plaque generated from the small plaque type genome. Thus, early genomic changes were supposed to be a mutation rather than selection. From the results of cloning experiments of the F and P gene, the critical mutations were demonstrated at early passage history. We speculate that the genome had a memory of the attenuation process during the passage in different cell lines. The mutated positions were genetically unstable and could be easily back-mutated in Vero cells.

Infectious virus was recovered in B95a cells and additional eight passages in B95a cells did not influence the biological phenotype without mutation in the P genes related to the *ts* phenotypes. However, in the Vero cells large plaques were observed at the first passage and cloned virus had Phe at position 278 of the F gene.

In summary, recombinant virus recovered from infectious cDNA clone was back-mutated to the parental Edmonston strain, losing *ts* phenotypes and small plaque inducibility after passages in Vero cells and we speculated that the genome had past mutation memory.

Acknowledgement

This study was supported in part by a Grant of the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Efficacy of inactivated trivalent influenza vaccine in alleviating the febrile illness of culture-confirmed influenza in children in the 2000–2001 influenza season

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Received 7 May 2004; received in revised form 8 February 2005; accepted 25 January 2006
Available online 28 February 2006

Abstract

During the 2000/2001 influenza season in Japan, children ranging in age from 6 months to 13 years with fever exceeding 37.5 °C were recruited. Vaccine efficacy was evaluated by comparing the rates of pre-seasonal vaccination between groups stratified by fever severity. Seven hundred and sixty one patients (33.1%), culture positive for influenza were enrolled for analysis. The numbers of patients for A/H1N1 and A/H3N2 were insufficient for statistical analysis. For influenza B the odds ratio for vaccinated children to have a maximum fever exceeding 39.5 °C was 0.52 (95% CI, 0.30–0.92) Our findings suggest modest impact of influenza vaccination on limiting severity of disease symptoms.
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Keywords: Inactivated influenza vaccine; Efficacy on febrile illness; Culture-confirmed influenza

1. Introduction

Influenza causes yearly epidemics and is a major cause of lower respiratory tract illness in young children. Annual influenza attack rates vary from 10 to 30% in adults and 20 to 50% in children [1,2]. Earlier studies suggest the risk of serious complications and hospitalizations due to influenza infection are high in young children [3–5].

The FDA approved a live attenuated intranasal vaccine in 2003. Because its safety has not been established in high-risk individuals, it is not available for children under 5 years of age [6]. They can only be given inactivated vaccine at present.

Inactivated influenza vaccine protects against the illness and reduces its severity in adults and older children [7–10], but evidence of its efficacy in early childhood is limited, especially in respect to alleviation of symptoms.

In Japan many cases of influenza-related encephalopathy have been reported recently [11]. Vaccination is expected to

Abbreviations: CI, confidence interval; OR, odds ratio; MDCK, Madin–Darby canine kidney; PBS, phosphate-buffered saline; CPE, cytopathic effects; PAP, peroxidase–antiperoxidase; CMH, Cockran–Mantel–Henszel; *F*, Fisher' exact test; ACIP, Advisory Committee on Immunization Practices; SAGPJ, Society of Ambulatory and General Pediatrics of Japan

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doi:10.1016/j.vaccine.2006.01.067

reduce the incidence and severity of complications, including influenza-related encephalopathy.

Inactivated influenza vaccine does not give an ideal immunization to prevent infection completely. It would be better to estimate the efficacies of vaccination on prevention and on alleviation of severity of illness separately. In this study we tried to estimate the efficacy of inactivated influenza vaccine in alleviating the febrile response in culture-confirmed influenza in children.

2. Materials and methods

2.1. Study design

This study was conducted as a case-control, multicentered study of twenty-one pediatric clinics during the 2000/2001 influenza season in Japan.

Febrile children were recruited consecutively until the planned number assigned to each institution was reached. Cases with culture-confirmed influenza were enrolled for analysis. Subjects were stratified to two or three groups based on the severity of fever. The proportion of pre-seasonal vaccination in each stratum was compared statistically.

The purpose of the study was explained to parents or guardians of all patients and signed consent was obtained.

2.2. Subjects

During 1 December 2000 to 30 April 2001 each collaborator started when the beginning of the outbreak was recognized and stopped when the allocated number was reached. Children ranging in age from 6 months to 13 years who had a febrile illness of over 37.5 °C that lasted for fewer than 3 days were recruited. To avoid potential selection bias, recruitment was performed consecutively and regardless of influenza-like symptoms. Patients given two doses of pre-seasonal vaccine with an adequate interval and those given no pre-seasonal vaccine were enrolled as vaccinated cases and as unvaccinated cases, respectively. The following were excluded: patients given only one dose of vaccine; those given two doses with less than two weeks between doses; those with illness onset within two weeks after the second vaccine; those having a history of underlying chronic illness; and those having an apparently different febrile illness.

2.3. Virus isolation

Nasal or throat swabs, or nasal aspirates were collected from all patients within 72 h after the onset and they were stored at −20 °C and subsequently transported to the laboratory for virus isolation.

Samples were centrifuged at 3000 rpm for 30 min at 4 °C and the supernatants were inoculated onto Madin–Darby (MDCK) cells in 24-well microplates. After removal of the specimens and washing with phosphate-buffered saline (PBS), the cells were covered with Eagle' minimal essential medium containing 2 μg/ml acetylated trypsin and 2.5 μg/ml amphotericin B. Cultures were incubated for 7 days at 35 °C and were observed for the development of cytopathic effects (CPE). The fluids of cultures with complete CPE were harvested and stored at −20 °C for the identification of isolated strains.

For subtype differentiation, the peroxidase–antiperoxidase (PAP) staining method was performed [12]. MDCK cells in 96-well microplates were inoculated with the isolated strains in triplet and plates were incubated for 20 h at 35 °C. The cells were fixed with absolute ethanol and then treated with two subtype-specific (H1N1 and H3N2) and B type-specific monoclonal antibodies, and rabbit anti-mouse immunoglobulin. The cells were treated successively with goat anti-rabbit immunoglobulin G antibody and PAP (rabbit antiperoxidase) complex. Finally, a peroxidase reaction with 0.01% H_2O_2 and 0.3 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in PBS was allowed to develop for 5 min. The cells were then rinsed with tap water and dried. The stained cells were observed under an ordinary light microscope.

Patients with influenza infection confirmed by virus isolation were enrolled as subjects for analysis.

2.4. Vaccination

The vaccine was inactivated trivalent HA split vaccine including Influenza A/NewCaledonia/20/99, A/Panama/2007/99 and B/Yamanashi/166/98, those were strains recommended by WHO and adopted by the Japanese government. Two doses of vaccine were administered more than two weeks apart. Different doses of vaccine were given according to Japanese standards; 0.1 ml for children 6–11 months of age, 0.2 ml for 1–5 years of age and 0.3 ml for 6–13 years of age.

2.5. Record of symptoms

Each parent or guardian of all patients was asked to record body temperature and other symptoms daily during the illness on formatted sheet.

2.6. Statistical analysis

Subjects were stratified to two or three strata by severity of febrile illness defined by degree of maximum body temperature during the illness or by the number of days with fever in each case. The vaccine effects were evaluated by comparing the rates of vaccinated children for each stratum of fever level.

First, we stratified subjects into two groups by the level of maximum body temperature during the illness in each case. Four trials of analyses for this purpose were performed using different cutoff points of body temperature including three points dividing the patients into equal quartiles (25% tile: 39.1 °C, 50% tile: 39.3 °C, 75% tile; 39.8 °C) and 39.5 °C as the dip point in a histogram of maximum body temperature.

Second, we stratified the patients into two or three groups by the number of days having a fever higher than several certain points.

The rates of pre-seasonal vaccination were compared between groups of different febrile severity.

The difference in proportions was evaluated using Fisher's exact test. Two-sided 95% confidence intervals were calculated on the odds ratios. Cochran–Mantel–Haenszel test was used to estimate the adjusted odds ratio. Proportional odds regression model was used for ordered categorical outcome such as severity of fever. All analyses were performed with SAS software, version 8.2 (SAS Institute Cary, NC).

3. Results

Twenty-one pediatricians in private clinics and hospitals in Japan participated in this study. All of 2814 children who met the inclusion criteria were registered and 2300 of them were enrolled. Others were excluded mainly because of incomplete information. Specimens for virus culture were collected from all patients and 761 cases (33.1%) proved to be culture positive for influenza and were included in the analysis. A/H1N1 was isolated in 167, A/H3N2 in 93 and B in 501 (Table 1).

The distribution of the patients' age was analyzed in the patients positive for virus isolation, A/H1N1, A/H3N2, B and negatives. In patients infected with influenza B, the proportion of older patients seemed to be greater, compared with the other groups.

In patients who were culture negative for influenza, there was no significant difference in the distribution of maximum body temperature during illness between the vaccinated and unvaccinated groups, which suggested that there was no major problem in sampling and sensitivity of virus culture.

There were insufficient cases of A/H1N2 and A/H3N2 for statistical analysis, so vaccine efficacy was analyzed only for influenza B.

We analyzed the distribution of patient's age with influenza B infection and compared the vaccinated and unvaccinated groups and the results are shown in Table 2. A difference in age distribution between the two groups was detected. Because the unvaccinated patients were somewhat older, adjustments for age were necessary for further analysis.

We set four different temperature points to divide the patients into two groups of higher and lower fever. At first the point was set at 39.5 °C representing the dip between two peaks in the histogram of maximum temperature and the other three points were set at temperatures dividing the patients into quartiles.

The rate of pre-seasonal influenza vaccination was 11.4% (20/176) in the higher fever group, maximum body temperature over 39.5 °C and 18.1% (58/320) in the lower fever group, maximum body temperature below 39.5 °C (Table 3).

The odds ratio for vaccinated children to have a maximum fever over 39.5 °C was calculated to be 0.58 (95% CI, 0.34–1.01), indicating no significant difference. After adjustment for age, it fell to 0.52 (95% CI, 0.30–0.92) with the Cochran–Mantel–Henszel test ($P = 0.024$), indicating a significant difference (Table 4).

When the cutoff point was set at 39.3 °C, a significant but smaller difference was detected (Table 5).

No significant difference was detected in analysis with the other two points.

In a proportional odds model analysis, the odds ratio for having a temperature exceeding 39.5 °C fell to 0.588 (95%

Table 1
The results of virus isolation and immunization status

Influenza type	Influenza vaccine		Total
	(+)	(−)	
A/H1N1	20	147	167
A/H3N2	12	81	93
B	78	423	501
Total	110	651	761

Table 2
Age distribution of influenza B

Age (years)	Influenza vaccine	
	(+) (%)	(−) (%)
0.5–3.0	20 (25.6)	75 (17.7)
3.1–6.0	36 (46.2)	156 (36.9)
6.1–12.9	22 (28.2)	192 (45.4)
Total	78	423

Table 3
The rates of vaccinated children in different fever groups; 37.5–39.5 °C and ≥39.6 °C of maximum body temperature in influenza B (cutoff point = 39.5 °C)

Vaccination	Maximum body temperature	
	37.5–39.5 °C (%)	≥39.6 °C (%)
Vaccine (+)	58 (18.1)	20 (11.4)
Vaccine (−)	262 (81.9)	156 (88.6)
Total	320	176

Table 4
Efficacies on the risk of high fever in vaccinated patients compared with unvaccinated patients (39.5 °C: dip of histogram)

	Odds ratio	95% CI	*P*-value
Crude	0.58	0.34–1.01	0.054 (*F*)
Adjusted on age	0.52	0.30–0.92	0.024 (CMH)

CI: confidence interval; *F*: Fisher's exact test; CMH: Cochran–Mantel–Henszel test.

Table 5
Efficacies on the risk of high fever in vaccinated patients compared with unvaccinated patients (39.3 °C: median)

	Odds ratio	95% CI	*P*-value
Crude	0.7	0.43–1.14	0.173 (*F*)
Adjusted on age	0.58	0.35–0.96	0.033 (CMH)

CI: confidence interval; *F*: Fisher's exact test; CMH: Cochran–Mantel–Henszel test.

CI, 0.38–0.91) by the vaccination, and also fell to 0.887 (95% CI, 0.84–0.94) by aging a year. The effect of the vaccination was statistically independent from that of aging.

We also analyzed the efficacy of vaccination on shortening the duration of fever due to influenza infection. We set different criteria from 37.5 to 40.0 °C by 0.5 °C intervals for counting the days of fever duration, defined as days of actual fever, which were not necessarily consecutive, because fever does not always develop every day of the illness.

In each fever-criteria we tried to compare the rates of vaccination between two or three groups stratified by the fever duration in days. No significant difference was observed in an analysis for vaccine efficacy to shorten the febrile duration.

4. Discussion

Influenza is common in childhood with the highest morbidity occurring in preschool children and an excess of school absence and hospitalization has been reported [1–5]. Although it is clinically difficult to distinguish influenza from other respiratory illnesses, which often circulate concurrently [13–17], limited data are available on laboratory-confirmed influenza in children [1,4,5,18,19].

Recently, reports of influenza-associated severe illnesses and deaths in otherwise healthy children have been increasing and emphasize the importance of protecting children from influenza [11,20–23].

Izurieta et al. reported that the rate of hospitalization for influenza among children younger than 2 years of age was approximately 12 times higher than for otherwise healthy children aged 5–17 years and approached the rate among children with chronic health conditions in the 5–17 years age group. They urged that routine influenza vaccination should be considered in these children [24].

Quach et al. reported on 182 hospitalized children with laboratory-proved influenza who received admission diagnoses of suspected sepsis (31%); lower respiratory infections (27%); and asthma or bronchiolitis (15%). Of these patients 34% were <6 months. Seventy percent of those hospitalized did not have any underlying medical disorders. They argued that extending vaccination to all young children, in addition to high-risk groups and pregnant women has the potential to reduce the impact of influenza on children [25].

Because young, otherwise healthy children are at increased risk for influenza-related hospitalisation. In 2002 the Advisory Committee on Immunization Practices (ACIP) began encouraging annual influenza vaccination of children 6–23 months of age, when feasible [26]. At its 2003 meeting, ACIP voted to recommend that children 6–23 months of age be vaccinated annually against influenza. This recommendation expands the age group for which vaccination is recommended [27].

Difficulty in the clinical diagnosis of influenza infection is re-emphasized because of its wide spectrum of manifestations in childhood and the potential confusion with other viral illnesses. The efficacy of influenza vaccine against infection and illness in childhood may be underestimated by including other respiratory virus infections circulating concurrently or may be overestimated by missing mild influenza infections [13–18]. Studies of the efficacy of influenza vaccine where the diagnosis was not confirmed virologically should be evaluated with great caution, especially in childhood.

Since inactivated influenza vaccine cannot completely prevent recipients from infection and illness, it is preferable to estimate effectiveness on prevention and that on alleviation of symptoms separately. In this study we focused on vaccine efficacy on alleviation. In previous studies, efficacy on alleviation in otherwise healthy children was estimated by complications that were indirect indices of severity [28–30]. As an objective index of severity we used fever, an established major component of influenza illness in children.

In this study all patients having a febrile illness of over 37.5 °C were recruited consecutively to avoid selection bias and all provided samples for virus isolation to exclude other viral infection and to avoid missing mild influenza infections. Only children with culture-confirmed influenza were included in the analysis. Statistical analysis was performed for influenza B only. In comparisons between groups stratified by maximum fever, odds ratios of vaccinated children for developing a fever exceeding 39.5 °C (dip of histogram) and 39.3 °C (median) were 0.52 and 0.58, respectively, after adjusting for age, indicating significant differences. Analysis by the other two points resulting from dividing into quartiles did not result in a statistical difference.

The vaccine strain and the circulating strain of influenza B were not well matched for this season. The 2000/2001 Japanese epidemic strains of influenza B were B/Sichuan and/Johannesburg and the vaccine strain influenza B was B/Yamanashi. Our results show that inactivated trivalent influenza vaccine was modestly effective in reducing the risk of developing a higher febrile illness from influenza B in a season when circulating strains did not match well with the vaccine strain. Despite a small beneficial effect in the year studied, influenza vaccination with better matching would be more effective.

There are statistical limitations to the study. Subjects who were recruited from outpatients might not represent strictly the target population. Statistical examinations for confounding factors were insufficient. In Japan the usual doses of influenza vaccine are the following: 0.1 ml for <12 months of age, 0.2 ml for 1–5 years of age, 0.3 ml for 6–12 years of age and 0.5 ml for >13 years of age. We have no data to refute the possibility that the results of this study were influenced by immunization dosage. The efficacy of inactivated influenza vaccine might be limited and insufficient for priming of influenza infection for infants.

Recently, trivalent cold adapted live attenuated intranasal influenza vaccine was approved in the USA. The overall efficacy of this vaccine in children was 93% for those presenting with culture-confirmed influenza [31], but it cannot be given to children under 5 years of age. An inactivated triva-

lent vaccine has been beneficial and necessary up to now. The necessity for universal immunization for children 6–24 months of age and for the identification and recall of children with chronic medical conditions for influenza vaccination has recently been stressed [32].

Further investigations are required to promote better understanding of the efficacy of vaccine against the illness of influenza A/H1N2 and A/H3N2, as well as B when strains of vaccine and circulating virus are better matched. But the general use of a rapid diagnostic test for the diagnosis, and of antiviral medication, will make it difficult to carry out future studies to estimate the efficacy of vaccine to alleviate illness.

5. Conclusions

In the 2000–2001 influenza outbreak in Japan, inactivated trivalent influenza vaccine was modestly effective in reducing the risk of developing a higher febrile illness from influenza B in children in a season when the vaccine strains were not well matched with circulating strains.

Acknowledgements

This study was supported partially by a grant from the Society of Ambulatory and General Pediatrics of Japan research foundation.

This study was approved and monitored by the Society of Ambulatory and General Pediatrics of Japan ethical committee.

The authors are grateful to the following general practitioners for providing data: Dr. Michiko Aoki, Dr. Yutaka Atsuta, Dr. Chiharu Igarashi, Dr. Tomochika Kato, Dr. Yasuhiro Kawasaki, Dr. Hirotaka Seki, Dr. Akira Takehiro, Hajime Takeuchi, Dr. Takahiro Tahara, Dr. Toshihiro Nishimi, Dr. Hiroyoshi Hashizume, Dr. Moriatsu Yoshida, Dr. Kouji Wagatsuma.

The authors also thank Professor Yoshio Hirota (Professor of Public Health, Osaka city University), Professor Kiyoshi Ichihara (Professor of Faculty of Health Sciences, Yamaguchi University School of Medicine) and Mr. Fujio Matsuo for helpful comments regarding statistical analysis. We are indebted to Professor Peter M. Olley (Professor of Pediatrics, University of Alberta at Edmonton) and Dr. John Ichiro Takayama (Director of the Department of Interdisciplinary Medicine, National Children's Medical Center and National Center for Child Health and Development, Tokyo) for their invaluable help in revising the manuscript.

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JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2006, p. 3268–3273 Vol. 44, No. 9
0095-1137/06/$08.00+0 doi:10.1128/JCM.00803-06
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Development of a New Method for Diagnosis of Rubella Virus Infection by Reverse Transcription–Loop-Mediated Isothermal Amplification

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Received 21 December 2005/Returned for modification 11 June 2006/Accepted 9 July 2006

We developed a useful method for the detection of rubella virus genome RNA by reverse transcription loop-mediated isothermal amplification (RT-LAMP) and compared the sensitivity of RT-LAMP with that of other virological tests: reverse transcription-PCR (RT-PCR) and virus isolation. The rubella virus genome was amplified by RT-LAMP from clinical isolates obtained between 1987 and 2004 with similar sensitivities to the Takahashi vaccine strain. The detection limit of RT-LAMP was compared with that of RT-PCR using the Takahashi vaccine strain. We detected rubella virus genome material corresponding to 30 PFU/ml in a culture fluid sample by RT-LAMP within 60 min after the extraction of RNA with equal sensitivity to RT-nested PCR. The positive result rates of RT-LAMP, RT-PCR, and virus isolation were also compared using throat swabs obtained from patients who were clinically diagnosed with acute rubella virus infection in 2004 in Tochigi, Japan. Among nine patients with clinical rubella, the positive result rates were three/nine (33.3%) for virus isolation, six/nine (66.7%) for RT-PCR, and seven/nine (77.8%) for RT-LAMP. Consequently, RT-LAMP for rubella virus would be expected to be a reliable rapid diagnostic tool in the clinical setting.

Rubella virus is an enveloped positive-strand RNA virus that is the sole member of the *Rubivirus* genus of the *Togaviridae* family. The rubella virus genome consists of less than 10,000 nucleotides and encodes five proteins, including three structural proteins, E1, E2, and the capsid protein. E1 is a structural glycoprotein with neutralizing and hemagglutinating epitopes (5, 22, 31). According to a report on the meeting organized by the World Health Organization (WHO) in 2004 to discuss the standardization of nomenclature for describing the genetic characteristics of wild-type rubella virus, the E1 gene sequence between genome positions 8731 and 9469 is recommended as a target site for routine genotyping analysis for molecular epidemiology (30).

Rubella virus infection is one of the communicable diseases in infants and children. Most patients with rubella develop mild symptoms and recover without any complications or sequelae. However, infection among young women who have no immunity against rubella virus during early pregnancy, especially within the first trimester, may cause fetal death or congenital rubella virus infection/syndrome (CRS). CRS is characterized by multiple malformations: deafness, eye abnormalities, congenital cardiac disease, neurological abnormalities, and so on (2, 31). Although the burden of CRS is not well characterized in all countries, WHO estimated that more than 100,000 cases of CRS occur every year in developing countries and 43% (91/214) of countries did not have a national immunization program for rubella in 2002 (2, 31). However, in the United States and several European countries, the indigenous circulation of rubella has been interrupted and CRS has been eliminated. This achievement of the elimination of CRS largely depends on vaccination programs with effective surveillance (2).

In Japan, nationwide outbreaks of rubella have not occurred since 1992-1993. The number of reported cases of clinical rubella decreased from 2,795 in 2003 and 4,248 in 2004 to 895 in 2005 from approximately 3,000 pediatric sentinel sites without requiring any laboratory confirmation (national surveillance data from the National Institute of Infectious Disease, Japan). Although only one case of CRS was reported annually during 1999 to 2003, 10 patients with CRS were reported to the national surveillance program in 2004 and two were reported in 2005. The issue of rubella and CRS still remains important in Japan for the elimination of CRS with the accelerated control of rubella. A single dose of monovalent rubella vaccine targeted to children aged between 12 and 90 months was introduced in a national immunization program in Japan in 1994. From April 2006, Japan has launched a two-dose immunization program with combined measles and rubella vaccine given to children aged 1 year and aged 5 or 6 years, before primary school entry.

There are some problems with diagnosing rubella and CRS from clinical information alone, because some infected individuals are asymptomatic or have mild symptoms. Different viruses such as measles, human parvovirus B19, and enterovirus cause symptoms similar to those of rubella (2, 25, 32). Also, misclassification of cases of CRS may occur, since some patients with CRS may have a single symptom or defect or may have a positive result for immunoglobulin M (IgM) enzyme immunosorbent assays (EIAs) without clinical symptoms (4, 5, 33). The development of efficient laboratory tests will enable the accurate diagnosis of rubella and CRS.

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Virus isolation is traditionally performed for the laboratory confirmation of rubella virus infection, but the sensitivity of virus isolation is low because of poor conditions of sample transport and inadequate timing of the sample collection. It takes 3 to 4 weeks to obtain the results of virus isolation after three blind passages in RK13 or Vero cells (5, 22). Serological tests such as the hemagglutination inhibition method are performed principally with paired sera from the acute and convalescent phases (5). Although IgM EIA is employed for diagnosis using a single serum sample, false-positive results of serum rubella IgM tests may occur in people with human parvovirus B19 infection, with a positive heterophile test for infectious mononucleosis, or with positive rheumatoid factor (1, 24, 25, 28, 29).

Recently, several molecular biological laboratory tests for the detection of rubella virus have been developed, such as reverse transcription-PCR (RT-PCR) (3, 6, 8, 13, 15, 22). Loop-mediated isothermal amplification (LAMP) was originally developed for DNA amplification, as reported by Notomi et al. (19) in 2000. This method was also applied for the detection of RNA genomes. LAMP amplifies targeted nucleotides with six primers within 1 hour under isothermal conditions without any temperature shifts such as those used for denaturation, annealing, and extension in the PCR cycling reactions (19). In this study, we developed an RT-LAMP method for the detection of the rubella virus genome and compared the sensitivity of RT-LAMP with those of RT-PCR and virus isolation using clinical samples.

(We presented this study at the plenary session of the 46th Annual Meeting of the Japanese Society of Clinical Virology, Fukuoka, Japan, June 2005.)

MATERIALS AND METHODS

Clinical samples and virus isolation. We obtained nine samples from patients diagnosed with acute rubella virus infection by some of the authors in our collaborating hospital between April and May 2004 in Tochigi prefecture, Japan, during a rubella community outbreak. Some of them were serologically diagnosed by IgM antibodies against rubella virus that were assayed using the Rubella IgM EIA kit (Denka Seiken, Niigata, Japan) per the instruction manual. Clinical samples of throat swabs were obtained from patients with acute rubella virus infection, and 0.1 ml of samples was inoculated on monolayers of RK13 cells in minimum essential medium containing 2% calf serum and adequate antibiotics. After three blind passages, the plate was stained with avidin-biotinylated enzyme complex, using polyclonal rabbit antibodies against rubella virus, monoclonal antibodies against rabbit IgG, and avidin-biotinylated enzyme complex staining kits (Vector Laboratories, Inc., California). The name of the isolated rubella virus was specified according to the WHO nomenclature (30).

The Takahashi vaccine strain developed at the Kitasato Institute, Japan (RV/Takahashi: AB03338), was used as the reference strain in this study. Also, we used four isolates as wild circulating strains: two strains (RVi/Tokyo.JPN/87CRS-w: AB238916 and RVi/Tokyo.JPN/87CRS-o: AB238917) isolated in 1987 in Tokyo, Japan; one strain (RVi/Tokyo.JPN/95CRS-n: AB238918) isolated in 1995 in Tokyo, Japan; and one strain (RVi/Kanagawa.JPN/04-s: AB238915) isolated from a patient with acute rubella virus infection in 2004 in Kanagawa, Japan. The E1 sequence data were registered with the DNA Data Bank of Japan under the accession numbers in parentheses.

RT-LAMP. The genomic RNA was extracted from 200 μl of virus culture fluids of rubella virus or clinical samples using a magnetic bead RNA purification kit (TOYOBO Co. Ltd., Osaka, Japan). RNA was resuspended in 30 μl of distilled water, and 5 μl was subjected to RT-LAMP and RT-PCR. RT-LAMP was carried out using autocycling strand displacement DNA synthesis performed using *Bst* DNA polymerase (New England Biolabs). The RT-LAMP primers were designed using the software program for LAMP primer design (Eiken Chemical Co. Ltd., Japan). The RT-LAMP primers were designed in the E1 region between genome positions 8476 and 8680. A diagram of the RT-LAMP primers is shown in Fig. 1. We synthesized six primers: outer primers (F3 and B3), inner primers (FIP and BIP), and two additional loop primers (F loop and B loop). FIP contained a sequence complementary to F1 linked with the F2 sequence, and BIP contained a B1 sequence linked with a sequence complementary to B2. These four primers amplified the targeted DNA. We synthesized the F loop primer between F1 and F2, and the B loop primer between B1 and B2, to enhance the sensitivity and reactivity (17, 18, 19).

For the RT-LAMP reaction, an RT-LAMP mixture was made up to a total of 25 μl, containing 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, 20 pmol each of F loop and B loop, 1.4 mM of each deoxynucleoside triphosphate, 0.8 mM betaine, 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 M $MgSO_4$, 0.1% Tween 20, 0.5 U of avian myeloblastosis virus reverse transcriptase, 8 U of *Bst* DNA polymerase (New England Biolabs), and 5 μl of sample RNA. The reaction mixture was incubated in an LA200 real-time turbidimeter (Teramecs, Japan) at 63°C for 60 min (16, 17, 18, 19).

As the RT-LAMP reaction progresses, the reaction by-products, pyrophosphate ions, bind to magnesium ions and form a white precipitate of magnesium pyrophosphate. Thus, the value of the turbidity is closely related to the amplification of targeted DNA (16).

RT-PCR. The purified RNA genome was converted to cDNA with avian myeloblastosis virus reverse transcriptase (Life Science Inc.) at 50°C for 1 h, using primer 9407–9426 (−) (5′-AGGGCGGCGGTGACGAACTT-3′). Sets of primers in the E1 region for the detection of rubella virus genes were designed as follows: 8110–8129 (+) (5′-GTCTCTTGATCACGCCCTCG-3′) and 8797–8816 (−) (5′-GTGTTGCAGAACGGGTGTTCA-3′) for the first PCR and 8476–8494 (+) (same as F3 RT-LAMP primer) and 8663–8680 (−) (same as B3 RT-LAMP primer) for the nested PCR. The RT-PCR was performed with 1.25 U of *Taq* DNA polymerase (TaKaRa BioMedicals, Tokyo, Japan) in a thermal cycler (TaKaRa BioMedicals, Tokyo, Japan) with 35 rounds of the following thermal cycling conditions; denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 min 40 seconds. The PCR products were confirmed by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide (10, 20, 27).

To conduct the sequence analysis using the PCR products from virus isolates, we made different sets of PCR primers designed as follows: 7981–8000 (+) (5′-CGAAGACGGCTGGACTTGCC-3′) and 9743–9762 (−) (5′-CTATGCAG CAACAGGTGCGG-3′) for the first PCR and 8110–8129 (+) and 9743–9762 (−) for the nested PCR. The nested RT-PCR products including the target site between genome positions 8731 and 9469 were used for routine genotyping analysis (30).

RESULTS

Sensitivity of RT-LAMP. The rubella virus genomic RNA was extracted from 200 μl of culture fluid containing $10^{5.5}$ PFU/0.1 ml of the Takahashi vaccine strain. The genome was serially diluted by 10-fold, and each dilution was subjected to RT-PCR and RT-LAMP. The results are shown in Fig. 2. The genome was amplified at least until the 10^{-5} dilution by both RT-LAMP and RT-PCR. The detection limit was calculated as 30 PFU/ml in culture fluid for both RT-LAMP and RT-PCR and estimated as 1 PFU of infectious particles in 5 μl of RNA materials in a single test.

As for the specificity of RT-LAMP for rubella, RNA samples extracted from other viruses (measles virus, mumps virus, respiratory syncytial virus [RSV], and influenza virus) were examined by RT-LAMP for rubella. These viruses were found to be negative for rubella RT-LAMP, demonstrating the lack of cross-reaction (data are not shown).

Detection of rubella virus genome by RT-LAMP. Nine patients who were clinically diagnosed with acute rubella virus infection were examined. They were 0 to 8 years old, and six (66.7%) were male. The results of virus isolation, RT-PCR, and RT-LAMP are summarized in Table 1. Rubella viruses were isolated from three patients (33.3%) and designated RVi/Tochigi.JPN/04-s, RVi/Tochigi.JPN/04-h, and RVi/Tochigi.JPN/04-i, their E1 region genome sequences having the accession