

Japanese Encephalitis Virus infection and Replication: Biological Roles of Nonstructural Protein NS4a and the 3'-Untranslated Region in Persistent Infection

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ABSTRACT

Over 30,000 Japanese Encephalitis (JE) cases occur each year worldwide. JE is caused by the JE Virus (JEV), serious human pathogen, and is responsible for 30% mortality and permanent neurological disabilities in 50% of survivors. JEV is an enveloped virus and its genome comprises ca.11kb of positive sense RNA. In this study, we examined JEV replication in several human cell lines, and we established a JEV-persistent infection system using KN73 hepatic cell lines. JEV obtained from the persistently infected cells contained mutations in the nonstructural protein NS4a and the 3'-Untranslated Region (UTR) of the genome. These mutant viruses had relatively weak virulence in mice. In addition, we recently isolated a new JEV in Japan, the Ishikawa strain. Based on the sequencing data, we found that the Ishikawa strain belonged to genotype 1 and it had a nucleotide deletion in the 3'-UTR, which differed from many of the JEV strains (genotype 3) isolated previously in Japan. JEV in the persistently infected system and the newly isolated Ishikawa strains both had relatively weak pathogenicity, but they were resistant to innate immunity. Those findings indicate that most of the recent JEV isolates in Japan are genotype 1, and this may explain the recent low incidence (<10 in a year) of JE cases in Japan. To avoid future outbreak of JE, it is necessary to continue vaccination, but it is also important to check for

changes in the viral pathogenicity of JEV as well as determine the ecological status of mosquitoes distributed in the field. Our results may facilitate the development of antiviral drugs.

Keywords: JEV; Persistent Infection; NS4a; 3'-UTR

ABBREVIATIONS

nt: Nucleotide; pi: Post Infection; moi: Multiplicity of Infection; ip: Intra-Peritoneal

INTRODUCTION

Japanese Encephalitis Virus (JEV) is wide spreads in Southeast Asia, Korea, China, and Japan. JEV is an important human pathogen that is responsible for over 30,000 annual cases of encephalitis with a mortality rate of 30% and permanent neurological disabilities in 50% of the survivors [1,2]. The normal transmission cycle of this virus occurs between mosquitoes and pigs and/or water birds, but it also has a zoonotic transmission cycle where swine serve as amplifier hosts, from which infected mosquitoes can transmit the virus to humans.

JEV is small (diameter = 40~60nm) enveloped flavivirus with a single-stranded positive-sense RNA genome of approximately 11 kb [3-6]. The viral structural proteins are encoded by the 5'third of the Open Reading Frame (ORF), and comprise the Capsid (C), membrane (M; formed by proteolytic cleavage of its precursor protein prM), and Envelope (E) proteins. The nonstructural protein (NS1, NS2a/2b, NS3, NS4a/4b and NS5) are encoded by the remaining 3' region of ORF. ORF is flanked by 5'- and 3'-untranslated regions (UTRs), which contain 95 and 583 nucleotide (nt), respectively [4-6].

In order to elucidate the actual viral pathogenicity, it is necessary to examine the biological roles of viral proteins well as host gene expression in cells and tissues during JEV infection.

Many studies have analyzed the molecular mechanism of JEV replication in cells. In particular, the viral E protein has an essential role in viral adsorption and infection, while the nonstructural proteins NS3 and NS5 have important roles in viral replication, as protease, RNA helicase, and RNA polymerase enzymes, respectively [7-10]. However, several aspects of JEV replication mechanism still remain unclear and the functions of proteins such as NS4a and NS4b have not yet been clarified.

In general, JEV produces the symptoms of acute infection with severe encephalitis, but in some conditions, there is a persistent infectious state in pigs. JEV has a wide host range and can replicates in many different cell lines ranging from mosquito to human cells, but their growth rate differs among the cell lines. This suggests that cellular factors such as innate immunity may be involved in the JEV replication process. Persistent infection systems are very useful for investigating the interaction between cellular factors and viruses [11-17]. It has been reported that JEV persists not only in pigs but also in human blood cells, even though the mechanism of viral persistence is not understood.

In this study, we report the establishment of a persistent JEV infection in KN73 cells derived from human liver cells, where mutant viruses obtained from the persistent infection system exhibited major changes in the amino terminal region of NS4a. In addition, these mutants had very weak pathogenicity compared with the parent viruses. These findings are consistent with a previous report that the role of dengue virus NS4a is important for virus replication [18].

Mosquito-born flaviviruses such as JEV are among the most important emerging and resurgent pathogens [1,2]. To avoid future JE outbreaks, it is necessary to carefully monitor changes in virulence, although we have the low incidence (<10 in year) of JE cases in Japan (Figure 1). In the last two decades, we have isolated JEV from mosquitoes collected in Ishikawa prefecture area in Japan. In this study, we report the isolation and biological characterization of JEV Ishikawa strains [19].

We consider that our results are useful for understanding the JEV replication mechanism, including the functions of NS4a and 3'-UTR, as well as for controlling JE infection.

MATERIALS AND METHODS

Cells and Viruses

The main cell lines used in this study were KN73 derived from human liver and IMR32 from human neuroblastoma cells. Vero (African green monkey kidney) and HEK293 (Human Embryonic Kidney) cell lines were also used for viral infection. The cell lines were infected with JEV strains, including JaGAR01 at a multiplicity of infection (m.o.i.) of one. After infection, the cells were cultured at 37°C in Dulbecco's minimum essential medium (MEM) or RPMI1640 medium supplemented with 10% Fetal Calf Serum (FCS).

To isolate JEV strains, we collected mosquitoes (*Culex tritaeniorhynchus*) by trapping using mosquito nets and dry ice. The collected mosquitoes were homogenized, and the extracts were applied to Vero cells in Eagle's MEM supplemented with 5% FCS.

Viral Titration

The virus yields in the cultured fluids were estimated using the plaque method with Baby Hamster Kidney (BHK) cells, as described previously [20,21]. BHK cell monolayer was infected with JEV and then cultured in the presence of 0.6% methylcellulose and 1% FCS in E-MEM. After 3~4 Days Post Infection (dpi), the infected cells were fixed using methanol and stained using 1% crystal violet. The viral titer was expressed as Plaque-Forming Units (PFU).

Immunocytochemistry Assay (IC) and Western Blot Analyses

To determine the expression of viral proteins in the JEV-infected cells, IC and western blot analyses were performed using anti-E and anti-NS3 monospecific rabbit sera [7,9,22]. In the IC assay, cells were fixed using acetone and then incubated with anti-E or anti-NS3 sera (1:500) for 1 h at 37°C. In the second reaction, cells were incubated with FITC- or peroxidase- conjugated

anti-rabbit IgG goat sera for 1 h at 37C, and then observed by fluorescence microscopy (Olympus, Japan). In the western blot analyses, JEV-proteins were detected using a peroxidase- conjugated secondary antibody against rabbit IgG and stained with diamino-benzidine.

Determination of Nucleotide Sequences

To prepare RNA from JEV-infected cells, cells were washed with phosphate buffered saline and then harvested by adding 50 mM Tris buffer (pH 7.5) containing 0.5% SDS and 1 mM EDTA. RNA was extracted from the JEV-infected cells using Isogen (Takara, Japan). To analyze the nucleotide sequences, the viral RNA was subjected to RT-PCR, where the primers comprised several oligonucleotides that corresponded to the sequences in the JEV genome. The PCR products were purified using agarose gels (BioRad, USA), and then directly sequenced following the dideoxy method using non-R1 sequencing procedure (ABI, BigDye terminator, USA) and a DNA sequencer (ABI Prism™ 310, USA). Multiple sequence alignments and secondary RNA structure analyses were performed using Genetyx software (Japan).

Viral Pathogenicity in Mice

ICR mice strains aged four weeks were purchased from Sankyo Lab (Tokyo, Japan) and inoculated intra peritoneally (ip) with JEV at 500PFU~50,000 PFU. The mice were observed after inoculation, and their survival rates were determined. Some mice were sacrificed and their brain tissues were immune stained using anti-E.

Preparation of Plasmids and Luciferase Assay

To analyze the biological functions of the 3'-UTR, pJEL3' plasmids were constructed containing luciferase gene and the 5'- and 3'-UTR fragments from the JEV genome. RNA was synthesized *in vitro* using pJEL3' and the SP6 RNA polymerase system. The synthesized RNA was transfected into cells with Lipofectamine 2000 reagent (Invitrogen, USA), and cell extracts were used for luciferase assay after 24 h (Promega, USA).

Microarray Analysis

DNA microarray analysis was performed to exclude differences in gene expression. Total RNA was reverse transcribed into cDNA using an Ambion® WT Expression kits (Applied Biosystems, USA), labeled with a Gene Chip® WT Terminal Labeling and Controls kit (Affymetrix, USA), and hybridized on a Gene Chip® Human Gene 1.0 ST Array (Affymetrix), which included 28869 probes. Digitized image data were processed using Gene Chip® Operating Software (Affymetrix). Following background correction and 50th percentile normalization, the microarray results were analyzed using Gene Spring software version 11.0 (Agilent Technologies, USA).

Real-Time PCR

As an endogenous reference, the copy number of the β -actin gene in each DNA extract was determined by real-time PCR using TaqMan β -actin Control Reagents.

Total RNA was reverse transcribed into cDNA using a Revertra RT kit (Toyobo, Japan) and oligo (dT) primers according to the manufacturer's instructions. Primers and TaqMan probes were prepared. Real-time PCR amplification was performed using an ABI 7900HT Fast system (ABI, USA) with a TaqMan gene expression assay (Applied Biosystems, USA). The relative quantity of each target mRNA was normalized relative against that of the internal control, β -actin.

RESULTS

Viral Replication in Human Cultured Cells

JEV can grow in various cell lines, ranging from mosquito to human cells. KN73 cells are derived from human liver cells, and they were easily infected with JEV, JaGAR01 strains. As shown in Figure 2, the JaGAR01 virus yields in KN73 cells reached to the maximum level (around 10^6 PFU/mL) at 48-72 hpi, which was lower than that in human neuroblastoma IMR32 cells (around 10^8 PFU/mL). The infection and replication of JEV were much easier in IMR32 cells, which are similar to neurons.

JEV RNA synthesis occurred as follows: First, negative stranded viral RNA (42S) was synthesized in the early stage of infection, as described previously [20]. The appearance of the positive RNA (42S) was delayed, but it was clearly detected at 12 hpi, and the amounts then increased gradually. The accumulation of 42 S (+) RNA was similar to results of the viral growth curve assay using plaque-forming methods (Figure 2). Most of the viral proteins, including NS3 (70KDa) and NS5 (100KDa), were detected, and they appeared to be aggregated in the perinuclear and membrane fractions [22], whereas the E protein was dispersed in the cytoplasm (Figure 3).

Establishment of the Persistent Infection System and Characterization of JK-1 Viruses

In the usual conditions, the JEV-infected IMR32 cells and most of the KN73 cells were killed via lysis, similar to many other JEV-infected cells (data not shown). However, some KN73 cells were still alive after 7 dpi, and they then grew gradually to form colonies (data not shown). During this period JEV also replicated slowly and the virus yields reached around 10^3 PFU/mL at 7 dpi~9 dpi. These persistent JEV (JaGAR01)-infected cells (JK-1) continued to grow, although their growth rate was lower than that of normal KN73 cells (data not shown). The release of virus from the JK-1 cells declined gradually. After one year, viruses (JK-1(1y)) were still obtained. ICR mouse strains were used to examine the pathogenicity of the mutant virus. Around two weeks after the infection, all of five mice inoculated ip with wild type JaGAR01 died, even when only 500 PFU of virus was injected. By contrast, five mice inoculated ip with mutant JK-1(1y) viruses survived, although 50,000 PFU of virus was injected (data not shown). These results indicate that the mutant viruses had low virulence in mice.

The expression levels of JEV-specific proteins, including E and NS3 proteins, decreased in the persistently infected cells (Figure 3). As shown by the western blot (Figure 3a) and

immunofluorescence assay (IFA) (Figure 3b), positive cells with E proteins comprised approximately 30 % of the total cells at 70 dpi. The western blot assays indicated that the expression levels of JEV specific proteins at one year after the first infection were much lower than those in the acute JEV-infected cells (data not shown). The expression levels of the viral proteins in JK-1 cells were maintained for longer than one year with passaging once a week. However, the release of virus from the JK-1 cells decreased gradually, and finally it was lower than the detection level of the plaque assay. The JEV derived from the persistently infected cells may have differed in terms of particle-formation.

To investigate mutations in JK-1 viruses in the persistently infected cells, genomic virus RNA extracted from cell-associated viruses was subjected to RT-PCR. The PCR products were sequenced directly using BigDye terminator (ABI). According to the sequencing analysis, we found that the JK-1 virus had mutations in the E protein but also in the NS4a and 3'-UTR, as shown in Figure 4. In particular, the amino (N) terminal region of NS4a contained many amino acid substitutions (Figure 4 and Table 1). Interestingly, the number of mutations in JK-1-NS4a (2y) increased after the longer culture period, as shown in Table 1. In addition, other JK-1 lines (R and B) cultured in different passage series contained several mutations in the N-terminal of NS4a. We also found that 8 nucleotides (10,445nt~10,452nt) were deleted at the 3' UTR of JK-1 (2y), JK-1(R), and JK-1(B) in the genomic RNA. The nucleotides deleted at 3' UTR might be related viral RNA replication and protein synthesis. We consider these issues later.

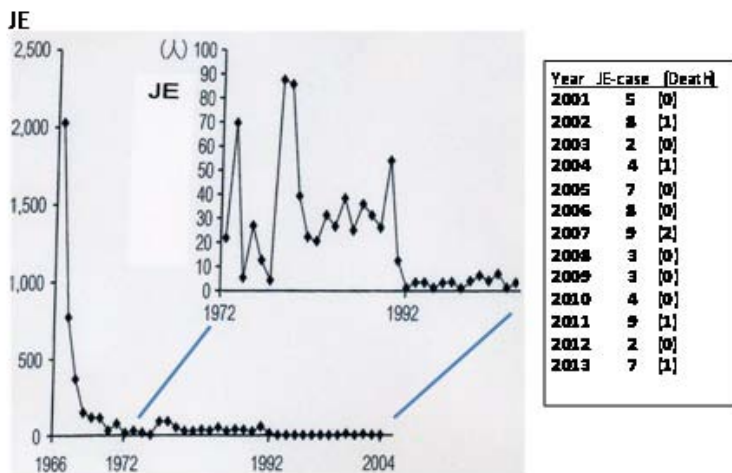


Figure 1: Cases of Japanese encephalitis in Japan (1966~2013).

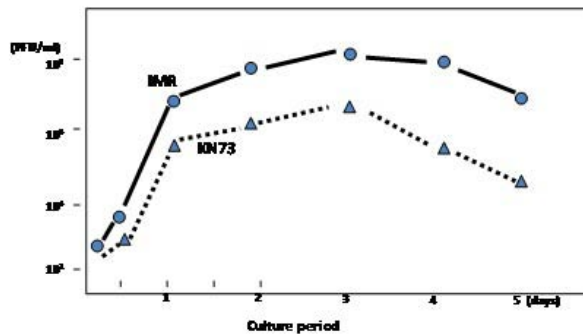


Figure 2: JEV replication in the infected cells. IMR32 and KN73 cells were infected with JEV at 1 m.o.i., and JEV in culture fluids was estimated by the plaque assay using BHK cells.

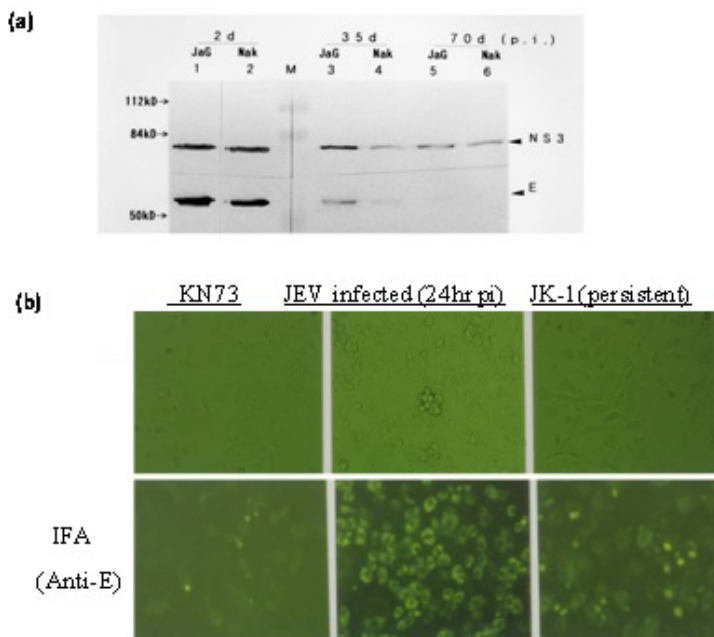


Figure 3: Expression of JEV proteins in the persistently infected cells. (a) E and NS3 proteins were detected by western blot analysis using anti-E and anti-NS3 sera. (b) Immunofluorescence assay was carried out using anti-E. JEV-uninfected or infected KN73 (24hpi), and persistently JEV-infected KN73 (JK-1) cells were used for the assay.

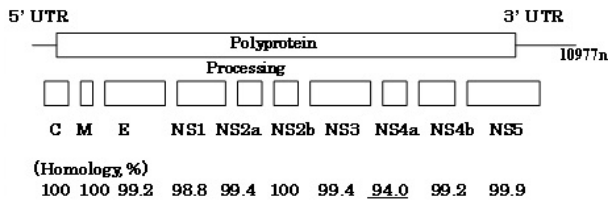


Figure 4: Comparison between parent JEV JaGAR01 and variant JK1(1y) genome. JK1(1y) viruses were obtained from the persistently JEV-infected JK1 cells.

Table 1: Substitution of amino acids of E and NS4a proteins. The amino acid sequences deduced from nucleotide sequences of various variant viruses were compared.

Strain/E	72	93	123	176	186	219	227	240	327	348
JaGAR 01	A	K	R	I	V	H	S	M	S	M
JK1 (1y)	A	K	R	I	V	Y	S	M	S	M
JK1m (2y)	A	K	R	T	V	Y	P	M	S	I
JK1 (R)	A	R	K	T	V	Y	P	M	S	M
JK1 (B)	T	K	R	T	A	Y	P	V	C	M

NS4a	2	6	7	12	14	18	20	21	22	24	27	29	30	43	44	45
JaGAR01	A	I	E	M	E	G	T	R	E	L	M	L	V	A	L	E
JK1 (1y)	A	I	D	M	E	R	T	R	G	I	V	I	F	A	L	Y
JK1 (2y)	A	I	D	M	E	R	T	R	G	I	V	I	Y	A	F	Y
JK1 (R)	D	M	Y	L	D	R	M	L	G	I	V	I	Y	S	F	Y
JK1 (B)	A	M	Y	L	D	K	M	L	D	I	V	I	Y	A	L	Y

Biological Features of Newly Isolated JEV Ishikawa Strains

JEV is an important human pathogen that causes acute meningoencephalitis, with a fatality rate of ca. 30%. Thus, it is important to monitor the distribution of JEV in Japan, although there have been less than 10 JE cases each year since 1992 (Figure 1). We found that the biological features of the Ishikawa strain isolated in 1994 differed from other JEV strains (e.g., JaGAR01) isolated in Japan before 1990. First, it was surprising that the Ishikawa strain (Ishikawa (94)) was classified as belonging to genotype 1 based on the sequencing analysis, whereas all of the JEV strains isolated in Japan before 1990 belonged to genotype 3. Since isolating Ishikawa (94),

we have isolated new JEV strains from mosquitoes (*Culex tritaeniorhynchus*) in the Ishikawa prefecture area of Japan, during the past 20 years. The JEV strains isolated in 2005, 2010, and 2012 possessed similar sequences to Ishikawa (94), and thus they belonged to genotype 1. The Ishikawa strains had a low replication rate in IMR32 cells but a normal rate in Vero cells (data not shown).

As shown in Figure 5, the virulence in mice of the Ishikawa (10) strain (isolated in 2010) (genotype1) was relatively lower than that of JaGAR01 (genotype 3). We confirmed that JEV replicated efficiently in neurons, although this is well known. As shown in Figure 6, more E protein was detected in the cytoplasm of neurons by immunostaining using anti-E. Thus, the E-protein expression level was higher in the brain of JaGAR01-infected mice, while lower in that of Ishikawa -infected mice (Figure 6).

The nucleotide sequence data indicated that the E protein region and 3'-UTR of the RNA genome in the Ishikawa strains differed from those of JaGAR01 (Genbank AB051292 and AF069076, respectively). The 3'-UTRs of the Ishikawa isolates contained a region where several nucleotides had been deleted, although the 3'-UTR of the JEV genome was previously considered to be a conserved region. Interestingly, the numbers of deleted nucleotides in the 3'-UTR were as follows; 13nt and 22nt (13+9) were deleted in Ishikawa (94) and Ishikawa (10), respectively (Figure 7). What are the effects of these deletions?

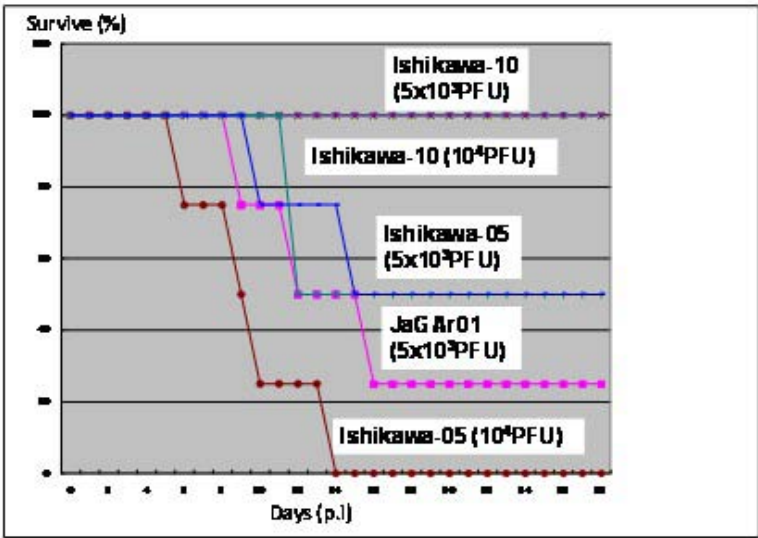


Figure 5: Pathogenicity of various JEV strains: Five ICR mice were inoculated (ip) with JaGAR01 and Ishikawa strains at 5,000 or 10,000 PFU, and observed in each experiment.

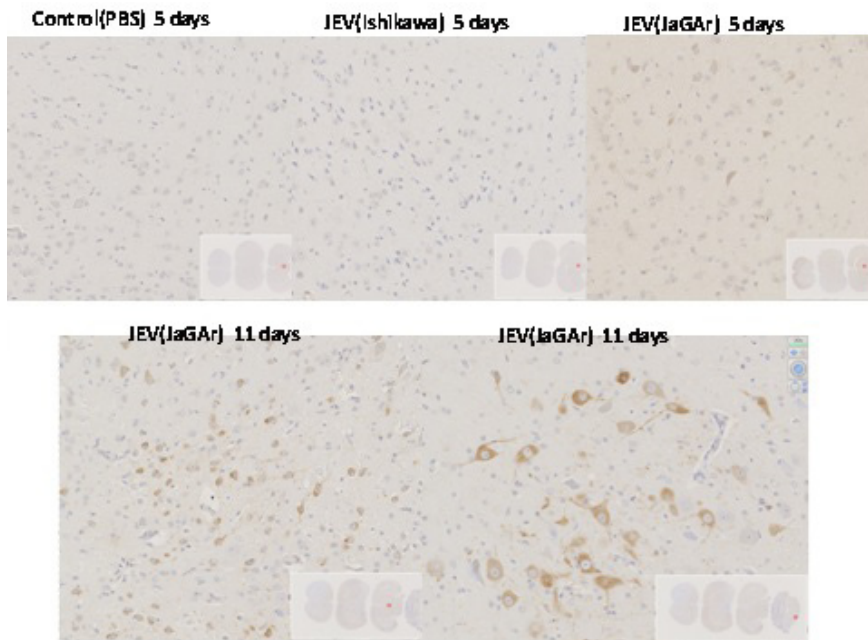


Figure 6: Expression of E protein in mouse brain infected with JEVs, JaGAR01 and Ishikawa

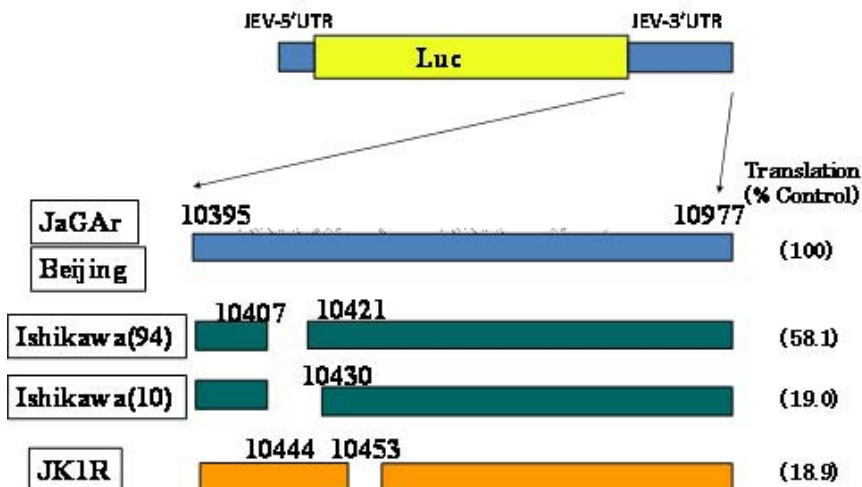


Figure 7: Effect of 3'-UTR of JEV genome to translation. Several plasmids containing various 3'-UTRs were constructed. RNAs were synthesized *in vitro* using the plasmids and SP6 polymerase system, and then transfected into cells. Cell homogenates were assayed for luciferase. Luciferase translation activity were measured and indicated as percent of control at right side.

Biological Function of 3'-UTR in JEV RNA

To understand the function of the JEV 3'-UTR, we constructed several plasmids containing 3'-UTRs, and we performed luciferase gene reporter assays (Figure 7). The results obtained indicated the translation activity, because the *in vitro* synthesized RNA was transduced into the cells. Ishikawa (94), with an RNA deleted sequence in the 3'-UTR, had an activity level of 58.1% compared with those of JaGAR01 and Beijing 3'-UTR as a control. The activity of the Ishikawa (10) RNA was much lower than that of Ishikawa (94). Interestingly, the JK1(R) RNA derived from the persistently JEV-infected cells had a relatively low activity of 18.9%, as shown in Figure 7.

The RNA structure of the 3'-UTR was determined using Genetyx software (Figure 8). It is generally considered that the sequence of the 3'-UTR is conserved in JEV. Indeed, the 3'UTR in the representative strains JaGAR01, Nakayama, and Beijing comprise 583 nt with almost the same sequences [5,6]. However the 3'-UTRs of the Ishikawa strains possessed deletions, i.e., 13 nt in Ishikawa (94) and 22 nt in Ishikawa (10), as shown by the red bar in Figure 8a. The RNA structure of 200 nt at 3'-UTR differed from that of JaGAR01 (Figure 8a). The JK1-RNA derived from the persistently JEV-infected cells also had a deletion site in the 3'-UTR and a different RNA structure, as shown in Figure 8b.

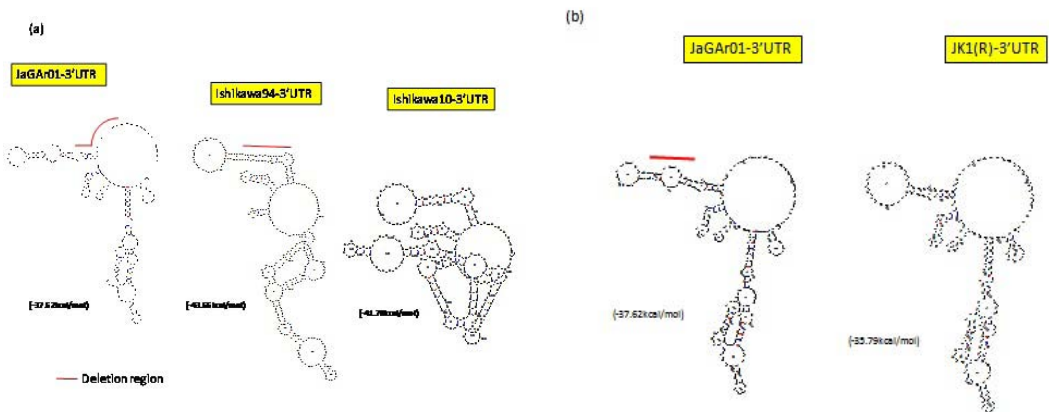


Figure 8: Secondary RNA structure at 3'-UTR of JEV genome. RNA structures were compared between JEV genomes including JaGAR01, Ishikawa (a) and JK1(R) (b). Red bar indicates the deleted nucleotide sites. RNA structure analyses were performed using Genetyx software.

Host Gene Expression in JEV-infected Cells

We performed DNA microarray analyses of the total RNA from JEV-infected cells, and the results are shown in Table 2. The expression levels of IFN-related and cytokine genes, e.g., OAS, IFI and CCL were upregulated greatly in the acute infection (24 hpi). These genes were also up regulated in the persistently infected cells, but their levels were different. The gene expression levels of infection-related genes such as IL1 and prostaglandin were also upregulated in the acute

infection, whereas they were downregulated in the persistently infected cells. The expression levels of several genes were downregulated by the JEV-infection, e.g., MTF, NR1H4 and RNF14.

The expression of the IFN-related gene IFIT1 was notable, because it was upregulated in the acute infection, whereas it changed little in the persistently infected cells (Table 2), and this might have been related to the viral pathogenicity. Thus, we used real-time PCR to confirm that infection by the low virulence virus elicited a low reaction from IFN-related genes such as IFIT1. The Ishikawa strain had relatively low virulence in mice (Figure 5). To examine the IFIT1 expression levels, we performed real-time PCR using IFIT1 probes (Figure 9), which showed that the IFIT1 levels in the cells infected with Ishikawa strains (94), (05), and (10) were lower than those in cells infected with JaGAR01. This trend was similar in IMR32 and HEK239, but the levels were different in the infected cells, as shown in Figure 9.

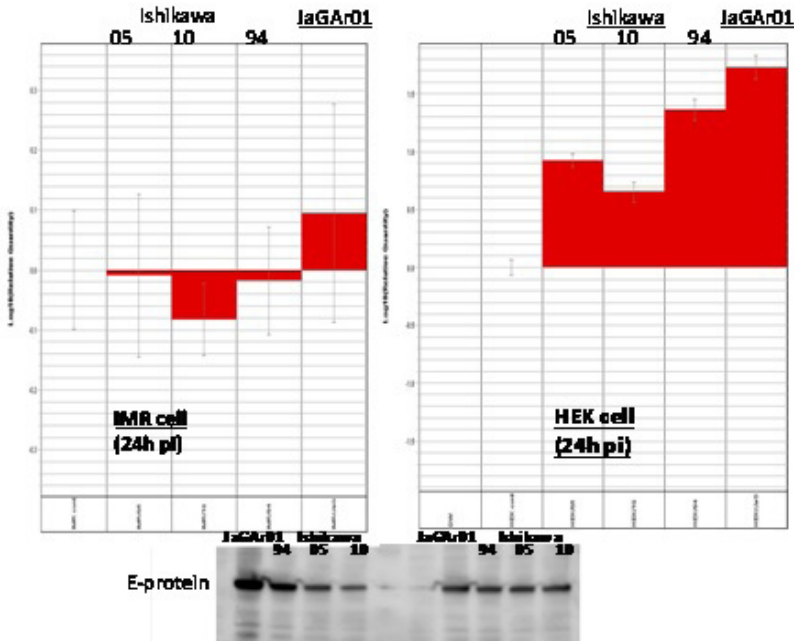


Figure 9: Expression of IFIT1 gene in JEV-infected cells (24hpi). IFIT1 gene expression levels were measured by real-time RT-PCR system and compared between the cells (IMR32 and HEK293) infected with JaGAR01 and Ishikawa strains. Western blot data at the bottom side indicate the expression of E protein in the JEV-infected cells.

Table 2: Difference in gene expression level among JEV-infected cells. Gene expression level was assayed by DNA microarray and relative amounts of gene expression were compared between acute and persistent JEV infection.

	Genes	JEVinf/KN73 (24h pi)	JK-1/KN73 (Persist)
	2'-5'-oligoadenylate synthetase-like isoform (OASL)	13.6 (Up)	37.4 (Up)
1	interferon-induced protein with tetratricopeptide repeats 1 isoform 1 (IFIT1)	128.8	1.69
	interferon-induced, hepatitis C-associated microtubular aggregation protein (IFI44)	12.6	62.4
	small inducible cytokine B11 precursor (CXCL11)	21.3	3.33
	small inducible cytokine A5 precursor (CCL5)	17.4	28.7
2	microphthalmia-associated transcription factor isoform 4 (MITF)	0.71 (Down)	0.33 (Down)
	nuclear receptor subfamily 1, group H, member 4 (NR1H4)	0.82	0.042
3	interleukin 1 receptor, type II precursor (IL1RB)	4.17 (Up)	0.21 (Down)
	prostaglandin-endoperoxide synthase 2 precursor (PTGS 2)	3.11	0.27
	cytochrome P450, family 3, subfamily A (CYP3A43)	1.13	0.068
4	ring finger protein 14 (RNF14)	0.29 (Down)	1.29 (Up)
	LOC143458	0.68	2.18
	nuclear receptor subfamily 1, group A, member 2 (NR4A2)	0.81	2.31
	Adenosine A2a receptor (ADORA2A)	1.09	2.78

DISCUSSION

First, it should be noted that JEVs still circulate in a wide area, including Japan, although the number of JE cases in Japan is currently < 10 each year [1,2,19,23]. In addition, it is necessary to carefully monitor changes in the pathogenicity of JEV strains, including mutants or new strains [23].

It is well known that JEV has various hosts, which range from insects to humans, and it can replicate in many different cultured human cell lines. In this study, we used IMR32 and KN73 cells for JEV multiplication, and we observed differences in JEV replication in both human cell lines. JEV grew quickly and released virus particles into the culture fluid in IMR32 cells, which lysed after several days post-infection. By contrast, JEV grew slowly, and finally established a persistent infection system in KN73 cells derived from human liver. In this persistent JEV infection system, the viral yields were maintained at a low level of less than 10 PFU/ml in the culture fluid. The viral yields level was maintained for a long period, and the expression of viral RNA and proteins in the cells continued for over 5 years. These results and the sequence analysis indicated that the mutant JEVs appeared at 35 days~40 days after the first infection, which suggests that viral mutation might have been related to the establishment of the persistent infection [13]. The results of the IFA and western blot analyses showed that the mutant viruses were deficient in terms of synthesis or the processing of viral proteins, which might influence the formation of infectious virus particles. The appearance of variant viruses, such as DI particles in persistent infection has been reported [14, 24]. We found that the E protein expression level was relatively lower than that of the NS3 protein, thereby indicating that the synthesis and processing of viral proteins was

changed during the replication of variant viruses, which might have been related to the mutation in NS4a (Table 1). It is likely that NS4a affects the function of NS3 which forms the replication complex on the ER, because NS4 is thought to provide an anchor for NS3 [25]. Thus, a possible cause of the persistent JEV infection may be related to the mutation affecting the hydrophobicity of the transmembrane protein NS4a, although the actual function of NS4a is not clear.

In addition, the mutation (deletion of 8 nt) in the 3'-UTR had a major influence on the translation of viral proteins. As shown in Figure 7, the luciferase assay clearly indicated that the translation activity of the mutant 3'-UTR was decreased to 18.9% of that in the control. A similar effect on translation was caused by the nucleotide deletion in the 3'-UTR was observed in the newly isolated Ishikawa strains, although the deletion site was different. We found that the 3'-UTR of Ishikawa (10) strain with a deletion of 22 nt had a relatively low activity compared with that of Ishikawa (94), which had a deletion of 13 nt. These results clearly suggest that the 3'-UTR of JEV is important for maintaining the translation activity during virus replication. It is also possible that the secondary RNA structure of the 3'-UTR in the JK1 and Ishikawa strains might be related to viral RNA synthesis and pathogenicity [3,6,26-30]. Similarly, it has been reported that structural features of the West Nile virus 3'-UTR have important effects on RNA synthesis [28].

The mutations in NS4a and the 3'-UTR appear to be important for viral pathogenicity. Indeed, the mutant JEV (JK1) and Ishikawa strains had lower virulence than JaGAR01. Interestingly, the virulence of Ishikawa (10) was relatively lower than that of Ishikawa (94) (Figure 5). It is well known that viral E protein is essential and that it has an important role in viral pathogenicity. Thus, several studies of the virulence of JEV have shown that E protein is important for pathogenicity [7,8,31], particularly arginine (R) at amino acid position 123 in the Eprotein [31]. However, we found that R did not differ between the mutant JK1 and Ishikawa strains, as shown in Table 1.

The low virulence of the mutant virus and Ishikawa strains appears to be related to complex elements, including viral RNA, proteins, and the host reaction [32,33]. Innate immunity is the first host reaction to a virus infection. The results of the microarray analyses showed clearly that the expression levels of IFN-related genes and cytokines were upregulated by the JEV infection. The expression of these genes in infected cells is expected in the immune system. However, during the persistent infection, the expression levels of some key genes did not differ or they were decreased compared with those in the uninfected cells, e.g., IFIT1 and cytokines. The expression of IFIT1 was particularly interesting because it increased 128.8 fold during acute infection with JEV, whereas it only increased 1.69 fold in the persistent JEV infection. These results suggest that the expression of innate immunity related genes was maintained at the normal levels during the persistent infection. Therefore, the reaction between the virus and host cells appears to be controlled in the persistent infection or in the infection with avirulent viruses. In these conditions, the virus life cycle can be maintained for a long time in cells. The results of real-time PCR analyses showed that the Ishikawa strains with low virulence activated lower levels of IFIT1 expression compared with

JaGAR01, although viral replication occurred at almost the same rate with both JEVs in HEK293 cells, and the expression level of IFIT1 differed between the cell lines. These results suggest that avirulent viruses can activate innate immunity, but only weakly, including IFIT1 [34,35]. It is noted that IFIT has RNA binding activity [36]. In addition, the activation of innate immunity by viruses probably differs among cells and tissues, e.g., low reaction in IMR32 cells and neurons infected with JEV.

Viral pathogenicity is thought to be related to innate immunity including IFN and cytokines [37,38]. The Influenza H1N1 pandemic in 1918 caused the death of more than 25 million people. One of the reasons for this high mortality and highly pathogenic avian influenza might be the immune reaction leading to a cytokine storm [39]. It has been reported that the HCV-protein NS3 influences the IFN pathway via the cleavage of IRF3 [40] or other way containing microRNA [41]. Thus, the interactions between viral proteins and the innate immunity-related proteins appear to be important.

The amino terminal region of JEV-NS4a was substituted rapidly during the culture of the persistent infection, as shown by our results. This may mean that the NS4a-mutant virus can avoid attack by the innate immunity system in cells. During JEV replication, NS4 may function as transmembrane protein and as an anchor for NS3 which has protease, ATPase, and RNA helicase activity [9,10,25]. NS4a is probably one of the key proteins involved in the regulation of the JEV replication rate and as well as coordinating with the innate immune system, thereby affecting the viral pathogenicity [18,42].

In this study, we obtained a JEV associated with low pathogenicity. However, it is necessary to carefully monitor changes in the pathogenicity of new JEV genotypes [19,43,44]. In the future, it is possible that more virulent forms of JEV may appear. Thus, vaccination is the most important method for preventing JEV infections, as demonstrated in Japan (Figure 1). However, it is also necessary to develop new drugs to combat JEV infection, e.g., RNAi [21, 45,46]. Therefore, it is important to elucidate the molecular mechanisms of viral reproduction and pathogenicity to facilitate the development of new treatments and drugs.

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