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Biophysical characterization of Japanese encephalitis virus (KV1899) isolated from pigs in Korea

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A virus strain, showing cytopathic effect in Vero cell, was isolated from plasma of a fattening pig in Gyeonggi province, Korea in October 1999. The evaluation of physicochemical/biological properties of the isolate showed that the virus, KV1899, inoculated suckling mouse showed paralysis and died within 7 days post-inoculation, the mouse brain suspension had hemagglutinating activity with goose RBC. Pathogenicity of isolate was carried out by intracranial and intraperitoneal inoculation of 3-4 weeks mice. The mice inoculated with isolate showed $10^{4.5}$ LD_{50}/0.03 ml and $10^{3.0}$ LD_{50}/0.5 ml according to the inoculation route. The isolate was identified as RNA and enveloped virus using IUDR and chloroform sensitivity test. The virus particles within the infected Vero cell were measured to be 40-50 nm in size by electron microscopy. The isolate was further characterized by immuno-fluorescence assay using Japanese encephalitis virus (JEV) specific monoclonal antibodies. Reverse transcription polymerase chain reaction (RT-PCR) revealed the presence of JE specific conserved sequences in this isolate. The artificially inoculated pigs had HI titer of 320 to 2,560 against JEV at 14 to 42 days post inoculation. We confirmed this isolate as Japanese encephalitis virus. It was the second isolation of JEV in pigs in Korea.

Key words: Japanese encephalitis virus, Isolation, Identification

Introduction

Japanese encephalitis (JE) is a zoonotic disease transmitted through the bite of *Culex* mosquitoes. In development of JE, pigs are considered the most important natural amplifying host among several animals [2]. Although adults and fattening pigs do not show clinical signs, infected pregnant sows may deliver stillborn and mummified fetus, and weak piglets with nervous system signs [8,21]. Most human cases of the disease are subclinical or mild but it can result in fetal encephalitis in some children and abortions in pregnant women [3].

JEV is a member of the genus *Flaviviruses* of the *Flaviviridae* family. The genomic RNA of JEV is single stranded and approximately 11 kb in length. It contains a single open reading frame (ORF) encoding a polyprotein. It has three structural proteins: nucleocapsid protein (C), membrane protein (M), envelope glycoprotein (E) and at least seven non-structural proteins (NS1-NS5). Recently, based on analysis of highly variable nucleotide sequence in the prM and E gene, several authors classified a number of JEV into 4 genotypes [1,4,17,26].

In Korea, JEV was first isolated from an American soldier of human JE case in 1946 [20] and the animal JEV, the Anyang strain, was isolated from a newly born piglet in 1969 [12]. The disease persisted thereafter at near epidemic level until Japanese encephalitis live vaccine (Anyang 300 strain) was developed for pigs in 1980. After an extensive vaccination program for pigs and mosquito control have been carried out, the number of JE case in pigs was reduced to 10-20 cases per year in Korea. Since the first isolation was identified in veterinary science, Japanese encephalitis cases have been reported in pigs for 30 years. But, there was nofurthermore isolation of JEV of the agents that caused abortion in swine.

We heard about a farm in that pregnant sow frequently delivered the mummified fetuses and weak piglets. So, we tried to isolate the causative agent from pigs in the farm located in Gyeonggi province, Korea. A virus was isolated in Vero cells showing specific cytopathic effects. After investigating the biological and physicochemical characterization of the isolate, we confirmed that this isolate belonged to JE virus. In this study, we described the biophysical characterization of the second isolate from the pigs.

Materials and Methods

Preparation of samples and virus isolation

Anticoagulant treated blood samples were collected from

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fattening pigs in Gyeonggi province in Korea. The collection was carried out from September to November in 1999. All the samples were centrifuged and prepared into 10% suspension in α-MEM containing 100 μg/ml kanamycin and 2.5 μg/ml fungizone. The suspension was centrifuged at 2,500 rpm for 10 min and the supernatant fluid was used for virus isolation.

The growth medium of Vero cell cultures consisted of α-MEM supplemented with 0.2 mM non-essential amino acid and 10% heat inactivated fetal bovine serum. The maintenance medium after inoculation was the same as growth medium except that the fetal bovine serum concentration was reduced to 3%. Growth medium was removed from the Vero cell in 24 well plates and 0.1 ml of the sample, diluted 100 fold, was inoculated into the wells. After 2 hr adsorption, the cells were washed by 2 ml PBS and covered by 1 ml/well of the maintenance medium and inoculated at 37°C in a CO_2 incubator for a week.

Hemagglutination (HA) and hemagglutination-inhibition (HI) test

HI test was carried out using the standard method [6] and was modified for microtiter plates. The viral antigens were prepared by sucrose-acetone extraction method from suckling mouse brains infected with JEV, Nakayama strain. HA titer was expressed by the reciprocal of the highest dilution of antigen that showed hemagglutination. The serum specimen was pre-treated with kaolin and washed goose RBC to remove non-specific inhibitor.

Physicochemical properties

The nucleic acid type of the isolate was determined by the inhibitory effects of 5-iodo-2-deoxyuridine (IUDR). After virus adsorption for 60 min, maintenance medium containing 50 μg/ml of IUDR was added. Chloroform/ether and heat treatment effects on the isolate were examined under the conditions shown in Table 2.

Electron microscopy

Cells were harvested using a rubber policeman at 72 hr post-inoculation of the isolate. After centrifugation at 1,500 rpm for 10 min, cell pellets were fixed with 2.5% glutaraldehyde in phosphate buffered saline (PBS) at 4°C for 2.5 hr and post-fixed with 1% osmium tetroxide in PBS at 4 for 2 hr. After dehydration in a graded series of ethanol and propylene oxide, the cells were embedded in spur resin. Ultrathin sections were made and stained with uranyl acetate and lead citrate, and were observed under a Hitach 7100 electron microscope (Hitach, Japan).

Indirect immunofluorescent assay (IFA)

Vero cell monolayer grown on cover slip was fixed in 80% chilled acetone for 20 min about 36 hr after virus inoculation. After washing out excess unbound reagent with PBS, the fixed cells were incubated with JEV specific monoclonal antibody against E protein [11] at 37°C for 30 min in humid chamber, and then stained with FITC conjugated anti-mouse IgG (Cappel, USA). After washing in PBS, the cells were examined by fluorescence microscope (Olympus, Japan).

Reverse transcription polymerase chain reaction (RT-PCR)

Viral genomic RNA was extracted from infected culture

Table 1. Oligonucleotide primers for PCR amplification

Primer designated	Oligonucleotide sequence (5'-3')	Expected size
JEMF (429-448)*	ATC ATG TGG CTC GCA AGC TT	619 bp
JEMR (1,029-1,048)	TCC TTC TAG CAC CAA GTA CA	
JEEF (960-979)	GTC GCT CCG GCT TAC AGT TT	1,541 bp
JEER (2,482-2,501)	GAT GTC AAT GGC ACA GCC GT	

*Numbers in parenthesis indicate the nucleotide sequence of K94P05 strain (GenBank accession no. AF045651).

Table 2. Physicochemical properties of the isolate

Property	Treatment	Virus titer**
	No treatment	6.8
Nucleic acid type	IUDR* 50 ug/ml	6.3
Chloroform stability	20%, 1 hour, 22°C	<1.5
Ether stability	10% 1 hour, 4°C	<1.5
Heat stability	22°C 60 min	6.3
	37°C 60 min	5.8
	56°C 60 min	<1.0
HA activity***	Sucrose-acetone extraction	1,024 HA unit

*5-iodo-deoxyuridine. **Log $TCID_{50}$/ml in Vero cells. ***Mouse brain antigen.

fluid using RNA isolation reagent (Ultraspec, USA) according to the manufacturers instruction. The precipitated RNA was dissolved in DEPC-treated water and stored at −70°C until used. The extracted RNA was denatured at 95°C for 5 min. The denatured RNA was incubated for 50 min at 50°C to obtain the first strand cDNA synthesis using reverse transcriptase reaction with reverse primers (Table 1). Oligonucleotide primers were selected on the basis of the submitted sequence for the K94P05 strain [17]. PCR amplification was carried out in 30 cycles using denaturation at 95°C for 45 sec, annealing at 50°C for 45 sec and extension at 72°C for 60 sec using a thermal cycler (Whatman, Germany). The final extension step was done at 72°C for 5 min. The PCR products were detected by electrophoresing 15 µl in 1.5% agarose gels (Gibco, USA) containing 0.1 µg/ml of ethidium bromide and TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.5).

Infections in experimental animals and artificial pigs

The suckling mice were inoculated by intracranial route with 0.03 ml of isolate and observed for 15 days. As soon as the suckling mice demonstrated illness, the mice were collected to check HA activity of brain emulsion. In addition, 3-4 weeks old mice (ICR strain) were inoculated by intracranial and intraperitoneal route with 0.03 ml and 0.5 ml of isolate in order to check pathogenicity. Clinical signs of the mice were observed for 15 days. Ten-healthy pigs of 8 week old for artificial infection were used. All of them, JEV antibody negative, were injected intramuscularly with 1 ml ($10^{3.3}$ $TCID_{50}$/ml) of the isolate of the third passage in Vero cells. During the observation period after virus inoculation, general clinical conditions were recorded daily. Serum samples collected from the experimental pigs were inactivated by heating at 56°C for 30 min before serological examination. Sera were collected every 2 weeks interval after virus inoculation.

Results

Isolation of JEV in cell culture

Out of the total 134 plasmas inoculated into Vero cell culture for virus isolation, one specimen produced obvious cytopathic effect (CPE) in three successive passages in Vero cells. CPE characterized by rounding, shrinkage and dislodgment from the growth surface was detected microscopically after an incubation period of 5 days. This isolate was cloned 3 times by limiting dilution method, designed as KV1899, which was obtained from plasma of a fattening pig in November 1999.

Physicochemical properties

Physicochemical properties of the virus are shown in Table 2. The addition of IUDR to the cell culture medium did not inhibit the growth of KV1899 strain. The infectivity of the isolate was affected by chloroform as well as ether and by treatment at 56°C for 60 min. But after exposure for 60 min at 22°C and 37°C, the virus was less affected in infectivity titer. Although the virus grown in cell culture didn't have hemagglutination with goose RBC, the mouse brain samples prepared by sucrose-acetone extraction showed 1,024 HA unit.

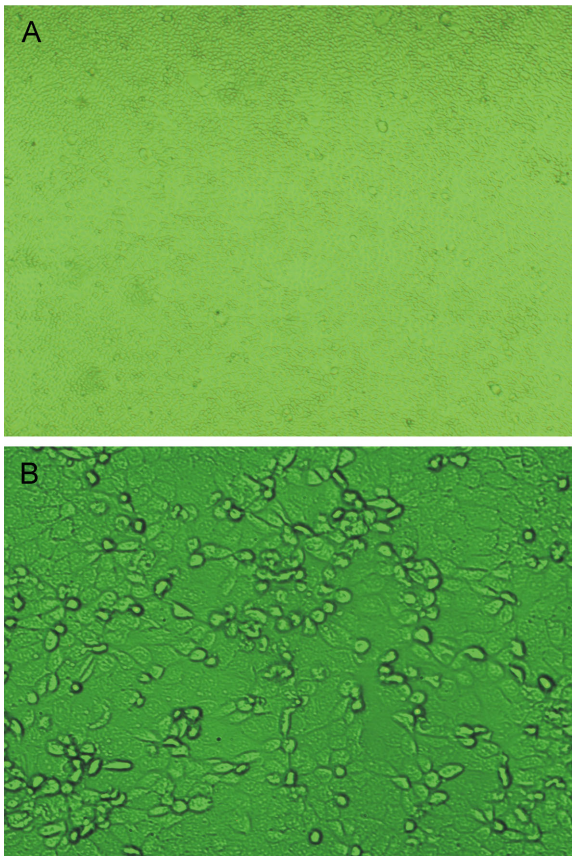

Fig. 1. Cytopathic effect (CPE) of Vero cell inoculated with JEV isolate (A: normal Vero cell, B: Vero cell infected with JEV isolate).

Indirect immunofluorescence assay (IFA)

The progress of viral replication in Vero cells was monitored by the appearance of cytopathic effects, IFA test, HA test, and electron microscopy. Although there were various morphologic changes in the cells such as rounding and floating of cells in the medium, these cytopathic changes were not enough to determine if viral growth had occurred. The immunofluorescence staining of infected cell cultures with JEV specific monoclonal antibody against E protein was the best indicator (Fig. 2). Several foci of fluorescence were noticed in the cytoplasm within 36 hr post inoculation.

Electron microscopy (EM)

In electron microscopy, virus particles were similar in size

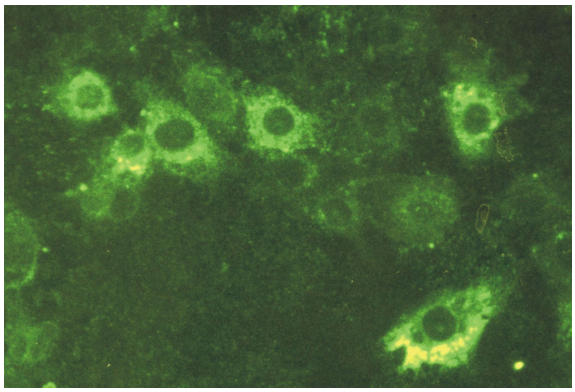

Fig. 2. Immunofluorescence of a JEV, KV1899 strain in Vero cell with monoclonal antibody against JEV.

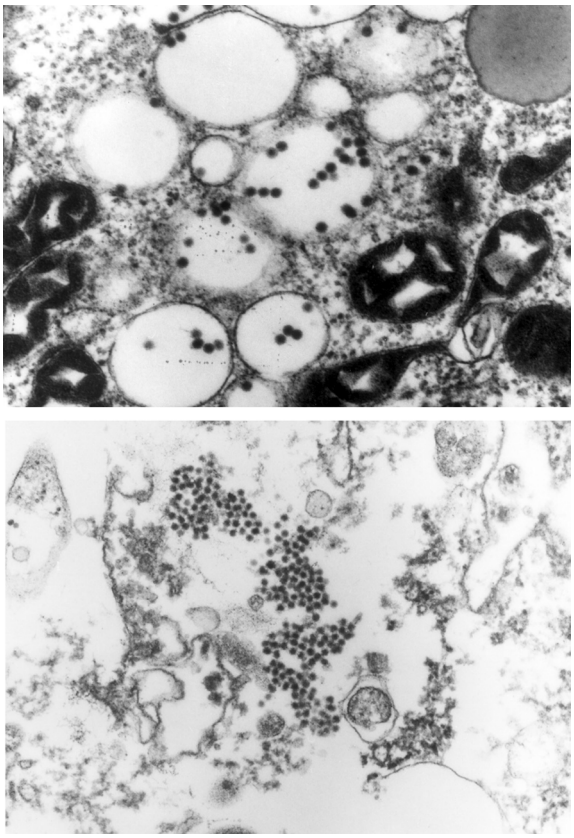

Fig. 3. Japanese encephalitis virus particles in cytoplasm of KV1899 isolate infected Vero cells (×46,000).

and the intact particles were covered with envelope. Most particles of the virus were spherical and ranged from 40 to 50 nm in diameter (Fig. 3). Apparently, virus particles that bud into vacuoles were trapped there upon disruption of the Vero cell. Virus containing vacuoles would be liberated by exocytosis or cell lysis.

Identification of the isolate by RT-PCR

Although physicochemical and biological methods could provide possible clue, molecular method was applied to get definitive answer of JEV isolate. The RT-PCR using specific primers of JEV could amplify membrane and envelope genes of the new isolate (Fig. 4). Expected sizes of M and E genes were detected with 619 and 1,541 base pairs respectively.

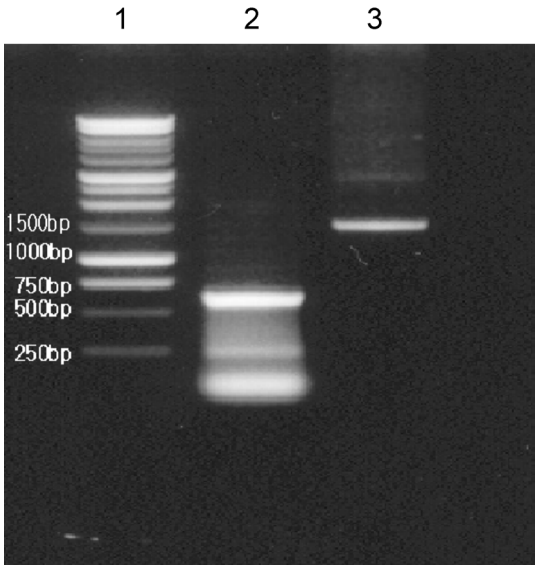

Fig. 4. RT-PCR products of KV1899 were separated by 1.5% agarose gel electrophoresis. M and E genes were amplified using JEV specific primers in lane 2 (M) and 3 (E). lane 1; molecular weight marker, lane 2; M, lane 3; E.

Experimental infections

Suckling mice were inoculated with isolate by intracranial route and observed for 15 days. All of the mice inoculated with KV1899 strain showed paralysis, signs of nervous system and died within 7 days post inoculation. The infected mouse-brain suspension of the isolate revealed 1,024 HA unit (Table 2). Pathogenicity of isolate was carried out on 3-4 weeks mice by intracranial (IC) and intraperitoneal (IP) inoculation. The mice inoculated with isolate showed $10^{4.5}$ LD_{50}/0.03 ml (IC) and $10^{3.0}$ LD_{50}/0.5 ml (IP) according to the inoculation route. In contrast, the response of mice by attenuated JEV strain (Anyang300) did not show clinical signs (Table 3). All the pigs, showing no HI antibody to JEV, were inoculated intramuscularly with 1 ml of the isolate at the third passage in Vero cell cultures. The artificially inoculated pigs did not show clinical signs throughout the observation period. All the inoculated animals had HI titer of 320 to 2,560 against JEV at 14 to 42 DPI. But there was no HI titer change in the control group (Fig. 5).

Discussion

A cytopathogenic agent was isolated in Vero cell culture from plasma of a fattening pig, which was selected from a

Table 3. Comparison of pathogenicity of isolate and attenuated vaccine strain in mice

Virus strain	Virus titer	Dose	Route	Lethal Dose
KV1899	$10^{6.5}$ $TCID_{50}$/ml	0.5 ml	IP*	$10^{3.0}$ $10^{4.5}$
		0.03 ml	IC**	
Anyang300***	$10^{5.5}$ $TCID_{50}$/ml	0.5 ml	IP	- -
		0.03 ml	IC	

*IP: intraperitoneal route. **IC: Intracranial route. ***attenuated strain of JEV in CEF.

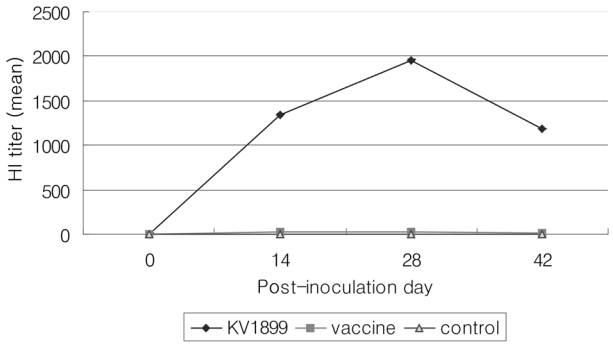

Fig. 5. Kinetics of antibody response of pigs experimentally inoculated with the isolate.

pig farm that had reproductive disorders such as stillbirth, mummified and dead neonatal piglets. Tomiak *et al.* reported that 6 isolates were isolated from 171 dead fetuses and the isolation rate might have been considerably low. This low isolation rate may be responsible for the physical and chemical properties of JE virus. In this study, only one isolate was isolated from 134 samples. The low isolation rate of JEV might be responsible for low incidence of JE in Korea and short viremia period in pigs. It was the second isolation of JEV in pigs in Korea.

JEV could be propagated in a variety of primary and continuous cell culture derived from human, monkey, swine, mosquito, avian tissue [2,10,22]. Anyang strain of JEV from a new born-piglet was cultivated by using chicken embryonic fibroblast cell [12,13]. In this study, the new isolate was propagated with typically clear cytophatic effect in Vero cells. The cells infected with isolate displayed cell rounding, shrinkage and dislodgement from the growth surface (Fig. 1). Therefore, CPE of the isolate in Vero cell cultures was employed for JE virus titration.

Tissue culture virus was stable against heating at 22°C and 37°C for 60 min. When it was subjected to heat at 56 for 60 min, viral infectivity was reduced below $10^{1.0}$ $TCID_{50}$/ml. Our results also demonstrated that heat liability of our isolate was in agreement with the result of Fuji strain [22].

JE virus could be purified from brain homogenate of infected mice by ultracentrifugation. The purified JE virus from brain homogenate using ultracentrifuge revealed homogeneous size of virus particles of 38 nm in diameter [24]. In this report, electron microscopy demonstrated the virus particles in cytoplasm of infected Vero cell (Fig. 3).

RT-PCR has been used to detect *flavivirus* rapidly and specifically in various biological samples [7,23]. In this report, JEV M and E genes of isolate were amplified from infected cell culture by RT-PCR. Expected sizes of M and E genes were detected with 619 and 1,541 base pairs, respectively.

Previous works [10,13] have reported experimental infections in pigs with JEV. Kodama *et al*. [10] described that, in colostrum-deprived pigs, Fumumoto strain showed general weakness and high temperature on the 6th and 7th days post inoculation (PI). Hemagglutination-inhibition and neutralizing antibody was produced rapidly after 8 days PI. In this study, the antibody response of the experimental inoculation of JEV isolate in fattening pigs is shown in Fig. 5. When 10 pigs were inoculated intramuscularly with the KV1899 strain, slight weakness was observed from 4 to 10 days post-inoculation. Response of colostum-deprived pigs to infection with attenuated Japanese encephalitis virus (m strain) showed low HI titer ranging 10 to 20. Titer of HI antibody produced by virulent Fumumoto strain was 640. HI antibody of KV1899 strain elevated rapidly on the 14th day. HI titer ranged from 640 to 2,560. In addition, 3-4 weeks mice inoculated isolate showed $10^{4.5}$ LD_{50}/0.03ml in intracranial route. Therefore, the new isolate, KV1899 strain could be considered as virulent as shown in wild type virus.

From the results of physiological, biological and molecular characterization, the isolate was identified as a member of *Flaviviridae* Japanese encephalitis virus. These characteristics were similar with those of other members of *Flaviviridae* [2] and with the results of other JEV described in previous studies [9,12,25]. Our physicochemical and morphological studies on the agent disclosed that the KV1899 isolate contained RNA genome, was inactivated by ether or chloroform as well as treatment at 56°C, and had a typical morphological feature of JEV in EM.

Characterization of the KV1899 strain at the physicochemical, biological and genetic identification would be an important step towards identifying those properties of the virus, which may aid in the design and construction of recombinant JEV vaccine that are based on the E, NS1 protein. It is necessary

to study further on the genetic characterization of the isolate.

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