

An Enveloped Virus Isolated from Field-caught
Culex tritaeniorhynchus by *Aedes albopictus*, Clone C6/36,
Cell Cultures, Causing Extensive Cytopathic Effect¹⁾

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Abstract: Homogenate of *Culex tritaeniorhynchus* captured in a field of Osaka Prefecture was inoculated to *Aedes albopictus*, clone C6/36 cells, and a virus was isolated which showed extensive cytopathic effect and produced large plaques on the cell line. The agent, tentatively named as Yokoshoji virus, grew to high titer in C6/36 cells but did not grow at all in mammalian cell lines which support the growth of arboviruses. The virus growth was not inhibited by inhibitors of DNA synthesis indicating RNA nature of the genome. The infectivity was sensitive to ether and deoxycholate and the virus did not show any cross reactions with grouping antisera against arboviruses or some pestiviruses. Spherical particles around 80 nm in diameter were revealed under an electron microscope. The density of the infective particles was 1.15g/cc and sedimented at 215 S in sucrose gradients. Only 2 structural proteins of 64k and 45k dalton molecular weights were revealed when ³H-leucine-labeled and purified virion was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The results as a whole indicate that the agent is an insect virus of mosquitoes.

Key Words: Mosquitoes, Enveloped virus, Cytopathic effect

Received for Publication, June 18, 1986

Contribution No. 1811 from the Institute of Tropical Medicine, Nagasaki University

1) Presented at the 5th International Congress of Virology, Strasbourg, France, August, 1981.

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INTRODUCTION

Japanese encephalitis (JE) virus is almost an exclusive arbovirus in Japan with medical importance. Epidemiological monitoring on the virus spread in nature has been carried out during epidemic summer season in Japan by antibody survey among swine, major amplifier vertebrates, and by virus isolation from field-caught vector mosquitoes, *Cx. tritaeniorhynchus*. The first author isolated a clone of *Ae. albopictus* cells, C6/36, with high sensitivity to arboviruses, such as dengue or chikungunya viruses (Igarashi, 1978), and utilized the cell line for the isolation of arboviruses from field as well as clinical specimens (Igarashi *et al.*, 1981a, b, c, d, e, f; 1982a; 1983). Through these studies, many filtrable agents, which apparently have never been described, could be detected from field mosquitoes by cytopathic effect (CPE) or plaque formation on C6/36 cells (Igarashi *et al.*, 1981a). An agent which produced small plaques were quite abundant in *Cx. tritaeniorhynchus* mosquitoes in Osaka Prefecture and some of its characters have been reported including morphological similarity to flaviviruses (Okuno *et al.*, 1984). Morphologically similar small-plaque forming agent was isolated also from leaf hoppers captured on East China Sea (Igarashi *et al.*, 1982b). Both these agents, however, did not have any serological cross reactions with known flaviviruses or with CFA (cell-fusing agent) isolated from a line of *Ae. aegypti* (Stollar & Thomas, 1975). In this report, we describe another filtrable agent also isolated from field-caught *Cx. tritaeniorhynchus* but produces large plaques and extensive CPE on C6/36 cells with possible characteristics of an insect virus of mosquitoes.

MATERIALS AND METHODS

Cells: *Ae. albopictus*, clone C6/36 cells (Igarashi, 1978) were grown at 28°C with cell growth medium consisting of Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 0.2 mM each of nonessential amino acids (Eagle, 1959). Two mammalian cell lines, BHK21 and LLC-MK2, were grown at 37°C with Eagle's medium supplemented with 10% calf serum.

Virus: The virus was isolated as a filtrable agent causing extensive CPE (Photo 1) and producing large plaques (Photo 2), from a pool of 17 engorged females of *Cx. tritaeniorhynchus* by inoculation to C6/36 cells as described before (Igarashi *et al.*, 1981a). The mosquitoes were captured on July 12, 1976, at Yokoshoji, Higashiosaka City, Japan, by Dr. Buei and his staffs at Osaka Prefectural Institute of Public Health using light traps. Therefore, the virus was tentatively named as Yokoshoji virus. The virus was plaque purified 3 times to prepare seed virus, and its infectivity was assayed by plaque titration on C6/36 cells and expressed as plaque forming units (PFU). Monolayer cultures of C6/36 cells in 60 mm diameter Petri dishes were inoculated with 0.2 ml/dish of virus specimens serially diluted in 10 fold steps. After 2 hours of adsorption, cells were covered by the first overlay medium of 1% Difco Special Noble Agar in the maintenance medium (cell growth medium from which FCS concentration was reduced to 2%). The dishes were incubated at 28°C for 5 days in humidified 5% CO₂ atmosphere. Then, the

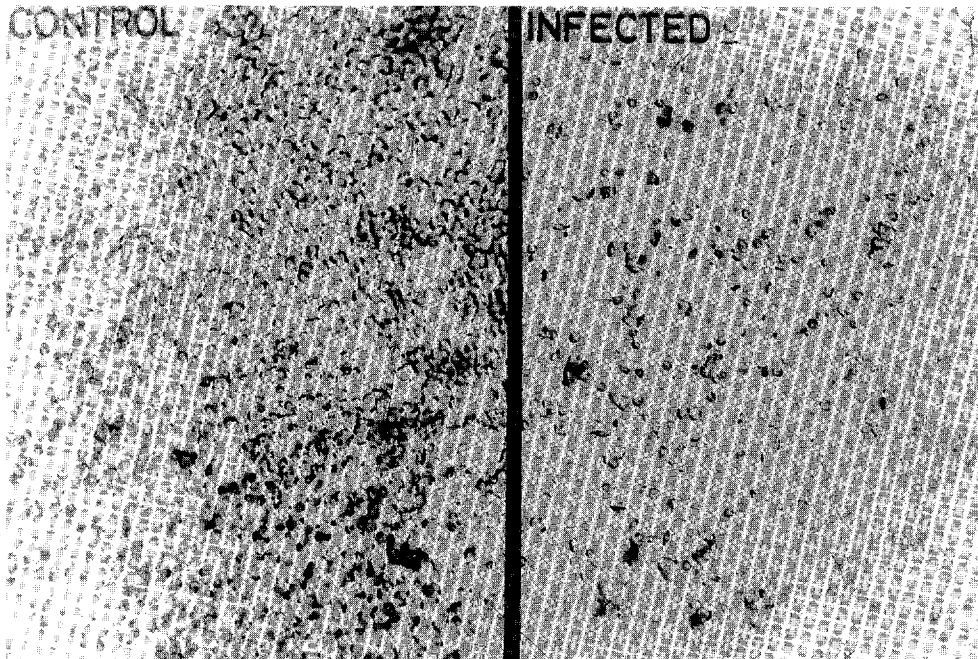


Photo 1. Light microscopic picture of C6/36 cells : uninfected control on the left and Yokoshoji virus infected on the right.

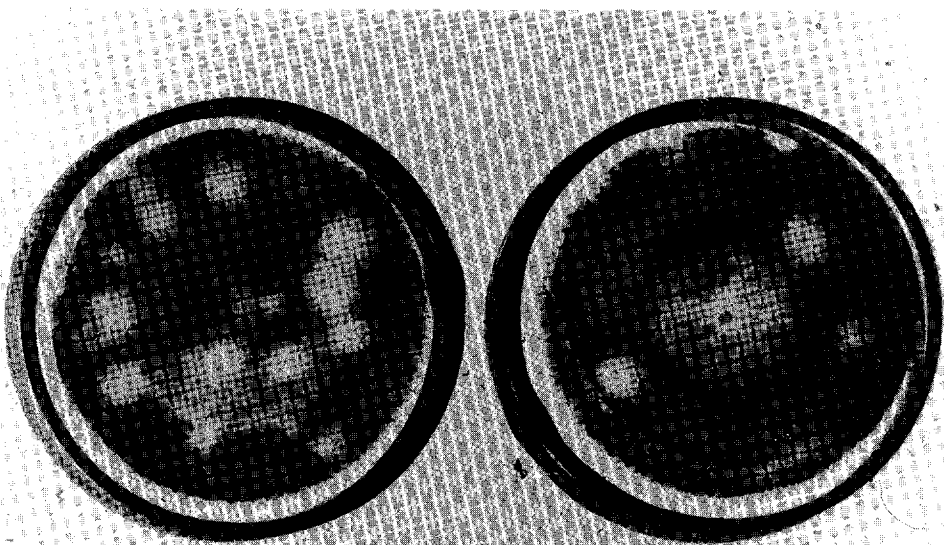


Photo 2. Plaques produced on C6/36 cells by Yokoshoji virus under agar overlay.

second overlay containing neutral red was introduced and the plaques were counted from the following day. As control, 3 alphaviruses; chikungunya, Sindbis and Getah viruses were used.

Virus growth experiment: Replicate cultures of C6/36 cells or other cell lines were inoculated with seed virus at high multiplicity of infection (MOI) over 10 PFU/cell. After 2 hours of adsorption at 28°C for C6/36 cells and at 37°C for mammalian cells, virus inoculum was removed and the cells were washed twice with PBS and incubated at various temperatures under the maintenance medium. At various time of incubation, duplicate cultures were harvested, their infected fluids were collected and cells were scraped into PBS and homogenized. Virus infectivity in the fluid and cell homogenate was assayed as described above.

Purification of the virus: Seed virus was inoculated to C6/36 cell cultures at low MOI around 0.1 PFU/cell. After 2 hours of adsorption, the cells were covered by the maintenance medium and incubated at 28°C for 2 days. Infected culture fluid was collected and centrifuged at low speed. The resulting supernatant was added with polyethylene glycol 6000 and NaCl to final concentrations of 6% and 0.5 M, respectively. The mixture was centrifuged at 10,000×g. for 30 min to precipitate the virus, which was taken up into a small volume of STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4) and centrifuged at low speed. The supernatant was loaded on a sucrose gradient (30–50% in STE) and centrifuged at 35,000 rpm for 20 hours at 4°C in an SW 50.1 rotor of a Beckman model L2–65B ultracentrifuge. Fractions were collected by an ISCO gradient fractionator model 640 and assayed for infectivity and refractive index for density determination. Peak fractions of the infectivity were pooled and used as purified virus.

SDS-PAGE: The seed virus was inoculated to C6/36 cells as described for the growth experiment. After virus adsorption and washing, the cells were incubated under leucine-free maintenance medium. At 6 hours after infection ³H-leucine was added to the medium at 100 µCi/ml and incubated for another 12 hours. The virus was concentrated and purified as described above, and was disrupted by 1% SDS and 1% 2-mercaptoethanol by heating at 100°C for 1 min. Polypeptides were separated by discontinuous SDS-PAGE (Laemmli, 1970). Radioactive polypeptide bands were revealed by fluorography (Bonner & Lasky, 1974).

Electron microscopy: Purified virus was dialyzed against 1% ammonium acetate, pH 7.0, at 4°C overnight, stained by 2% uranyl acetate and observed under a Hitachi model HU-12 electron microscope.

Serological tests: Grouping immune ascitic fluids against various arboviruses were obtained from National Institute of Health, USA. Antisera against several pestiviruses, such as bovine diarrhea virus, hog cholera virus and equine arteritis virus, were supplied by Dr. Y. Inaba, National Institute of Animal Health, Japan. Rabbit antiserum against CFA was supplied by Dr. V. Stollar, Rutgers Medical School, New Jersey, USA. The new virus isolate, Yokoshoji virus, was examined for its reactivity with these immune ascitic fluids and antisera by complement-fixation and neutralization tests.

RESULTS

Growth characteristics of the virus

Fig. 1 shows typical growth curve of the virus in C6/36 cells at 28°C. After 4–6 hours of incubation, infectivity rapidly increased to reach more than 10^7 PFU/ml after 12 hours of infection. The infectivity in the culture fluid was always higher than that in the cell homogenate. On the other hand, infectivity of Yokoshoji virus did not increase when the virus was inoculated to BHK21 or LLC–MK2 cells even at 28°C, although these cell lines could support the growth of arboviruses quite well (data not shown). Also the virus did not give any pathogenic effect on suckling mice after intracerebral inoculation, nor did it produce antibodies in the inoculated animals.

Table 1 shows 48 hours yield of Yokoshoji virus, and 2 alphaviruses, chikungunya and Getah, in C6/36 cells at 3 different temperatures of incubation. The growth of Yokoshoji virus appears to be more temperature-sensitive than 2 alphaviruses, because its yield at 37°C was significantly lower than that at 28°C, while the yields of 2 alphaviruses were similar at these temperatures.

Table 2 shows the effect of various inhibitors of DNA synthesis on the yield of Yokoshoji virus. All the 5 different kinds of inhibitors did not apparently affect the virus yield, while these inhibitors gave significant reduction in the yield of herpes simplex

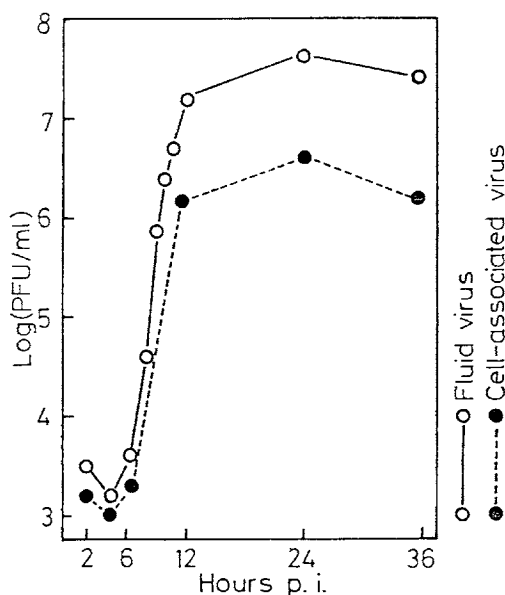


Fig. 1. Growth of Yokoshoji virus in C6/36 cells at 28°C. Replicate cultures of C6/36 cells were infected with the virus at high MOI. Virus infectivity in the infected fluid (○—○) and cell homogenate (●.....●) was assayed as described in the text.

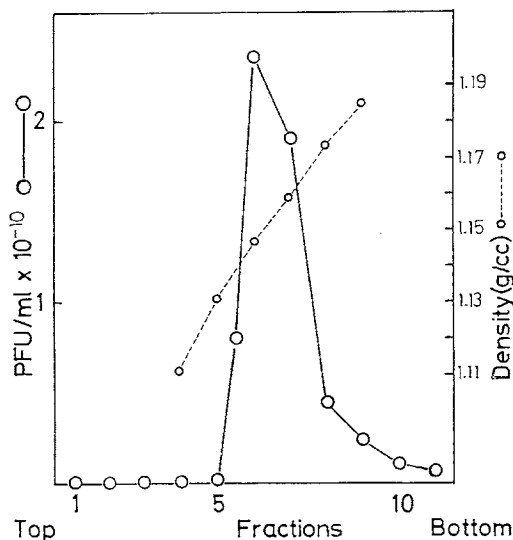


Fig. 2. Sucrose density gradient sedimentation of Yokoshoji virus. Concentrated virus preparation was fractionated by sucrose density gradient centrifugation to determine infectivity and density of the fractions as described in the text.

virus at these concentrations (data not shown). The result indicate RNA nature of the virus genome.

Characteristics of Yokoshoji virus particles

Table 3 shows the result of stability test on Yokoshoji virus. The virus infectivity was sensitive to ether and deoxycholate, indicating the presence of envelope on the virus particles. The result also showed that the virus infectivity was rather heat-labile.

Fig. 2 shows fractionation of Yokoshoji virus by sucrose density gradient equilibrium centrifugation. The virus infectivity distributed rather broadly around the density of 1.15 g/cc.

Fig. 3 shows the result of sucrose gradient velocity sedimentation of Yokoshoji virus and Sindbis virus run in parallel. Again, the infectivity of Yokoshoji virus sedimented forming relatively broad peak with estimated sedimentation coefficient of 215

Table 1. Effect of incubation temperature on the virus yield in C6/36 cells

Temperature (°C)	48 hours yield: log (PFU/ml)		
	Yokoshoji	chikungunya	Getah
28	6.0	7.8	8.7
34	5.4	7.9	8.4
37	4.2	8.5	8.2

Table 2. Effect of inhibitors of DNA synthesis on the growth of Yokoshoji virus in C6/36 cells

Inhibitors	24 hours yield log (PFU/ml)
None	6.9
5-Iodo-2' -deoxyuridine, $10^{-4.5}$ M	6.6
5-Fluoro-2' -deoxyuridine, $10^{-4.5}$ M	7.4
Hydroxyurea, 10mM	6.4
Cytosine arabinoside, 30 μ g/ml	6.3

Table 3. Stability test on Yokoshoji virus

Treatment	Residual infectivity log (PFU/ml)
Control	7.0
Ether (20%, 4°C, overnight)	5.4
Sodium deoxycholate (0.5%, 37°C, 1 hour)	3.7
Heating (56°C, 30 min)	4.0

S compared with the value of 280 S for Sindbis virus (Westaway *et al.*, 1985a).

Photo 3 shows structural proteins of Yokoshoji virus and Getah virus as control analyzed by the fluorography of ^3H -leucine labeled and purified virions. There were only 2 polypeptides in Yokoshoji virus with estimated molecular weights of 64k and 45k daltons. At present we do not have any data on the localization of these structural proteins inside the virion.

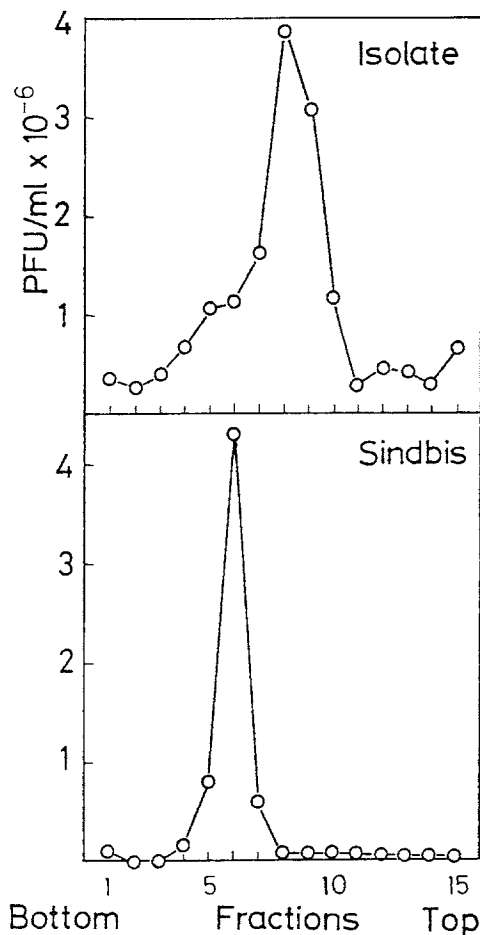


Fig. 3. Sucrose gradient velocity sedimentation of Yokoshoji virus and Sindbis virus. Culture fluid from C6/36 cells infected with Yokoshoji virus (top panel) and Sindbis virus (bottom panel) was sedimented through 15–30 % sucrose gradient in STE buffer in an SW 50.1 rotor of a Beckman L2 – 65B ultracentrifuge at 37,000 rpm for 60 min to determine infectivity of each fraction.

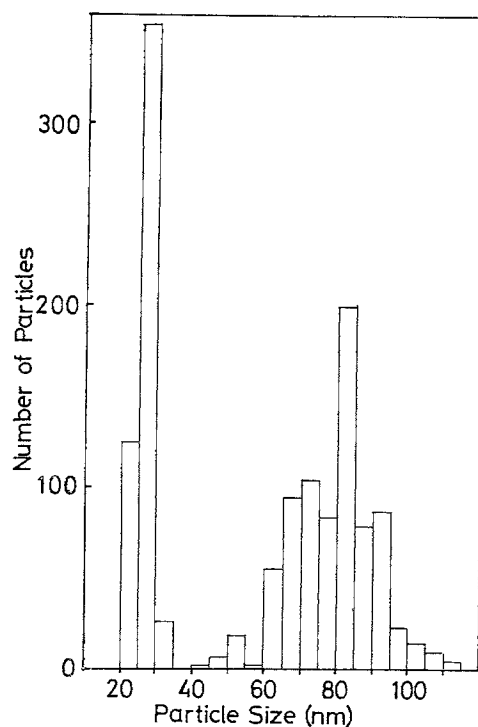


Fig. 4. Size distribution of purified Yokoshoji virus preparation observed under an electron microscope.

Photo 4 shows negatively stained purified Yokoshoji virus observed under an electron microscope, and Fig. 4 shows size distribution of the particles. Spherical particles around 80 nm in diameter were observed with apparent envelope structure. The particle size distribution in Fig. 4 showed relative heterogeneity and indicating the presence of less than 30 nm particles. These findings were also shown in Photo 4.

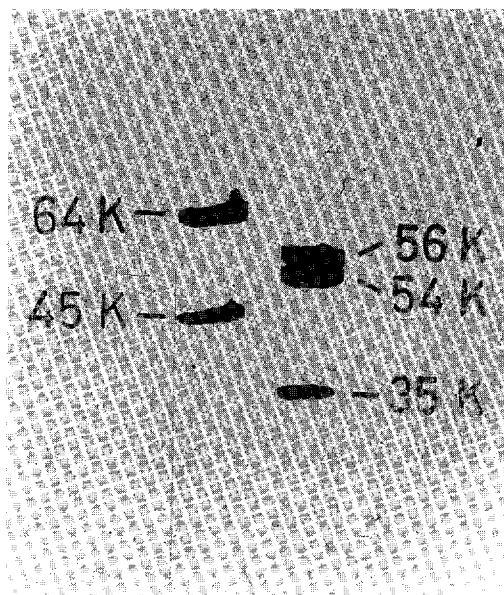


Photo 3. SDS-PAGE of Yokoshoji virus on the left and Getah virus on the right to reveal structural proteins by fluorography.

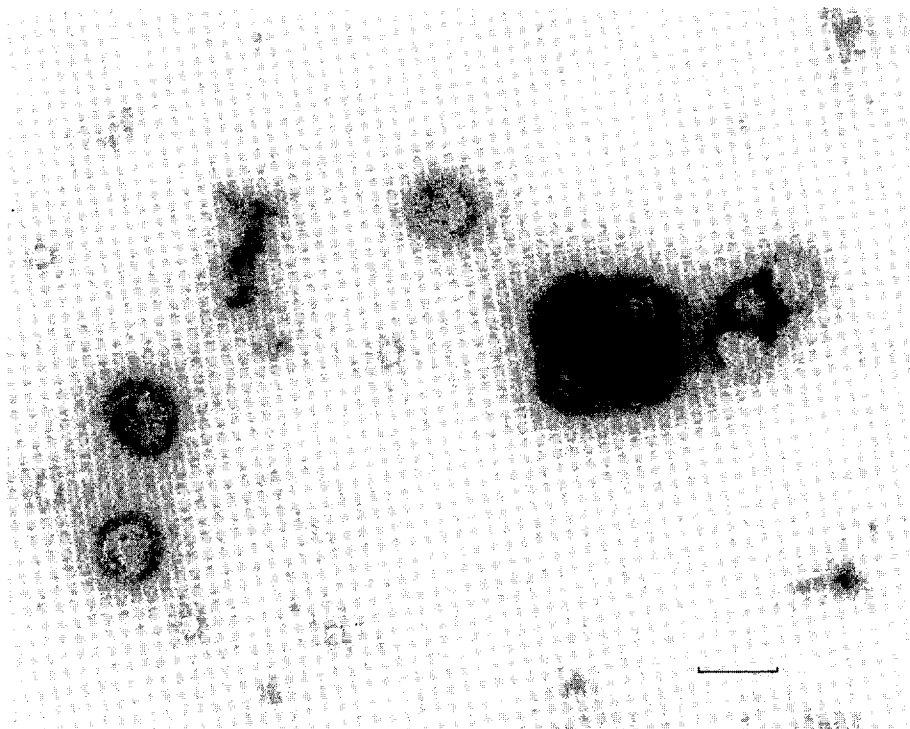


Photo 4. Electron micrograph of purified Yokoshoji virus preparation. Bar : 100 nm.

DISCUSSION

Our data indicate that enveloped particles around 80 nm diameter most probably represent the infective particles of Yokoshoji virus. At present we don't have any idea about the small round particles observed in the purified preparation of Yokoshoji virus. Although we did not directly analyze the genome of Yokoshoji virus, the resistance of its growth to various inhibitors of DNA synthesis indicated its RNA nature. The structural protein of Yokoshoji virus consisted of only 2 polypeptides, the molecular weights of which were quite different from those of togavirus (Westaway *et al.*, 1985a) or flavivirus (Westaway *et al.*, 1985b) in their size. Neither we could find any positive reactions between Yokoshoji virus and known arboviruses, some pestiviruses or CFA by serology. In spite of its rapid growth to high titer in C6/36 cells, Yokoshoji virus did not show any indication to grow in mammalian cell lines, which can support the growth of various arboviruses. The result indicates that Yokoshoji virus could very probably be a kind of insect virus of mosquitoes. Further characterization will be necessary on its genome RNA, localization of 2 structural polypeptides, and nature of small round particles observed in the purified preparation.

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野外採集コガタアカイエカからヒトスジシマカ培養細胞クローンC6/36を用いて分離され強い細胞病変を示す外被膜を有するウイルス

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大阪府の野外で採集されたコガタアカイエカの乳剤をヒトスジシマカ培養細胞クローンC6/36に接種して、この細胞に強い細胞病変を示し、大きなブランクを作るウイルスを分離した。仮にヨコショウジウイルスと名づけたこのウイルスはC6/36細胞では良く増殖するがアルボウイルスが良く増殖する哺乳類細胞では増殖しなかった。このウイルスの増殖はDNA合成阻害剤で抑制されないでウイルス遺伝子はRNAであると考えられる。ウイルス感染価はエーテル及びデオキシコール酸に感受性であり、既知のアルボウイルスとの交叉反応は認められなかった。精製ウイルスの電子顕微鏡観察によって外被膜を有する直径約80nmの球型粒子が観察された。蔗糖密度勾配遠心法によって感染性粒子の密度は1.15 g/cc, 沈降係数は215 Sと推定された。トリチウムロイシン標識精製ウイルスをドデシル硫酸ナトリウム存在下のポリアクリルアミドゲル電気泳動で分析すると分子量64 Kと45 Kの二つのポリペプチドがウイルス構造タンパクとして存在することがわかった。これらの所見を総合するとこのウイルスは蚊の昆虫ウイルスの一種であろうと考えられる。