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Larvicidal Effect of an Insect Virus of Mosquitoes: An attempt to use as a potential agent of biological control on mosquitoes

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Abstract: Effect of a mosquito insect virus infection on several laboratory−colonized mosquito larvae were investigated. Larvicidal effect was dependent on the virus dose, mosquito species and also the stage of the larvae. When 4th instar larvae were infected, cumulative mortality of mosquitoes were from 13 to 72% (highest with *Anopheles sinensis* and lowest with *Culex pipiens molestus*), in contrast to 0−8% in the control (highest with *Cx. p. molestus* and lowest with *Aedes albopictus*). In the case of *Ae. aegypti*, cumulative mortality was highest when the virus was infected to the 4th instar larvae and the less mortality was recorded when earlier instar larvae were infected. This tendency was also observed for *Cx. tritaeniorhynchus* and *An. sinensis*. However, opposite tendency was observed for *Ae. albopictus*, and *Cx. p. molestus* with higher mortality when the lst instar larvae were infected than the 4th or 3rd instar. The higher mortality resulted in the lower pupation or emergence rate. The dead larvae appeared to have shrunk and dehydrated. Virus growth was observed not only in dead larvae but also in apparently healthy ones. No transovarial transmission of the virus was observed from the adult females emerged from virus−infected larvae to the next generation. No pathological effects or appreciable antibody production were observed by intracerebral inoculation of Yokoshoji virus to weaning mice. Antibody survey among swine and bovine population in Nagasaki Prefecture did not reveal any indication of Yokoshoji virus infection in nature.

Key words: Insect virus, Mosquitoes, Larvicidal effect

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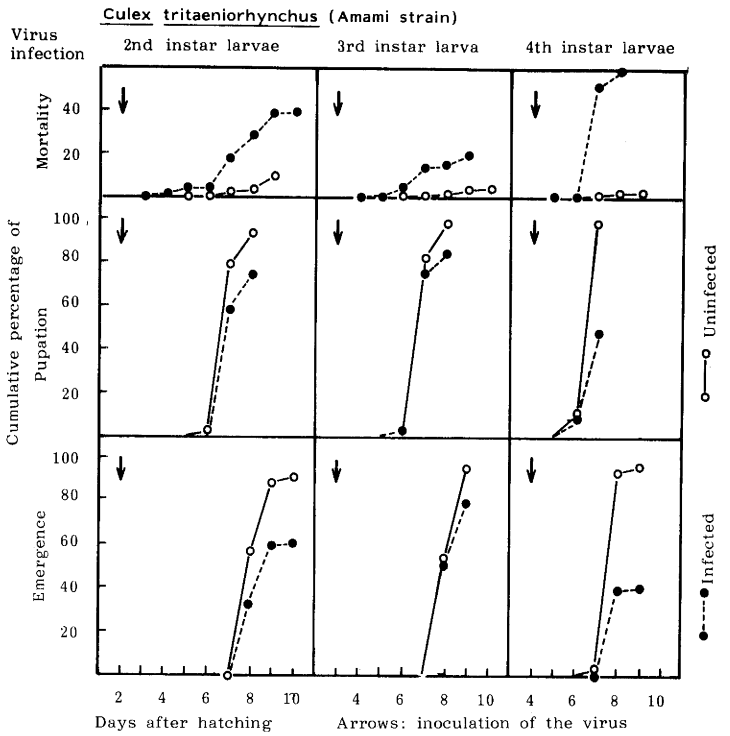

Fig. 5. Effect of Yokoshoji virus infection on *Cx. tritaeniorhynchus.*

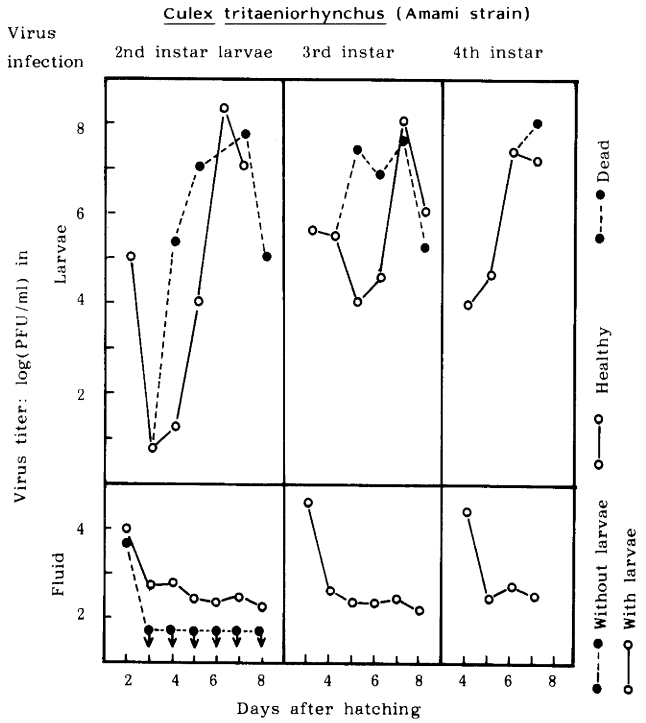

Fig. 6. Growth of Yokoshoji virus in infected *Cx. tritaeniorhynchus.*

after virus infection to mosquito larvae: (1) a few ml of the infected fluid, (2) ten individuals of healthy larvae, (3) all the dead larvae, and (4) all the newly metamorphosed pupae, which were transferred into another glass container with cover and water. Newly emerged adults from this container were again transferred into netted cages. Specimens (1), (2), and (3) were used for assay of virus infectivity. The specimens (2) and (3) were homogenized in 2 ml of PBS containing 0.2% of bovine plasma albumin and centrifuged at 3000 rpm for 15 min. The resulting supernatant was filtrated through 0.22 μm pore size membrane filter along with the specimen (1). The virus titer in the filtrated specimens was assayed by plaque titration as described in the accompanying paper (Igarashi *et al.*, 1986). The numbers of dead larvae, metamorphosed pupae and adults were counted every day to calculate mortality, pupation and emergence rates.

Animal experiment with Yokoshoji virus, preparation of standard antiserum and antibody assay: In order to see the effect of Yokoshoji virus infection to vertebrates, infective virus stock was intracerebrally inoculated to weaning mice. The mice were observed for 21 days to see any pathological symptoms, followed by the bleeding for antibody assay. Purified Yokoshoji virus prepared from infected C6/36 cell culture fluid as described in the accompaying paper (Igarashi *et al.*, 1986) was emulsified with an equal volume of Freund's complete adjuvant and intramuscularly inoculated to rabbits twice with 1 month interval. Rabbits were bled 1 week after the second immunization and the sera were used as the standard anti-Yokoshoji virus sera. Antibody titers agasinst Yokoshoji virus was measured by indirect Micro ELISA (Voller *et al.*, 1976). Basic condition of the assay was set up by checkerboard titration using varying concentration of purified virus as antigen and varying dilution of horseradish peroxidase (HRPO)-conjugated anti-rabbit IgG goat IgG (Cappel Laboratories, USA) using 1:100 dilution of standard antivirus serum and preimmune serum. For the antibody screening, test sera were diluted 1:100 and reacted with the antigen-coated microplate, followed by appropriate HRPO-conjugated IgG and peroxidase reaction.

Statistical methods. The methods described by Snedecor (1952) were followed.

RESULTS

Effects of virus infection on laboratory colonized mosquitoes

Fig. 1 shows cumulative mortality, pupation and emergence rates of *Ae. albopictus* infected with Yokoshoji virus and control mock-infected series. Larvae were infected at various stages after hatching as indicated by vertical arrows. Significant difference was observed between the infected and control in terms of their survival or mortality rate of larvae, resulting in the differences in the pupation and emergence rates. The highest mortality was recorded when lst instar larvae were infected and the cumulative mortality decreased as the older larvae were infected. Fig. 2 shows virus titer in dead and apparently healthy larvae as well as in the water where the larvae were maintained in this experiment. Virus titer in dead larvae reached to the plateau levels around 10^8 PFU/ml after 4, 2, 3, and 2 days of infection to the lst, 2nd, 3rd and 4th instar larvae, respectively. Virus growth was recorded, not only in dead larvae, but also apparently healthy ones as well, though virus titer in healthy larvae tended to be less than in dead ones.

Virus titer in the water decreased quite rapidly but was maintained at low levels until the end of the experiment, which could probably be supported by continuous supply of the virus from infected lervae.

Figs. 3 and 4 were similar results with *Ae. aegypti* as Figs. 1 and 2 for *Ae. albopictus*. However, the effect of the larval stage on the mortality (or survival) by virus infection was quite opposite, that is, higher mortality was recorded when older stage of larvae were infected. Here also, virus growth was recorded not only with dead larvae, but also with apparently healthy ones. No significant difference was observed between the virus titer in dead and healthy larvae when the 4th instar larvae were infected. While, the virus titer in healthy larvae were significantly lower than those in dead ones when younger larvae were infected. The decline of virus titer in the water in Fig. 4 followed similar trend as in Fig. 2.

Figs. 5 and 6 show similar results with *Cx. tritaeniorhynchus* but using only 2nd, 3rd and 4th instar larvae. No special trend was observed for the larval stage and mortality, however, the highest mortality was recorded when the 4th instar larvae were infected. Again, virus growth in apparently healthy larvae were noticed. As shown in the left lower panel of Fig. 6, virus titer in the water without infected larvae rapidly depleted to undetctable level even 1 day after the infection, supporting the previous description that low levels of infective virus in the water was supplied by the infected larvae.

Figs. 7 and 8 show similar results with *Cx. p. molestus* In this case difference in the mortality between the infected and mock−infected series was observed only when the lst instar larvae were infected. In this system, the virus titer, both in dead and healthy larvae, was less than the values in the previous experiments. Curiously, the infection to the lst instar larvae, which showed difference between infected and mock−infected, resulted in the continuous decrease in the virus titer of the larvae along with the days after infection, both in dead and healthy ones. Probably, this species of mosquitoes was not so sensitive to Yokoshoji virus infection, especially for older larvae.

Figs. 9 and 10 are similar results as the previous figures but were obtained when the 3rd and 4th instar larvae of *An. sinensis* were infected. In this case greater difference between the infected and mock−infected was observed when the 4th instar larvae were infected than the 3rd ones. The virus titer, both in dead and healthy, was higher when the 4th instar larvae were infected than the 3rd ones.

The above results were summarized into Table 1, showing that the virus infection resulted in the highest mortality when the lst instar larvae were infected for *Ae. albopictus* and *Cx. p. molestus*, in contrast to *Ae. aegypti*, *Cx. tritaeniorhynchus*, and *An. sinensis*, in which infection to the 4th instar larvae gave the highest motality. The highest cumulative mortality observed for virus−infected mosquitoes was between 56 to 72% when proper stage of the larvae were used in contrast to 10−38% for relatively resistant stages. Mortality in mock−infected series was generally lower than in the virus−infected series, 0.5 to 14% for the most susceptible stage and 0−16% for the least susceptible ones.

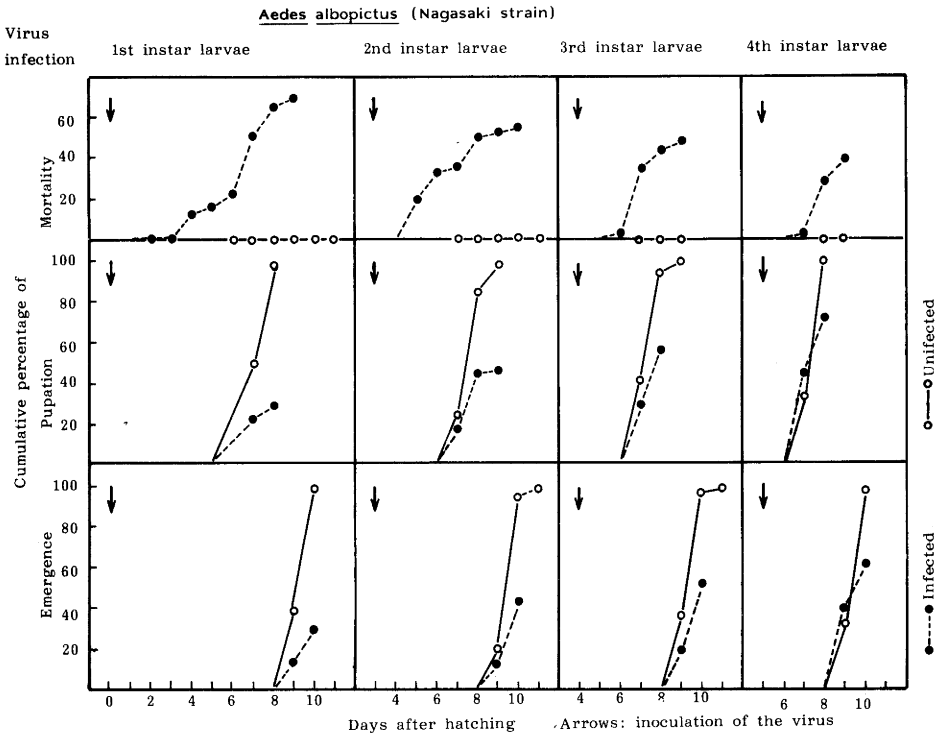

Fig. 1. Effect of Yokoshoji virus infection on *Ae. albopictus*.

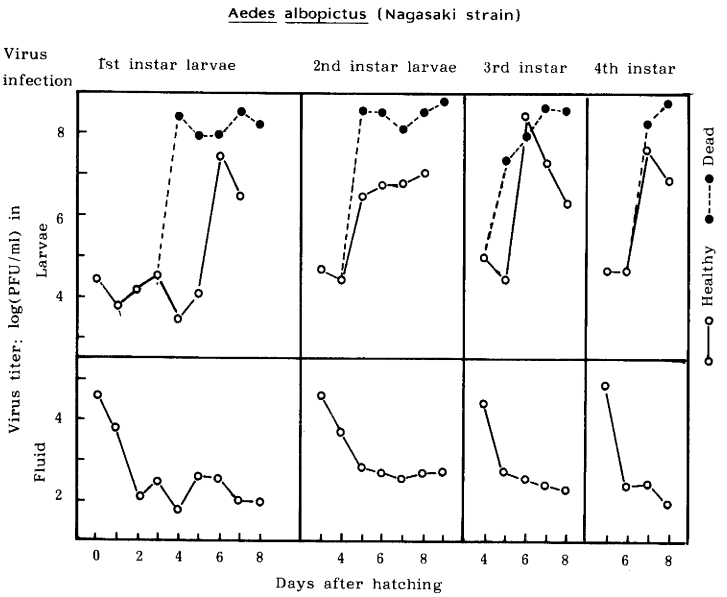

Fig. 2. Growth of Yokoshoji virus in infected *Ae. albopictus*

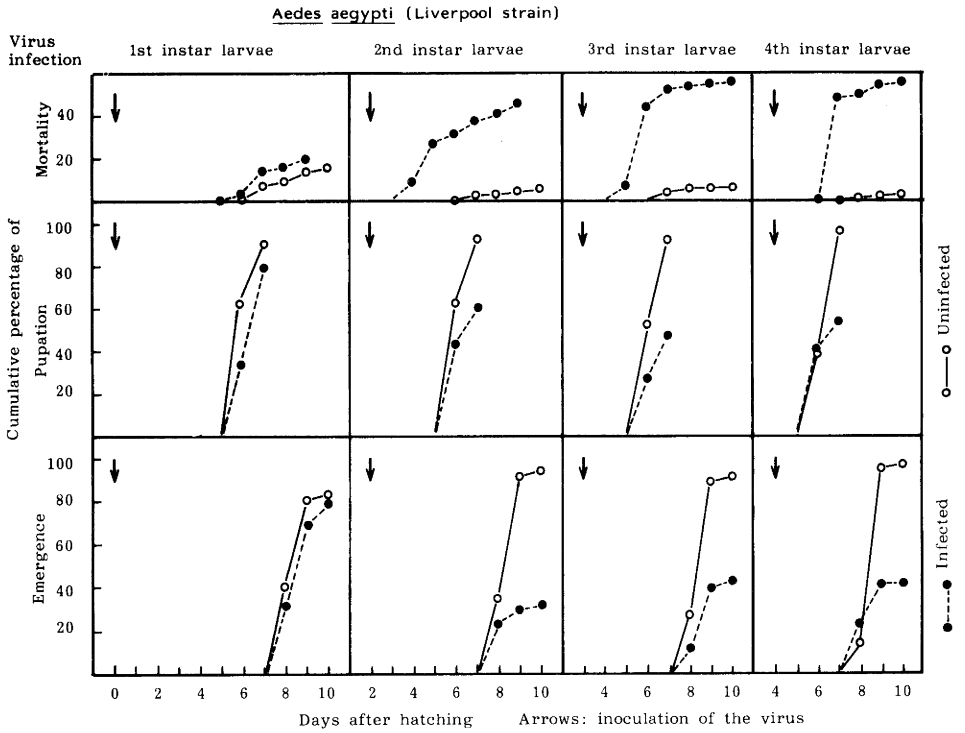

Fig. 3. Effect of Yokoshoji virus infection on *Ae. aegypti*.

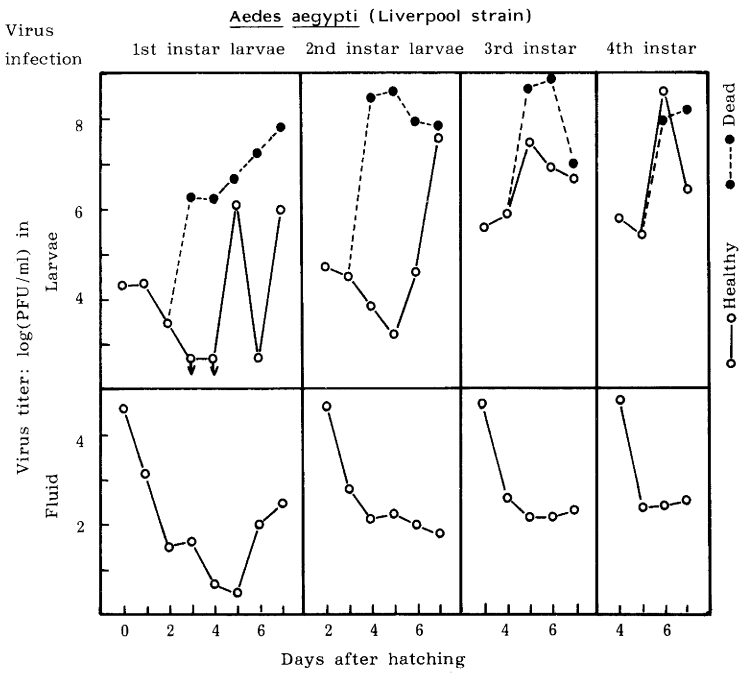

Fig. 4. Growth of Yokoshoji virus in infected *Ae. aegypti*.

INTRODUCTION

Impact of various vector-borne diseases on medical or public health aspects in most of the tropical countries around the world has been well documented (Newson, 1976; Peters and Gilles, 1981). As the most direct way to control such vector-borne diseases, vector control with chemical insecticides has extensively been practised. However, the measure was encountered by the problems of (1) emergence of the resistance to the insecticides among vectors and (2) environmental pollution with chemically stable insecticides (Burges, 1981). As an alternative, biological control on vectors has recently been re-evaluated with high priority, for example using natural enemies, insect pathogens, or bacterial toxins. In contrast to other insect pathogens, use of insect viruses as biological control agents were rather limited except baculoviruses for the forest pest control (Summers *et al.*, 1975).

In this report we describe the infection of an insect virus of mosquitoes with tentative name of Yokoshoji virus to several laboratory colonized mosquito larvae in order to see its effect on the survival, pupation and emergence of mosquitoes as well as the virus growth in the infected mosquitoes. Limited scales of experiment were also performed under simulated field conditions. Preliminary tests on the safety to use the virus as biological control agent were also examined, such as animal experiment and antibody survey.

MATERIALS AND METHODS

Virus: The origin of the virus, tentatively named as Yokoshoji virus, its infectivity titration on *Aedes albopictus* clone C6/36 cells (Igarashi, 1978), some of its basic characteristics as well as the method of cell culture have been described in the accompanying paper (Igarashi *et al.*, 1986). The virus was grown to high titer (2×10^8 PFU/ml) in C6/36 cells to make large stocks as infected culture fluid and stored at −70℃ until used in the experiments.

Laboratory colonized mosquitoes: Following species and strains of mosquitoes have been maintained through generations in the Department of Medical Zoology, Nagasaki University School of Medicine and were used in this study. *Ae. albopictus*, Nagasaki strain; *Ae. aegypti*, Liverpool strain; *Ae. aegypti*, Bangkok strain; *Culex tritaeniorhynchus*, Amami strain; *Cx. pipiens molestus*, Yanagawa strain, and *Anopheles sinensis*, Nagasaki strain.

Virus infection to mosquito larvae: After hatching from eggs, larvae were reared in enamel pans (26×20 cm) and fed daily with 1:1 mixture of Brewer's yeast and powdered mouse pellet. Approximately 300 larvae were transferred to a glass beaker and 5 ml of stock virus was added to final volume of 25 ml. Control larvae were similarly treated with uninfected C6/36 cell culture fluid instead of infective virus. The larvae were allowed to swim in the virus suspension for 2 hours at 28℃, followed by dilution with water and transfer to the enamel pans with 1 cm depth of water. Rearing was continued as described above at 28℃.

Sampling and data recording: Following specimens and data were taken every day

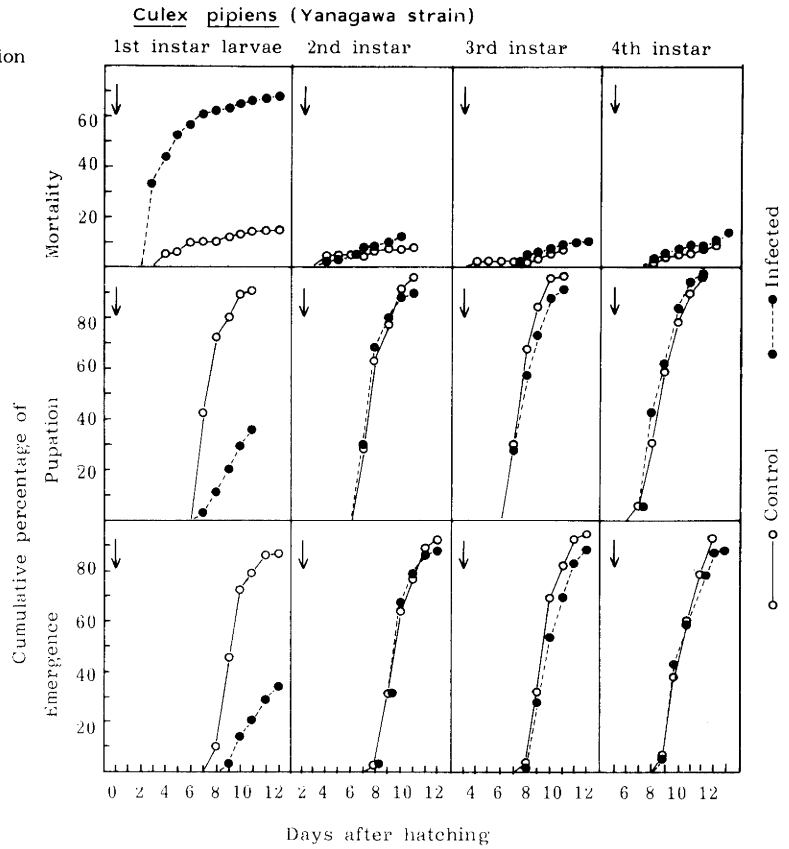

Fig. 7. Effect of Yokoshoji virus infection on *Cx. p. molestus*,

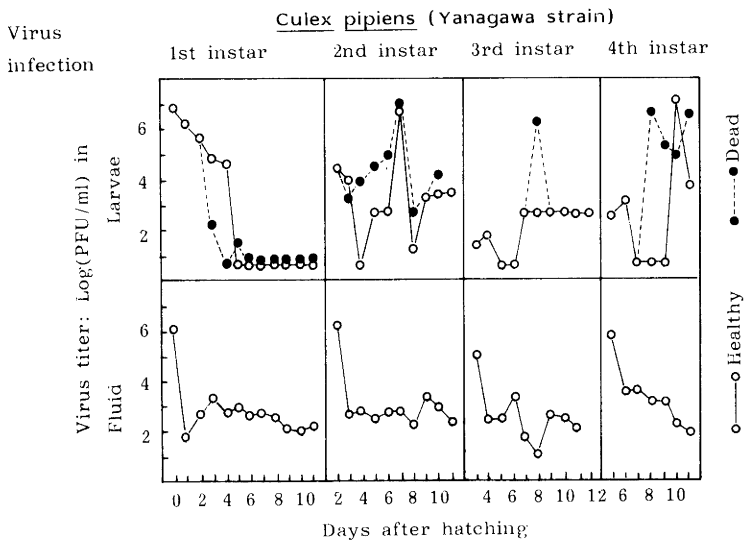

Fig. 8. Growth of Yokoshoji virus in infected *Cx. p. molesuts*,

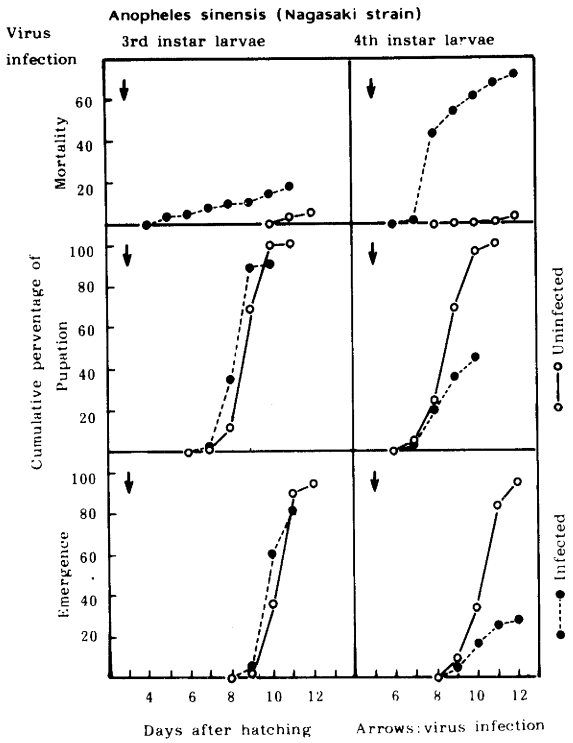

Fig. 9. Effect of Yokoshoji virus infection on *An. sinensis*.

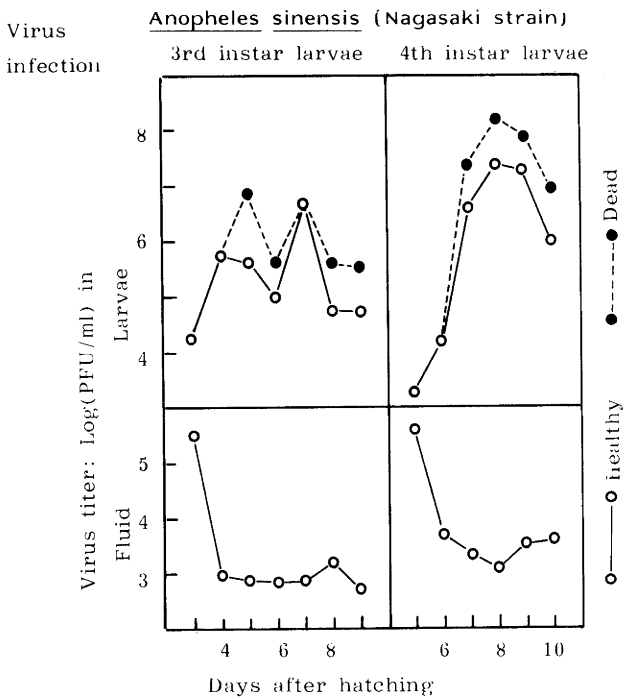

Fig. 10. Growth of Yokoshoji virus in infected *An. sinensis*.

Since all the species of mosquitoes were tested for the virus infection at the 3rd and 4th instar larval stages, the effect of virus infection to the 4th instar larvae were compared among different virus species. As shown in Table 2, the highest mortality rate of 72% was observed for *An. sinensis* while the lowest value of 13.4% for *Cx. pipiens*.

Effect of virus dose on the mortality, pupation, and emergence rates as well as the transovarial transmission

Using the 4th instar larvae of *Ae. albopictus*, the effect of infecting virus concentration on the mortality, pupation and emergence was investigated, and the results were summarized in Fig. 11 and Table 3. The higher the infecting virus titer, the higher mortality was recorded as expected resulting in the lower pupation and emergence. In this experiment, possibility of transovarial transmission of the virus from adult female emerged from infected larvae were examined by assaying virus titer in the next generation larvae and adults. The results were completely negative indicating that Yokoshoji

Table 1. Effect of virus infection on different larval stages of mosquito species

Mosquito species	Experimental series in which								
	Highest mortality (%) was observed					Least mortality (%) was observed			
	Larval atage (Instar)	Mortality		Virus titer		Larval stage (Instar)	Mortality		Virus titer	
		Infected	(Control)	Healthy	Dead		Infected	(Control)	Healthy	Dead
Ae. albopictus	1st	69.4	(0.5)	6.5	8.5	4th	38.1	(0)	7.8	8.8
Ae. aegypti	4th	56.7	(3.4)	8.6	8.2	1st	20.6	(16.2)	6.1	7.8
*Cx. tritaeniorhynchus**	4th	59.0	(3.0)	7.4	8.0	3rd	19.5	(4.1)	7.6	8.1
Cx.p. molestus	1st	67.5	(14.1)	6.9	2.3	3rd	10.4	(6.5)	1.9	6.3
*An. sinensis***	4th	72.2	(4.9)	7.4	8.2	3rd	18.3	(6.0)	5.8	6.9

* 1st instar larvae were not tested
** 1st and 2nd instar larvae were not tested
Virus titer was shown in log (PFU/ml)

Table 2. Effect of virus infection on the 4th instar larvae of several mosquito species

Mosquito species	Cumulative %(control)			Maximum virus titer in larvae	
	Mortality	Pupation	Emergence	Healthy	Dead
Ae. albopictus	38.1(0)	72.3(100)	61.9(100)	7.8	8.8
Ae. aegypti	56.7(3.4)	56.6(99.2)	43.3(96.6)	8.0	8.2
Cx. tritaeniorhynchus	59.0(3.0)	49.1(99.6)	41.0(97.0)	7.4	8.0
Cx. p. molestus	13.4(7.9)	95.4(97.0)	86.6(92.1)	7.0	6.7
An. sinensis	72.2(4.9)	44.8(100)	27.9(95.1)	7.4	8.2

Virus titer was shown in log (PFU/ml)

virus was not easily transmitted by transovarial route. The virus titer, both in dead and healthy, tended to be lower in pupae compared with the larvae, and further lower in adult compared with pupae. The data, together with the negative transovarial transmission data, appear to indicate that Yokoshoji virus infection in mosquitoes is more likely self−cured, or the virus infection may be limited to certain parts of the body which may be eliminated through metamorphosis.

Effect of virus infection on Ae. albopictus larvae kept under simulated field conditions

The data mentioned in the previous section were obtained purely under laboratory condition. In order to test the possibility to use Yokoshoji virus as a biological control agent, the effect of virus infection on mosquito larvae were examined under simulated

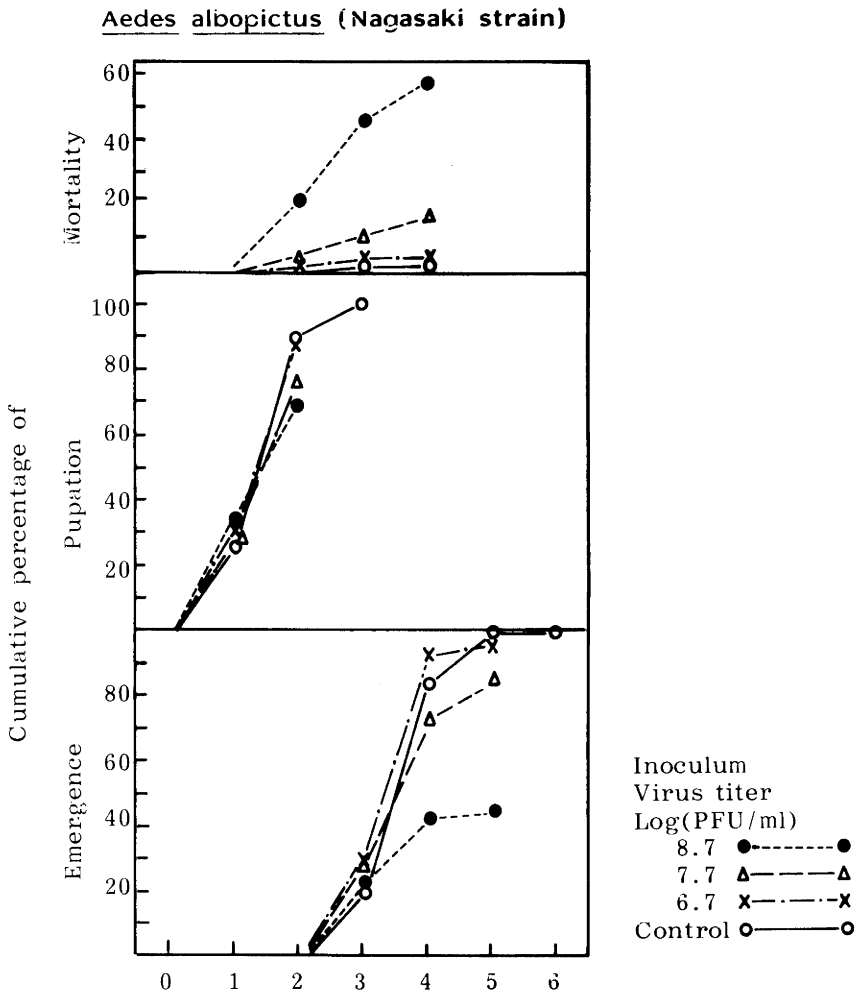

Fig. 11. Effect of varying concentration of Yokoshoji virus on *Ae. albopictus*.

Table 3. Effect of infecting virus concentration on *Aedes albopictus*

Experimental seris	Virus titer	Cumulative %			Infection rate in pools of 5 larvae
		Mortality	Pupation	Emergence	
A	8.7	56.0	68.1	44.0	11/11
B	7.7	15.6	75.8	84.4	5/8
C	6.7	3.5	84.7	96.5	1/5
D	0	0.6	100	99.4	0

Experimetal series	Virus titer in log (PFU/ml)						Presence of the virus in the next generation	
	Larvae		Pupae		Adults			
	Healthy	Dead	Healthy	Dead	Healthy	Dead	Larvae	Adult
A	5.8−8.8	6.4−8.0	3.5−7.8	3.2−8.1	−	3.0	0/1000	0/398
B	6.4−7.7	7.0	5.0	4.3	−	−	0/1000	0/666
C	3.7	4.5	−	−	−	−	0/1000	0/391
D	−	−	−	−	−	−	0/1000	0/567

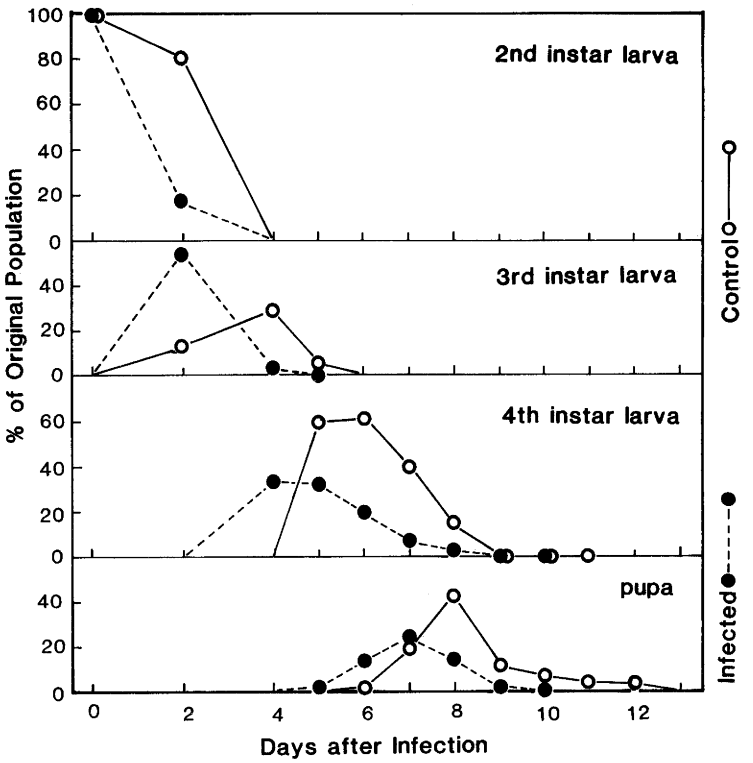

Fig. 12. Effect of Yokoshoji virus on the molting of *Ae. albopictus* under simulated field condition.

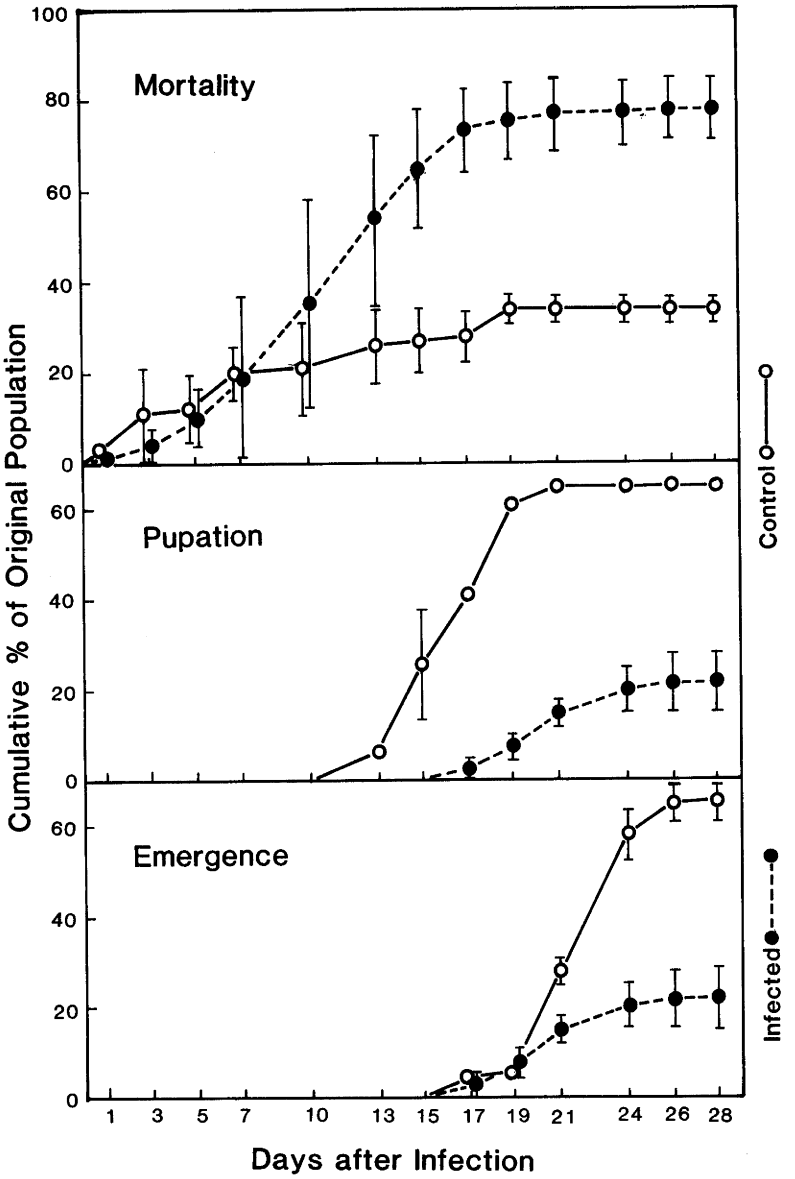

Fig. 13. Effect of Yokoshoji virus on *Ae. albopictus* under simulated field condition. Each point shows average of 10 containers with standard deviation shown by vertical bars.

field condition. Twenty replicate containers with water and rice glass stalks were prepared under an open−air roof in the campus of Nagasaki University Medical School. Each container was supplied by 20 individuals of 2nd instar *Ae. albopictus* larvae, and 10 containers were supplied with concentrated virus solution directly added to the water in the container so that the initial virus titer becomes around 10^6 PFU/ml, while remaining 10 containers were supplied with control cell culture fluid. Every day after the virus infection, each container was examined for the number of different stages of larvae and pupae in order to record molting and pupation rate as well as to record mortality and pupation. After pupation was noticed, containers were covered by fine net so that newly emerging adult can also be counted. The experiment was repeated twice and Figs. 12 and 13 show results of one of the experiments. As shown in Fig. 12, virus infection

Table 4. Cumulative rates of mortality, pupation, and emergence of *Ae. albopictus* (Okinawa) infected or uninfected, with Yokoshoji virus

	Number of specimens	Cumulative % of original population*		
		Mortality	Pupation	Emergence
Infected	10	83.4±6.1	23.3±11.4	17.9±6.8
Uninfected	2	21.0±9.9	57.0±11.3	57.5±5.3

* Average with standard deviation.
The test was performed in Ryukyu University, Department of Medical Zoology, in Okinawa, from October 4 to 14, 1982.

Table 5. Antibody response in mice intracerebrally inoculated with Yokoshoji virus, as determined by ELISA

Serum specimens tested	Ratio of ELISA-OD in virus antigen to control*
Inoculated	1.775 ± 0.156 (n=30)
Control	0.975 ± 0.067 (n=30)

* Average of 30 individual mice with standard deviation.
Serum dilution at 1 : 100.

Table 6. Antibody surveillance of Yokoshoji virus antibedies among swine and bovine populations in Nagasaki Prefecture

Animal species	Number of specimens	Ratio of ELISA-OD (virus antigen to control) Average with standard deviation (range)
Swine	330	1.151 ± 0.183 (0.583 − 1.575)
Bovine	319	1.074 ± 0.132 (0.810 − 1.450)

resulted in delayed molting of the larvae, increased larval mortality, reduced pupataion and emergence rate compared with the controls.

Similar experiment was conducted in Okinawa in collaboration with Ryukyu University School of Hygiene, and the result was summarized in Table 4. Although the number of control containers was only 2 compared with 10 for the virus−infected, the difference between the infected and control was significant in morality, pupation, and emergence.

Effect of Yokoshoji virus infection to mice

A group of 30 weaning mice was intracerebrally inoculated with Yokoshoji virus stock using 0.02 ml/brain. No pathological findings were observed throughout 21 days of observation period. Mice were bled and their sera were individually examined for anti −Yokoshoji ELISA titers along with 30 individual sera obtained from control uninfected mice, and the results were shown in Table 5. There was slight but definite antibody formation in the infected series compared with the control, however, this antibody response could be explained by the direct effect of inoculated virus as the antigenic stimulus.

Antibody surveillance among swine and bovine population

Arboviruses are known to be maintained in nature through alternative growth in susceptible vertebrates and vector arthropods in nature (World Health Organization, 1967). Antibody surveillance among swine population has routinely been used to monitor the spread of Japanese encephalitis virus, typical arbovirus in Japan. Suppose Yokoshoji virus shares similar characteristics as arbovirus, it should have left some trace among popular vertebrates as detectable antibodies. More than 300 each of swine and bovine sera collected in Nagasaki Prefecture in 1981 were examined for the possible presence of detectable anti−Yokoshoji virus antibodies, however, the results were almost negative as shown in Table 6.

DISCUSSION

Several insect viruses have been described for mosquitoes, such as nuclear polyhedrosis virus for *Cx. tarsalis*, cytoplasmic polyhedrosis virus for *Ae. taeniorhynchus* and *An. quadrimaculatus*, and iridescent virus for *Ae. taeniorhynchus*, *Ae. cantans*, *An. annulipes*, *An. fulvus pallens*, *Ae. vexans*, *Ae. detritus*, *Ae. stimulans* and *Psorophora ferox* (Smith, 1976). The genome of these viruses are all DNA, in contrast to Yokoshoji virus, which apparently possesses RNA as its genetic material (Igarashi *et al.*, 1986). Nobody has extensively tried to use possible insect virus of mosquitoes as biological control agent on mosquitoes. The experimental results shown in this report indicated some potentiality of Yokoshoji virus for such purpose, however, its infection did not result in high mortality of mosquito larvae. One of the drawback of this virus may be its relative lability in the water so that long−term effect on larvae was not expected.

Regarding the safety of Yokoshoji virus on vertebrates and humans, we did not find any adverse effects as far as tested. Oral administration of concentrated virus to several volunteers did not result in any adverse reactions.

Although the virus was first isolated from field-caught mosquitoes in Osaka Prefecture, antibody surveillance among swine and bovine in Nagasaki Prefecture did not show any positive indication of natural infection with this virus. Since the field of virus isolation is different from those of antibody survey, the negative result is difficlt to interpret. However, basic characteristics of Yokoshoji virus indicating it as possible insect virus of mosquitoes shown in the accompanying paper (Igarashi *et al.*, 1986) will more likely give negative serology among vertebrates. Laboratory experiments shown in this paper did not support transovarial transmission of this virus among mosquitoes, leaving natural cycle of this virus still misterious.

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昆虫ウイルスの蚊幼虫に対する殺虫効果：蚊の生物学的防除としての可能性について

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蚊の昆虫ウイルスの一つを数種類の実験室内累代継代蚊の幼虫に感染させた時の殺虫効果を調べた．殺虫効果はウイルスの濃度，蚊の種類，及び幼虫の成熟度によって異なった．四令幼虫にウイルスを感染させた場合，累積死亡率はチカイエカの13%からシマハマダラカの72%であったが対照群の死亡率は0－8%であった．ネッタイシマカの場合，四令幼虫にウイルスを感染させた時最も高い死亡率を示し若令幼虫に感染された時の死亡率は低かった．この傾向はコガタアカイエカ及びシナハマダラカでも認められたが，ヒトスジシマカとチカイエカの場合には逆の傾向が見られ，第一令幼虫に感染された方が三又は四令幼虫に感染させた時よりも高い死亡率を示した．死亡した幼虫は脱水し縮小した外観を呈したが，ウイルス増殖は死亡した幼虫だけでなく一見健康に見える幼虫にも認められた．ウイルス感染幼虫から羽化した雌成虫から卵を介して次代の蚊にウイルスが伝達される経卵感染は証明されなかった．この昆虫ウイルス（ヨコショウジウイルス）をマウス脳内に接種しても病的変化は認められなかった．長崎県下のブタとウシの抗体調査ではヨコショウジウイルスの自然感染を示す結果は得られなかった．

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