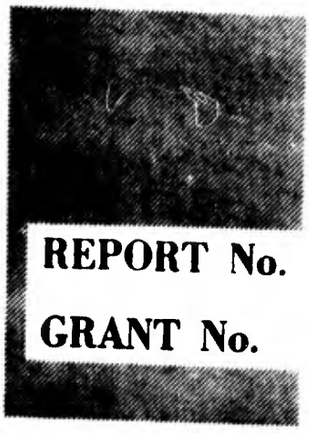

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REPORT No. FE-343-5 & FE-343-6 (Annual Report)

GRANT No. DA-CRD-AFE-S92-544-67-G78

# MULTIPLICATION AND ANTIBODY FORMATION OF JAPANESE ENCEPHALITIS VIRUS IN SNAKES

by

Ho Wang Lee, M.D., Ph.D.

Associate Professor
College of Medicine
Seoul National University
Seoul, Korea

December 1970


U. S. ARMY RESEARCH AND DEVELOPMENT GROUP
FAR EAST
APO San Francisco 96343

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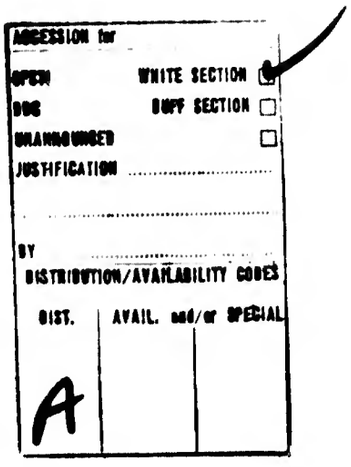


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REPORT No. FE-343-5 & FE-343-6 (Annual Report)

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DA Project/Task Area/Work Unit No. 3A061102B71Q 00 097FE

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## ABSTRACT

In studies designed to investigate the role of snakes in the ecology of Japanese encephalitis (JE) virus in Korea, 747 snakes representing 4 different species were collected during 1966 and 1967. Two strains of JE virus were isolated from the snakes, **E.** rufodorsata CANTOR caught in the rural area near Seoul. Plasma samples from 1,775 snakes collected in Korea during 1965 and 1969 were assayed for serologic evidence of infection with JE virus by hemagglutination inhibition test. Hemagglutination inhibiting antibodies were demonstrated in 54% of the snakes. Plasma samples from 683 snakes collected during 1968 and 1969 were assayed for serologic evidence of JE infection by an in vitro plaqueneutralization method. Neutralizing antibodies were demonstrated in 2.8% of total snakes. That snakes are effective as reservoir hosts for JE virus is discussed.

## INTRODUCTION

The ecology of Japanese encephalitis virus (JEV) is not completely clear yet, especially about overwintering mechanisms of the virus in the temperate zone like Korea. One of the hypotheses is that the virus overwinters in the hibernating cold blooded animals.

Recently, the author has reported that JE virus can artificially overwinter in the snakes (1) and that injection of the virus induces antibody formation and proliferation of the virus (2,3).

The present report concerns the isolation of JE virus from naturally infected snakes collected in Korea during 1966 and 1967 and the incidence of neutralizing and hemagglutination inhibition (HI) antibodies to JE virus in 1,775 snakes collected from 1965 to 1969.

## MATERIALS AND METHODS

### Snakes

The snakes employed are following 4 species of non-poisonous common snakes in Korea. Their weight ranged between 30 and 60 g, and the length from 50 to 70 cm. Total number of snakes used is 1,775.

1) Elaphe rufodorsata CANTOR
2) Natrix tigrina lateralis BERTHOLD
3) Elaphe schrenckii STRAUCH
4) Dinodon rufozonatum rufozonatum CANTOR

Snakes were collected throughout a year, but were not able to capture from December to February and the main collecting sites were near the rice paddies of suburban areas of Seoul.

### Viruses

The following 3 viruses were used for preparation of virus hemagglutinins and as controls for identification of the isolated viruses.

1) JEV M5/596 (4). 17th suckling mouse passage.
2) West Nile (WN) virus, 12th mouse passage.
3) Western equine encephalitis (WEE) virus, 2nd mouse passage in our laboratory.

West Nile and Western equine encephalitis viruses were kindly supplied from Dr. K. Y. Kim, N.I.H. Seoul, Korea which he obtained from Dr. Smadel in 1954.

### Isolation of Virus

Plasma of snake was obtained by cardiac puncture with 1 ml syringe and 26 gaze needle wetted with 100 units of heparin. The 0.2 ml of 1:10 dilution of plasma was adsorbed on primary chick embryo cells cultured in 2 oz prescription bottle for plaque formation (5).

## Plaque neutralization and inhibition test

Plaque neutralizing and inhibiting antibodies to JE virus were measured in primary chick embryo cells (5) and analysis of the results are based on the porterfield's methods (6,5). Control antiserums to JE, WN and WEE viruses were groups of 5 rabbits immunized by intracutaneous inoculation of suckling mouse brain suspension of the above described viruses.

## Hemagglutination inhibition test

Modified Buescher et al's method (7) was employed except that virus strain M5/596 was used to make hemagglutination antigen. In the test performed in 1967, acetone extraction of plasma was done twice and since 1968 acetone extraction was done three or four times.

# RESULTS

## Isolation of Japanese encephalitis virus

In 1966 and 1967, 204 and 543 snake plasmas were tested for the isolation of virus in primary chick embryo cells with agar overlay.

There were four different species in the collected snakes and among them, there were 184 and 510 Elaphe rufodorsata CANTOR. Two strains of JE virus were isolated from the snake S-6-182 collected at pupyung, 20 miles away from Seoul on October 19, 1966 and the snake S-7-283, caught at Jangwidong near Seoul on July 16, 1967 (Table 1). And 0.2 ml of 1:10 diluted plasmas formed 4 and 5 plaques respectively.

Identification of the virus was done by plaque neutralization and inhibition test in primary chick embryo cells (5,6) and as controls, JE, WN and WEE viruses were employed.

## Frequency of HI and neutralizing antibodies to JE virus

In 1965, 210 snake plasmas were tested for HI antibody to JE virus. As in table 2, 56% of the E. rufodorsata CANTOR contained HI antibody to JE virus. This is the first demonstration of antibody to JE virus in snakes collected in nature. The highest titer was 1:320 and most of them belonged between 1:10 to 1:40. In 1966, 204 snakes were caught and about 60% of E. rufodorsata CANTOR had HI antibody to JE virus.

There were four different species in the snakes, but most of them were E. rufodorsata CANTOR since this species inhabit mainly near the rice paddies. In 1967, 510 F. rufodorsata CANTOR were tested and found that 42% of them contained HI antibody to JE virus.

As the table shows 540 snakes were caught in 1968, and 70% of them had HI antibody to JE virus. This higher proportion compared with previous years is a result of three times performance of acetone extraction, because we found that the proportion becomes higher when the acetone extraction was done three or four times than twice and we did so since

1968. 278 snakes were caught in 1969 and about 54% of them possessed HI antibody to JEV (Table 2).

In 1968 and 1969, neutralizing antibody test to JE virus with snake plasma was done. As shown in table 3, antibody was demonstrated from 19 out of 683 snakes.

## DISCUSSION

The author has isolated one strain of JE virus from the snake collected in Korea on October 19, 1966 for the first time. At that time HI antibody to the virus was proved to be in the plasma of the snake and the virus was isolated. So it became more interesting to study the relationships between JE virus and snakes in the nature.

Artificial injection of JE virus into the snake caught in Korea induces antibody production and proliferation of the virus was proved by viremia, but the results were not so regular as in warm blooded animals (2,3). And so, various factors affecting JE virus multiplication and antibody formation in the snake are still being studied.

In 1967, plasmas were obtained from 543 snakes collected in the field by cardiac-puncture. And one of the snake plasmas diluted 1:10 produced 5 plaques in primary chick embryo cells and subsequently they were identified as JE virus by plaque neutralization test.

Because of the snake plasma is very toxic to the cells (8), it can not be used to the cells and mice without dilution. So it must be, at least, diluted 1:10 to produce plaque formation in primary chick embryo cells. It can be speculated that if undiluted plasma is used, larger number of the virus plaque is expected to be produced. If another system of cells, which has strong resistance to toxicity of snake plasma and has better capability of producing plaque formation, or such a kind of method is discovered, larger number of the virus can be isolated. Virus isolation from plasmas of the snakes caught in 1968 and 1969 is in progress now.

As the results indicate in Table 2, about 50% of the total snakes collected in the field in 1965 and 1966 had possessed HI antibody to JE virus.

In 1967, we compared the proportion of HI antibody to the virus with snakes caught by months and found that antibody containing proportion in the plasma is relatively low before July, pre-epidemic season, about 60-70% during July, August, September, October, epidemic season, in November the proportion fell to 28%.

This phenomenon seems to have some relations with the fact that main vector mosquitoe of JE in Korea, Culex tritaeniorhynchus (9), appears after June, reaches to the peak in August and September, and disappears after November and that the main breeding site of the mosquitoes is rice-paddies, the same place where E. rufodorsata CANTOR breeds, and consequently snakes have many chances to be involved with the vector mosquitoes.

During the last two years, we tried to collect snakes during winter season but in vain.

After detecting the HI antibody to the virus in the snake plasma, to solve the question whether this antibody is non-specific material or not, various methods were employed to remove non-specific inhibitors to JE virus hemagglutinin, and removal of acetone soluble non-specific lipids by repeated acetone extraction increased HI antibody titer to JE virus. So, since 1968, we performed three or four times acetone extraction of plasma before the test. As the result of this repeated acetone extraction, 72% and 60% of E. rufodorsata CANTOR appeared to be positive in 1968 and 1969 respectively.

However, these figures did not correlate with the incidental frequency of encephalitis cases in human because in 1967, 810 among 2,691 patients died and in 1968, 396 among 1,226 patients died in Korea.

In fact, the relationship between antibody containing proportion in the snakes and the epidemic in human is yet a problem to be studied from now on. And as we see in table 2, one third of HI positive snake plasmas showed titer 10.

Because HI antibody to JE virus was considered non-specific on account of cross-reaction in the group however, JE virus is the only arbovirus that exists in Korea so far, snake plasmas collected in 1968 and 196 were tested for neutralizing antibodies to JE virus. As the results indicate in Table 3, only 19 snake plasmas out of 683 snakes diluted 1:10 was proved to be positive, and this corresponds only to 2.8%.

The results of the experiments on the proliferation of JE virus and antibody production (2,3) have clarified that HI and neutralizing antibodies do not exist at the same time in the snake as do exist in the warm blooded animals usually, and their existing time is irregular and the number of snakes that produce antibodies are very few after aritficial inoculation of the virus at 21° C and 4° C.

As a matter of fact, present data were obtained by the processes used in antibody test of warm blooded animals. So if we employ new sensitive methods which can prove antibodies of cold blooded animals, better results are expected to be obtained.

According to the results so far obtained in the survey, snakes may play as a reservoir of JE virus in the endemic area.

## SUMMARY


1. Two strains of JE virus were isolated from the snakes, E. rufodorsata CANTOR, caught in the rural area near Seoul in 1966 and 1967.

2. As a results of HI antibody test to JE virus with 1,868 snake plasmas from 1965 to 1969, about 51% of the snakes collected in nature was positive.

3. The result of neutralizing antibody test to JE virus with the plasmas of 683 snakes collected in 1968 and 1969 appeared to be positive in 19 snakes, 2.8% of total snakes.

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Table 1

Isolation of JE virus from non-poisonous snakes in Korea collected during 1966 and 1967

| Species of snake | No. of JE virus isolation / Total No. tested | |
|---|---|---|
| | 1966 | 1967 |
| Elaphe rufodorsata CANTOR | 1*/184 | 1**/510 |
| Natrix tigrina lateralis BERTHOLD | 0/11 | 0/22 |
| Elaphe schrenckii STRAUCH | 0/6 | 0/10 |
| Dinodon rufozonatum rufozonatum CANTOR | 0/3 | 0/1 |

*: JEV S-6-182 (10/19/66 Pupyung, Kyungido)
**: JEV S-7-283 (7/16/67 Changwidong, Seoul)

Table 2

Occurrence of HI antibodies to JE virus in snakes collected in Korea from 1965 to 1969

| Species of snake | HI titer | No. of snake | | | | | No. of HI positive / No. of tested | | | | | % of HI positive to JEV | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1965 | 1966 | 1967 | 1968 | 1969 | 1965 | 1966 | 1967 | 1968 | 1969 | 1965 | 1966 | 1967 | 1968 | 1969 |
| E. rufodorsata CANTOR | < 10 | 68 | 56 | 297 | 146 | 100 | | | | | | | | | | |
| | 10 | 14 | 44 | 68 | 111 | 52 | | | | | | | | | | |
| | 20 | 32 | 66 | 103 | 142 | 74 | | | | | | | | | | |
| | 40 | 27 | 17 | 39 | 87 | 23 | | | | | | | | | | |
| | 80 | 9 | 1 | 3 | 39 | 2 | | | | | | | | | | |
| | 160 | 4 | | | | | | | | | | | | | | |
| | 320 | 1 | | | | | | | | | | | | | | |
| | | | | | | | 87/155 | 128/184 | 213/510 | 379/525 | 151/251 | 56 | 60 | 42 | 72 | 60 |
| N. tigrina lateralis BERTHOLD | < 10 | 43 | 10 | 22 | 8 | 21 | | | | | | | | | | |
| | 10 | 6 | 1 | | | | | | | | | | | | | |
| | 20 | 1 | | | | | | | | | | | | | | |
| | | | | | | | 7/50 | 1/11 | 0/22 | 0/8 | 0/21 | 14 | 9 | 0 | 0 | 0 |
| E. schrenckii STRAUCH | < 10 | 3 | 5 | 10 | 6 | 6 | | | | | | | | | | |
| | 10 | 1 | 1 | | | | | | | | | | | | | |
| | | | | | | | 1/4 | 1/6 | 0/10 | 1/7 | 0/6 | 25 | 17 | 0 | 14 | 0 |
| D. R. rufozonatum CANTOR | < 10 | 1 | 1 | 1 | | | | | | | | | | | | |
| | 10 | | 1 | | | | | | | | | | | | | |
| | 20 | | 1 | | | | | | | | | | | | | |
| | | | | | | | 0/1 | 2/3 | 0/1 | | | 0 | 67 | 0 | | |
| Total | | 210 | 204 | 543 | 540 | 278 | 95/210 | 132/204 | 213/543 | 380/540 | 151/278 | 45 | 65 | 39 | 70 | 54 |

Table 3

Occurrence of plaque neutralizing antibodies to JE virus from snakes in Korea collected during 1968 and 1969

$$\frac{\text{No. of positive}}{\text{Total No. tested}} = \frac{19}{683} = 2.8\%$$

| Species of snake | No. of neutralizing antibody positive / No. of tested | |
|---|---|---|
| | 1968 | 1969 |
| Elaphe rufodorsata CANTOR | $\frac{9}{399}$ | $\frac{10}{244}$ |
| Natrix tigrina lateralis BERTHOLD | $\frac{0}{7}$ | $\frac{0}{21}$ |
| Elaphe schrenckii STRAUCH | $\frac{0}{6}$ | $\frac{0}{6}$ |

## LIST OF PUBLICATIONS


1. Lee, H. W. and Kee, R. S., 1968. Multiplication and antibody formation of Japanese encephalitis virus in snakes. I. Antibody responses to the virus and serum. J. Kor. Soc. Microbiol., 3: 43-49.

2. Lee, H. W., 1968. Multiplication and antibody formation of Japanese encephalitis virus in snakes. II. Proliferation of the virus. The Seoul J. Med., 9: 154-161.

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Seoul National University
Seoul, Korea

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MULTIPLICATION AND ANTIBODY FORMATION OF JAPANESE ENCEPHALITIS VIRUS IN SNAKES (U)

Annual Report No. 5 and 6, May 1969 - May 1970

Ho Wang Lee

10 February 1971

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FE-343-5 and FE-343-6

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DD FORM 1473

Unclassified

| 14. KEY WORDS | LINK A | | LINK B | | LINK C | |
|---|---|---|---|---|---|---|
| | ROLE | WT | ROLE | WT | ROLE | WT |
| Snakes | | | | | | |
| Viruses | | | | | | |
| Isolation | | | | | | |
| Japanese B encephalitis | | | | | | |
| Antibody | | | | | | |
| Viremia | | | | | | |
| Overwintering | | | | | | |
| Immunology | | | | | | |
| Neutralization | | | | | | |
| Hemagglutination-inhibition test | | | | | | |
| Korea | | | | | | |