Low Susceptibility of Common Snakes in Japan to Japanese Encephalitis Virus[1]

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The reservoir(s) of Japanese encephalitis (JE) virus in the interepidemic season has not been clarified yet. In recent years, however, the role of garter snakes as the reservior of Western equine encephalomyelitis (WEE) virus has been discussed in the United States. Thomas et al. (1,2) reported that garter snakes were susceptible to WEE virus and experimentally infected snakes could circulate the virus in their blood for long periods including winter months. Gebhardt et al. (3, 4, 5) demonstrated that WEE virus could be isolated frequently from the blood of wild garter snakes caught in nature in early spring and transmitted to snakes by the bite of only one mosquito of *Culex tarsalis* infected.

In the present paper, the possible role of common native snake of Japan as a natural host of JE virus has been studied and discussed by examination on virus isolation from sera of wild snakes and hemagglutination inhibition (HI) test using sera of them, and making an attempt to establish experimental infection of common snakes with JE virus.

Materials and Methods

Snakes: Wild snakes were captured in the suburbs of Nagasaki city during a period from April 14 to September 28, 1967, corresponding to the epidemic season of Japanese enceph-

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alitis extending to the beginning and the stadium of it in Japan. The captured snakes were transferred to the laboratory and bled by snipping or cardiac punctures within 4 days. The sera were separated by centrifugation at 3,000 r. p. m. for 10 minutes at 4°C and stored at −75°C until use for virus isolation and HI test.

Virus isolation : For the virus isolation from wild snakes or virus recovery from experimentally infected snakes, the sera were diluted with phosphate buffered solution containing 0.75 percent bovine serum albumin and antibiotics (pH7.4) at 1 : 2 in the case of ***Elaphe quadrivirgata***, 1 : 4 in the cases of ***Rhabdophis tigrinus tigrinus***, ***Elaphe climacophora*** and ***Natrix vibakari***, 1 : 8 in the case of ***Agkistrodon halys***, because undiluted sera of these snakes had resulted undesirable death of suckling mice by intracranial inoculation (9). One litter of suckling mice were inoculated intracranially and intraperitoneally with 0.02 ml each of diluted sera respectively. The recovered virus were identified to JE virus by HI and CF test after second mouse passage.

Infection of snakes : The strain used for this study was JaGAr 01 strain of JE virus originated from *Culex tritaeniorhynchus* and that after 11 or 12 suckling mice brain passages. Virus inoculum was prepared by suspending the infected suckling mice brain in 10 percent in a phophate buffered solution containing 0.75 percent bovine serum albumin and antibiotics (pH7.4).

Snakes were inoculated intraperitoneally with 0.2 ml of virus suspension of the titer of $10^{1.5}$ to $10^{6.5}$ suckling mice LD $_{50}$ per 0.02 ml. The snakes inoculated virus were then divided into 4 groups : the first group was kept throughout at room temperature (32.7°C − 17.1°C), the second at 25°C constantly, and the third and the fourth groups were kept at 15°C and 6°C for 14 days before being kept at room temperature. Virus recovery from blood of them was tried by snipping their tails at intervals of 1 to 7 days during the experimental periods of 27 to 40 days, except the fourth group bled on 15th, 16th, 17th, 21st and 28th day after virus inoculation.

Hemagglutination - inhibition (HI) and neutralization (NT) test : The procedures of HI test were based on the method described by Clarke and Casals using the 8 units of antigens prepared by the aceton-ether extraction method (6). Neutralizing antibodies were examined by the modified method of 80 percent plaque reduction test by using stable line of porcine kidney cell cultures reported by Kato and Inoue (7) and the antigens tested was suckling mouse brain suspension. The sera of wild or virus inoculated snakes were all diluted to 5 times with PBS for NT test.

Results and Discussion

JE virus isolation and HI test on the sera of wild snakes :

As shown in Table 1, no virus could be isolated from the sera of 305 snakes of 6 species captured in nature. However, the fact deserves some notice that bleeding from snakes was carried out just once for all during the examination and the sera of the snakes were diluted to a certain extent for avoiding the lethal effect on suckling mice. Gebhardt et al. (4) emphasized that the virus had appeared cyclically in the blood of garter snakes infected

with WEE virus. On refferring to this statement, it should be impossible to deny that JE virus may have been isolated from the blood of snakes if the bleeding was tried several times during the examination. The development of HI antibodies against JE virus was examined in 270 sera of 305 snakes. As seen in Table 1, the HI antibody at low titer was found in 6 out of 270 sera, or 2.2 percent irrespective of species of the snakes. NT test of these 6 HI positive sera could not carried out owing to the scarcity of sera. Lee (8) reported the high incidence (14 to 56 percent) of HI antibodies against JE virus in non-poisonous common snakes in Korea. There

Table 1. JE virus isolation and HI antibody detection from the sera of various snakes

Species	virus isolation		HI test against JE virus	
	No. of sera tested	No. of virus isolated	No. of sera tested	No. of sera positive
Rhabdophis tigrinus tigrinus	213	0	186	3(10, 10, 40)*
Elaphe climacophora	28	0	24	0
Elaphe quadrivirgata	49	0	47	2(10, 20)
Natrix vibakari	6	0	4	0
Elaphe conspicillata	4	0	4	0
Agkistrodon hayls	5	0	5	1(10)
Total	305	0	270	6

* The figures in parenthesis show each HI antibody titer expressed by the reciprocal of serum dilution.

Table 2. Experimental infection of snakes with JE virus

Species	No. of snakes JE virs inoculated	Temperature exposed	Inoculum dosis of virus	Observation periods (days)	No. of snakes JE virus recovered	No. of sera turned to HI antibody rising / No. of sera tested	No. of sera turned to NT antibody rising / No. of sera tested
R. tigrinus tigrinus	11	room temp.	$10^{2.5-7.5}$	29-36	2	0/11	0/1
	8	25°C	$10^{3.5-5.5}$	31	0	0/ 8	
	7	15°C	$10^{3.5-5.5}$	37	0	1/ 7	0/3
	4*	6°C	$10^{5.0}$	35	0	0/ 2	
E. quadrivirgata	5	room temp.	$10^{3.5-7.5}$	30-35	1	0/ 8	0/1
	8	25°C	$10^{3.5-6.5}$	31-34	0	0/ 5	0/5
	2	15°C	$10^{3.5}$	37	0	0/ 2	
	3**	6°C	$10^{5.0}$	32	0	0/ 2	
E. climacophora	4	room temp.	$10^{3.5-7.5}$	29-40	0	0/ 4	0/3
	5	25°C	$10^{3.0-6.5}$	31-34	0	0/ 5	0/2
	2***	6°C	$10^{5.0}$	32	0	0/ 1	
E. conspicillata	2	room temp.	$10^{5.5-7.5}$	30	1	0/ 2	
	1	25°C	$10^{4.5}$	34	0	0/ 1	
N. vibakari	2	room tump.	$10^{7.5}$	17	1	0/ 2	
Total	64				5	1/60	0/15

* Two of 4 died during the exposure at 6°C
** One of 3 died during the exposure at 6°C
*** One of 2 died during the exposure at 6°C

is no evidence at present, however, to show clearly whether HI antibodies of this low titer are really due to JE virus infection, and whether the difference in incidence of HI antibodies between common snakes in Japan and Korea is responsible for the species of snakes.

Experimental infection of snakes with JE virus : Sixty-four snakes which had been confirmed previously not to be NT antibody positive were inoculated intraperitoneally with 0.2 ml of virus suspension ranging from titers of $10^{1.5}$ to $10^{6.5}$ suckling mice LD_{50} per 0.02 ml.

As indicated in Table 2, JE virus could be recovered from only 5 of 64 inoculated snakes,

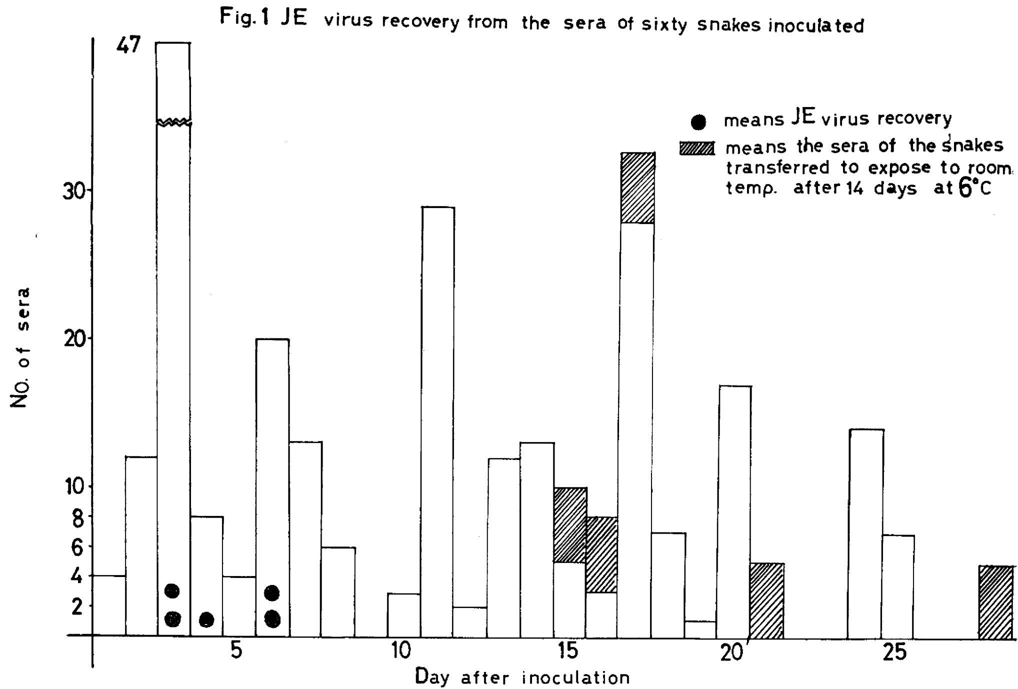

Fig. 1 JE virus recovery from the sera of sixty snakes inoculated

Table 3. Five cases of JE virus recovery

No.	Species	Inoculum dosis of virus and route		Temperature exposed	Day after inoculation JE virus recovered	Observation periods (days)	HI antibody titer of last bleeding serum	NT antibody titer of 1st bleeding serum
S 10	*N. vibakari*	$10^{7.5}$	ip.	room temp.	4	died 13th	10* >	5 >
S 40	*E. conspicillata*	$10^{7.5}$	ip.	〃	6	died 16th	N. T**	N. T
S 42	*R. tigrinus tigrinus*	$10^{7.5}$	ip.	〃	3	32	10 >	5 >
S118	*R. tigrinus tigrinus*	$10^{2.5}$	ip.	〃	6	30	10 >	5 >
S136	*E. quadrivirgata*	$10^{3.5}$	ip.	〃	3	30	10 >	5 >

* titer of serum of 11th day
** not tested

that is, two of *R. tigrinus tigrinus* and each one of *E. quadrivirgata*, *E. conspicillata* and *N. vibakari*, and no virus was obtainable from the fourth group kept at 6°C for 14 days before bleeding. Four of 64 inoculated snakes has died during the exposure at 4°C. Arranging the experiment of virus recovery from the sera of 60 snakes by the day after inoculation of virus, as illustrated in Fig. 1, it had been made successfully from two of 47 snakes on the third day, from one of 8 snakes on the fourth day, and from two of 20 snakes on the sixth day respectively. It is noteworthy that the virus isolation was possible in the range from the third to the sixth day of inoculation.

From Table 3 showing particulars of data concerning 5 virus-carrying snakes, no relations were seen among the time of virus recovery, the dose of the inoculated virus and the species of snakes.

Infectivity of the recovered virus had not been examined, however, it is considered to be very low by reference to the fact that only one or two mice died in each litter inoculated. But we can not conclude that JE virus did not propagate in snakes, because virus recovery from tissues of snakes were not attempted, and it is conceivable that a latent infection could have been initiated with subsequent viremia after 27-40 days after inoculation of virus.

The responses of HI antibodies and NT antibodies to virus inoculation have been presented in Table 2. Due to insufficient quantities of sera as a result of successive bleeding, only 15 sera was paired for NT antibody test. HI antibody developed at low titer in just one of 60 snakes and no response of NT antibody could be observed in 15 paired sera including the one of HI antibody response positive.

Summary

No virus could be isolated from sera of 305 wild snakes captured in nature, but hemagglutination-inhibition antibody was found in 6 of 270 snakes only at low titer.

Sixty-four snakes were inoculated with JE virus, at various temperatures and attempted for virus recovery from the blood of them at intervals of 1-7 days during the experimental periods of 27-40 days. In only five cases the virus was recovered once for all on the third day (two cases), on the fourth day (one case) and on the sixth day (two cases) after inoculation.

HI antibody developed in just one of 60 snakes at low titer, however, no response of NT antibody could be observed in 15 paired sera. These findings seem to imply that the common wild snakes in Japan are low susceptible to JE virus and they are in turn considered to play a minor role in epidemiology of Japanese encephalitis.

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日本脳炎ウイルスに対する日本産蛇の感受性の検討

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摘　　　要

1967年4月から10月にかけて採取されたヤマカガシ213匹，アオダイショウ28匹，シマヘビ49匹，ヒバカリ6匹，ジムグリ4匹，マムシ4匹合計305匹から日脳ウイルスの分離を試みたがすべて陰性であった．これらの蛇のHI 抗体保有はアオダイショウ3匹，シマヘビ2匹，マムシ1匹に 10乃至40倍という低い値であったがこれが真にHI 抗体か否かはなお検討の要が考えられた．

59匹の蛇を用い実験的感染を試みた．感染には $10^{2.5}$MLD$_{50}$の日脳ウイルスを腹腔内に接種し，25°C，室温，15°C，4°Cの温度条件で飼育，また途中でウイルスの活性化を目的とし低温飼育から高温飼育に環境を変化させることも行なった．ウイルス接種後，数日おきに採血し，ウイルス血症の有無，HI抗体の上昇を検査した．ヤマカガシ2匹が，ウイルス接種後3日目と6日目に，シマヘビ1匹は3日目に，ヒバカリ及びジムグリのそれぞれ1匹が4日目と6日目に血液からウイルスが分離されたがそれ以後はウイルスの分離は出来なかった．平均30日後のHI抗体上昇の検査ではヤマカガシ28匹中1匹のみに10倍という低い値を示した．

以上の事実から蛇の日脳ウイルスに対する感受性は予想以上に低いことが考えられるが蛇の日脳ウイルス増巾に果す役割は，ウイルス感染系路，飼育条件，抗体様成分の解析を吟味し検討した後明かにされるであろう．しかし今後，蛇以外の冷血動物に関しても広汎な実験を行う必要がある．