VARIABILITY OF STRAINS OF JAPANESE ENCEPHALITIS VIRUS IN REGARD TO THE PH-DEPENDENCY IN HEMAGGLUTINATION*

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One hundred and twelve strains of Japanese encephalitis virus (JEV) isolated in Japan during the past 25 years were tested for hemagglutination (HA) with the suckling mouse brain antigen. In the pattern HA tests of a checkerboard system employing graded pH, most of the strains showed about equal titers in the pH range of 6.0 to 7.0, the maximum titers being obtained usually at pH 6.8. In contrast, 7 strains which had received more frequent passages than the rest indicated a different pattern in which the HA titers at the neutral pH were much lower than those at the acid pH, resulting in demonstration of the peak titers at pH 6.2 to 6.4. Analyses of the phenomenon have suggested, however, that the pH-dependency of JEV HA is a genetically stable property, being characteristic to each strain. It is highly probable that the pH-dependency of JEV HA may be an expression of the physicochemical properties of the virus particle which control its adsorbing action onto red cells.

INTRODUCTION

The arbovirus HA has the tendency of pH-dependency (Sabin and Buescher, 1950; Chanock and Sabin, 1953, 1954a, 1954b; Sweet and Sabin, 1954). The HA with the antigen prepared by the method of Clarke and Casals (1958) from suckling mouse brains infected with Nakayama-NIH strain of JEV and goose red cells shows the highest titer at pH 6.4 than at any other pH between 6.0 and 7.0.

In 1958, Oya (Unpublished) noted that antigens from 2 freshly isolated JEV strains showed a higher HA titer at pH 6.8 than at pH 6.4. Since a good number of JEV strains isolated in this or other laboratories during the last 25 years were available, attempts were made to observe variability among these strains in the pH-dependency employing the pattern HA test. A limited trial was also made to test the genetical stability of this property.

MATERIALS AND METHODS

Viruses: A total of 112 strains including sublines were subjected to the present study. The name or number and history of each strain are illustrated in Tables 1 and 2. Strains H-58 and I-58 were isolated by Ohtahara et al. (1959). Strains Konishi,

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Okamoto and Yamashita were kindly given by Dr. Kono of the Kyoto University. Most of the other strains have been maintained in this department.

Source of antigen: All of the strains were passed at least once in the suckling mouse brain. The inoculum at each passage was a 10^{-2} to 10^{-3} diluted suspension of the preceding passage material made in phosphate buffered saline supplemented with 2% antibody-free calf serum. After harvest, the brains were kept in a dry ice cabinet until processing.

Preparation of antigen: The antigens were prepared by acetone-ether (AE) or sucrose-acetone (SA) extraction. A crude alkaline aqueous antigen was also employed. Details of the techniques for antigen preparation appeared elsewhere (Clarke and Casals, 1958). The concentrations of infected suckling mouse brains in the AE, SA and crude antigens were 25, 33 and 10%, respectively. When necessary, the antigen was lyophilized in ampules and stored at -20°C .

Red blood cells for HA: Except two of the present experiments, goose cells were employed. The stock goose cell suspension was prepared according to the technique of Clarke and Casals (1958) so as to show an optical density of 0.450 when diluted 1:40. The stock goose cell suspension made in dextrose-gelatin-veronal stored for less than 2 weeks was used by diluting 1:24 in the virus adjusting diluent (VAD). The one-day-old chick red cells were prepared and used in the same manner.

The antigen diluent, egg albumin borate saline (EA): For an economical reason, egg albumin soluble (Difco) was substituted for bovine albumin recommended earlier (Clarke and Casals, 1958). Egg albumin, initially employed by Oya (1958, unpublished) gives HA patterns comparable to bovine plasma albumin. Usually, a stock egg albumin solution (stock EA) was prepared with a mortar and pestle at 4%. The turbid suspension was kept in a refrigerator after pH adjustment. Before use, the stock EA was shaken vigorously, and centrifuged at 3,000 rpm for 7 minutes. The supernatant was mixed with 9 volumes of borate-buffered saline of pH 9.0. It was used within 3 days after dilution.

Procedures for HA tests: HA tests were performed by the method of Clarke and Casals (1958). The freeze-dried antigen was reconstituted shortly before test and diluted 1:200. Then serial two-fold dilutions in EA were made in test tubes up to 1:51,200. Each dilution of the antigen was distributed into depressions of a lucite tray in the amount of 0.4 ml, to which was added an equal volume of a goose or one-day-chick red cell suspension in VAD, pH 6.0-7.0. A 2 ml or Cornwall pipette was used to add the cell suspension. The tray was allowed to stand in an incubator at 37°C for an hour. An inevitable fluctuation of electrically measured pH at the HA test was within 0.03.

Criteria for reading: The reciprocal of the highest dilution of an antigen giving a partial or complete agglutination was taken as the endpoint. When HA titers at different pH were identical, the optimum pH for the antigen was expressed by the highest pH giving the highest titer.

RESULTS

The pH-dependency in HA of 68 JEV Strains Isolated in 1961:

Table 1 shows the results of HA tests with SA antigen prepared from suckling mouse brains infected with each isolate in 1961 (Oya *et al.*, unpublished). Most of the HA test were repeated twice or more with the result of giving identical HA patterns. The infected suckling mouse brain was used exclusively as the antigen source.

Although the HA titers of each strain varied from 1:3,200 to 1:51,200, the optimum pH was always around 6.8. None of the strains showed the highest HA titer at a pH lower than 6.6. The HA titers at 6.6, 6.8 and 7.0 were very close with each strain listed. All the strains appeared to be uniform with respect to the optimum pH for HA around 6.8, although they were isolated during a 2-month-period (Oya et al., unpublished).

The pH-dependency of HA Demonstrated with 44 JEV Strains Isolated during the Period of 1935-1960:

The pH-dependency and the titers of HA tests with these 44 antigens are presented in Table 2. A total of 8 antigens possessed the optimum pH either at 6.4 or at 6.2. Three of these were substrains of Nakayama and the remainders were strains Kalinina, 2869, G-1-Late, H-58 and I-58. The other strains showed the optimum pH around 6.8.

In Table 3, HA patterns of 10 JEV strains are compared. Strains Nakayama-NIH and I-58 differ not only in the optimum pH but also in the shape of the pattern from the strains given in the right half of the table. The HA pattern of JaGAr#01 strain is flat between pH 6.4 and 7.0. On the other hand, the HA titer of Nakayama-NIH antigen was 1:12,800 at its optimum pH (6.4), 16-fold higher than that at pH 7.0. An extremely sharp pH-dependency was observed with I-58 strain showing as high a titer as 1:6,400 at pH 6.2, whereas at pH 7.0 no agglutination occurred even at 1:200.

Inability of Mouse Brain Passage to Modify the Characteristics of the pH-dependency of JEV Strains.

A question arose as to whether the successive passages of a strain through the adult mouse brain have brought the optimum pH of HA to a more acid side since none of the strains passed through the suckling mouse brain gave an optimum pH at 6.4 or 6.2, whereas those which possessed an optimum pH at 6.4 or 6.2 had been passed at least 20 times through adult mouse brains. As can be seen in Tables 2 and 4, G-1-Early strain which had been passed 7 times in the adult mouse brain showed an optimum pH at 6.8. On the other hand, G-1-Late strain undergoing 189 adult mouse brain passages gave an optimum pH at 6.4, although there was no marked difference in the HA pattern. These tests were repeated more than 5 times giving consistent results.

One of the isolates in 1960, JaGAr 15460, was passed through the adult mouse brain to find whether its optimum pH would shift from 6.8 to 6.4. After every 20 passages, AE antigen was prepared from infected mouse brains. The results are given in Table 5, in which no appreciable change of the optimum pH is shown.

Suckling mouse brain passages were carried out with strains which showed their optimum pH at 6.4 or 6.2. As noted in Table 5, the optimum pH of Nakayama-NIH and I-58 strains did not shift by the additional 12 or 16 suckling mouse brain passages.

The results indicated that the optimum pH of HA is characteristic to each strain, genetically stable and not influenced by passing the virus serially through the adult or suckling mouse brain in the stated manner.

The pH-dependency in HA of JE Viruses Obtained by Serial Limiting Dilution Passage or by Plaque Purification in Chick Embryo Cell Cultures:

Attempts were made to find whether the preparation of JEV strains in the preceding experiments were genetically homogeneous or not, since the pH-dependency appeared to have been caused by a mixed population of two viruses which were distinct from each other in pH-dependency. The pH-dependency of the AE antigen prepared from suckling mouse brains infected with clone-purified viruses of each strain was tested.

Table 1. pH-dependency in HA of JEV strains which were isolated during the year of 1961.

Strain Name			Isolation			HA**	
			Date	Source	Place	Titer	Highest out. pH
1	JaGAr	11161	Aug. 9	Mosq. (CT)*	Gunma-Akabane	3,200	6.6
2	"	11761	July 27	"	Gunma-Itakura	6,400	6.8
3	"	12261	" 28	"	Gunma-Kiryu	3,200	6.8+***
4	"	12361	" 28	"	"	6,400	6.8
5	"	14761	Aug. 4	"	"	3,200	6.8
6	"	14861	" 3	"	Gunma-Itakura	6,400	6.8
7	"	16561	" 3	"	"	3,200	6.6
8	"	17861	" 9	"	Gunma-Akabane	6,400	6.8
9	"	18161	" 9	"	"	6,400	6.8+
10	"	18361	" 9	"	"	6,400	6.8+
11	"	19061	" 10	"	Gunma-Itakura	3,200	7.0
12	"	19161	" 9	"	Gunma-Akabane	3,200	6.8
13	"	19361	" 9	"	"	6,400	6.6
14	"	19461	" 10	"	Gunma-Itakura	6,400	6.8
15	"	19661	" 11	"	Gunma-Kiryu	12,800	6.8
16	"	20061	" 4	"	"	25,600	6.6
17	"	20561	July 27	"	Gunma-Itakura	51,200	6.6
18	"	20661	Aug. 18	"	Gunma-Kiryu	12,800	6.8
19	"	21161	" 18	"	"	12,800	6.6
20	"	21361	" 10	"	Gunma-Itakura	25,600	6.6
21	"	21561	July 28	"	Gunma-Kiryu	12,800	6.8
22	"	21761	Aug. 4	"	"	12,800	6.8
23	"	21861	July 28	"	"	3,200	6.6
24	"	22361	Aug. 18	"	"	3,200	6.6
25	"	23161	" 9	"	Gunma-Akabane	25,600	6.8
26	"	23661	" 11	"	Gunma-Kiryu	25,600	6.6
27	"	24161	" 16	"	Gunma-Akabane	25,600	6.8
28	"	25861	" 17	"	Gunma-Itakura	25,600	6.8
29	"	27061	" 17	"	"	6,400	6.8
30	"	27361	" 4	"	Gunma-Kiryu	6,400	6.6
31	"	27861	July 27	"	Gunma-Itakura	6,400	6.6
32	"	28161	Aug. 18	"	Gunma-Kiryu	3,200	6.8
33	"	28261	" 4	"	"	3,200	6.6
34	"	30761	" 10	"	Gunma-Itakura	6,400	6.6
35	"	31861	" 4	"	Gunma-Kiryu	12,800	7.0
36	"	32161	" 10	"	Gunma-Itakura	6,400	6.6
37	"	32461	July 28	"	Gunma-Kiryu	12,800	6.6
38	"	32861	" 27	"	Gunma-Itakura	6,400	6.6
39	"	33461	" 21	"	Gunma-Kiryu	6,400	6.6

Table 1. (continued)

Strain Name			Isolation			HA**	
			Date	Source	Place	Titer	Highest opt. pH
40	JaGAr	33961	Aug. 11	Mosq. (CT)	Gunma-Kiryu	25,600	6.6
41	"	34061	July 21	"	"	12,800	6.6
42	"	34961	" 28	"	"	12,800	6.8
43	"	35461	" 27	"	Gunma-Itakura	25,600	6.8
44	"	35661	" 26	"	Gunma-Akabane	25,600	6.8
45	"	36161	" 27	"	Gunma-Itakura	6,400	6.6
46	"	36261	" 12	"	Gunma-Akabane	3,200	6.6
47	"	36961	Aug. 3	"	Gunma-Itakura	3,200	6.8
48	"	49561	" 10	"	"	6,400	6.6
49	"	49761	" 11	"	Gunma-Kiryu	12,800	6.8
50	"	50161	" 30	"	Gunma-Akabane	6,400	6.8
51	"	50261	" 30	"	"	25,600	6.6
52	"	51961	Sept. 1	"	Gunma-Kiryu	3,200	6.8
53	"	52561	" 7	"	Gunma-Itakura	3,200	6.6
54	"	54561	" 7	"	"	6,400	6.6
55	"	58161	Aug. 4	"	Gunma-Kiryu	3,200	6.8
56	"	58261	" 24	"	Gunma-Itakura	3,200	6.8
57	"	58461	July 28	"	Gunma-Kiryu	3,200	6.8
58	"	58561	" 28	"	"	6,400	6.8
59	"	60061	" 21	"	"	6,400	6.8
60	"	60561	" 28	"	"	6,400	6.6
61	"	60661	" 28	"	"	3,200	6.8
62	"	60761	Aug. 4	"	"	25,600	6.6
63	"	60961	Sept. 28	"	Gunma-Itakura	12,800	6.6
64	"	61561	July 27	"	"	12,800	6.6
65	"	72061	Aug. 4	"	Gunma-Kiryu	12,800	6.6
66	"	72361	July 28	"	"	3,200	6.8
67	"	101861	Aug. 18	"	"	12,800	6.6
68	JaTH	161		Human brain	Tokyo	12,800	6.8

* *Culex tritaeniorhynchus* mosquito

** All antigens were prepared by SA methods with the third suckling mouse brain passage.

*** The HA was not performed at pH 7.0.

The JaGAr 15460 strain was purified by 5 successive limiting dilution passages through adult mouse brains. The pH-dependency of the AE antigen thus prepared did not differ noticeably from that of the parent, as shown in Table 6, the optimum pH being 6.8.

Viruses plaque-purified in the chick embryo cells of I-58, JaGAr#01, Nakayama-NIH and Nakayama-Yakken strains were given by Dr. Akira Oya of this laboratory. The antigens prepared with those plaque-purified viruses had the optimum pH identical to that possessed by their parent strains except Nakayama-NIH strain (Table 6). The parent strain showed the optimum pH at 6.4; the optimum pH for the plaque-purified Naka-

Table 2. pH-dependency in HA of JEV strains which were isolated during the period 1935-1960

Strain Name	Isolation			Passage Level*	Antigen	HA	
	Year	Place	Source			Titer	Highest opt. pH
1 Nakayama-NIH	1935	Tokyo	human CSF**	AMBr(##)SMBR(1-12)	AE	12,800	6.4
2 Nakayama-RFVL	"	"	"	AMBr(+)SMBR(10)	"	25,600	6.6
3 Nakayama-Yakken	"	"	"	AMBr(+)SMBR(1)	"	12,800	6.4
4 Nakayama-OP	"	"	"	AMBr(+)OE(15)	"	1,600	6.4
5 Kalinina	"	"	human brain	AMBr(61+)SMBR(1)	SA	6,400	6.4
6 2869	1947	Chiba	horse brain	AMBr(34)SMBR(2)	AE	6,400	6.4
7 G-1-Early	1949	Tokyo	human brain	AMBr(7)SMBR(3)	"	25,600	6.8
8 G-1-Late	"	"	"	AMBr(189)SMBR(1)	"	12,800	6.4
9 Sekiya	1952	"	"	AMBr(6)SMBR(1)	"	12,800	6.6
10 Nakai	"	"	"	AMBr(5)SMBR(1)	"	12,800	6.6
11 Mochizuki	1953	Okayama	"	AMBr(4)SMBR(1)	"	12,800	6.8
12 Kikuchi	1954	Tokyo	"	AMBr(4)SMBR(1)	"	25,600	6.6
13 Igaya	1955	"	"	AMBr(3)SMBR(2)	"	12,800	6.6
14 Ikeda	"	"	"	AMBr(5)SMBR(1)	"	6,400	6.6
15 Otsubo	1956	"	"	AMBr(1)SMBR(1)	"	12,800	6.6
16 Shimada	"	"	"	AMBr(8)SMBR(2)	"	25,600	6.8
17 Tanaka	1957	"	"	AMBr(3)SMBR(1)	"	25,600	6.6
18 TOP	"	"	"	OE(54)HKTC(6)SMBR(1)	"	3,200	6.6
19 TOP54HK28(Oya)	"	"	"	OE(54)HKTC(28)SMBR(1)	"	3,200	6.6
20 H-58	1958	Okayama	"	AMBr(19)SMBR(1)	"	25,600	6.4
21 I-58	"	"	"	AMBr(18)SMBR(1)	"	6,400	6.2
22 Hotta	"	·Tokyo	"	AMBr(2)SMBR(1)	"	6,400	6.6
23 Yachida	"	"	"	AMBr(2)SMBR(1)	"	25,600	6.8

Table 2. (continued)

Strain Name	Isolation			Passage Level*	Antigen	HA	
	Year	Place	Source			Titer	Highest opt. pH
24 Nakagawa	1959	Tokyo	human brain	SMBr(2)	AE	12,800	6.6
25 Sekiguchi	"	"	"	SMBr(3)	"	12,800	6.6
26 Kitano	"	Osaka	"	AMBr(9)SMBr(1)	"	25,600	6.8
27 JaGAR#01	"	Gunma	mosquitoes(CT)***	HKTC(3)SMBr(2-5)	"	12,800	6.8
28 JaGAR#02	"	"	mosquitoes(CT)	CETC-SMBr(2)SMBr(1)	"	6,400	6.8
29 Konishi	1960	Kyoto	human brain	SMBr(5)	"	3,200	6.8
30 Okamoto	"	"	"	SMBr(4)	"	12,800	7.0
31 Yamashita	"	"	"	SMBr(3)	"	1,600	6.6
32 JaTH 160	"	Tokyo	"	SMBr(2)	SA	3,200	6.8
33 JaTH 260	"	"	"	SMBr(2)	"	3,200	6.6
34 JaGAR 15460	"	Gunma	mosquitoes(CT)	SMBr(2)	"	25,600	6.8
35 JaGAR 16760	"	"	"	SMBr(2)	"	6,400	6.8
36 JaGAR 18660	"	"	"	SMBr(2)	"	6,400	6.8
37 JaGAR 20660	"	"	"	SMBr(1)	"	400	6.8
38 JaGAR 29760	"	"	mosquitoes(CP)****	SMBr(2)	"	6,400	6.8
39 JaGAR 29860	"	"	"	SMBr(2)	"	3,200	6.8
40 JaGAR 46160	"	"	mosquitoes(CT)	SMBr(2)	"	12,800	6.8
41 JaGAR 46360	"	"	"	SMBr(2)	"	6,400	6.8
42 JaGAR 47160	"	"	"	SMBr(2)	"	3,200	6.6
43 JaGAR 48060	"	"	"	SMBr(2)	"	3,200	6.8
44 JaGAR 48160	"	"	"	SMBr(2)	"	6,400	6.6

* AMBr=adult mouse brain passage SMBr=suckling mouse brain passage OE=one-day-old- hen's egg passage HKTC=primary hamster kidney culture passage CETC-SMBr=plaque purification procedures
 ** cerebrospinal fluid *** CT=*Culex tritaeniorhynchus* **** CP=*Culex pipiens*

Table 3. A comparison of HA patterns given by 10 JEV strains

	pH	200*	6,400		pH	200*	6,400
I-58 AMBr(18) SMBr(1)	6.0	+	+	+	+	+	0
	6.2	+	+	+	+	+	0
	6.4	+	+	+	+	+	0
	6.6	+	±	0*	+	+	0
	6.8	0*	0	0	0	0	0
	7.0	0	0	0	0	0	0
H-58 AMBr(19) SMBr(1)	6.0	+	+	+	+	+	0
	6.2	+	+	+	+	+	±
	6.4	+	+	+	+	+	0
	6.6	+	+	+	+	+	0*
	6.8	+	+	+	+	0*	0
	7.0	±	0*	0	0	0	0
Nakayama- NIH AMBr(††) SMBr(3)	6.0	+	+	+	+	±	0
	6.2	+	+	+	+	+	0
	6.4	+	+	+	+	+	0
	6.6	+	+	+	+	±	0
	6.8	+	+	+	+	±	0
	7.0	+	+	+	0	0	0
Kalinina AMBr(61+) SMBr(1)	6.0	+	+	+	+	0*	0
	6.2	+	+	+	+	+	0
	6.4	+	+	+	+	+	0
	6.6	+	+	+	+	±	0
	6.8	+	+	+	+	±	0*
	7.0	+	+	±	0	0	0
2869 AMBr(34) SMBr(2)	6.0	+	+	+	+	0*	0
	6.2	+	+	+	+	±	0
	6.4	+	+	+	+	+	0*
	6.6	+	+	+	+	±	0
	6.8	+	+	+	+	±	0
	7.0	(+)(+)	0*	0	0	0	0
Nakayama- RFVL AMBr(+) SMBr(10)	6.0	+	+	+	+	+	0
	6.2	+	+	+	+	+	0
	6.4	+	+	+	+	+	0
	6.6	+	+	+	+	+	0
	6.8	+	+	+	+	+	±
	7.0	+	+	+	+	+	0
Hotta AMBr(2) SMBr(1)	6.0	+	+	+	±	0*	0
	6.2	+	+	+	±	0*	0
	6.4	+	+	+	+	±	0
	6.6	+	+	+	+	±	0
	6.8	+	+	+	+	±	0
	7.0	+	+	+	±	0	0
Kitano AMBr(10) SMBr(1)	6.0	+	+	+	+	±	0
	6.2	+	+	+	+	±	0
	6.4	+	+	+	+	±	0
	6.6	+	+	+	+	±	0
	6.8	+	+	+	+	+	0
	7.0	+	+	+	+	+	0*
JAGA#01 HK(3) SMBr(2)	6.0	+	+	+	+	+	0
	6.2	+	+	+	+	+	0*
	6.4	+	+	+	+	+	0
	6.6	+	+	+	+	+	0
	6.8	+	+	+	+	+	±
	7.0	+	+	+	+	+	0*
JaTH 160 AMIP-Br(4)** SMBr(1)	6.0	+	+	+	+	+	0
	6.2	+	+	+	+	+	0*
	6.4	+	+	+	+	+	0*
	6.6	+	+	+	+	±	0
	6.8	+	+	+	+	+	0*
	7.0	+	+	+	+	+	0*

+: Positive agglutination (+): Partial agglutination
±: Trace agglutination 0*: Almost negative 0: Negative

* Antigen dilution (1:)

** The passage by inoculating adult mouse i. p. and harvesting the brain of infected mouse.

yama-NIH strain was slightly more alkaline, i. e., 6.6. This was confirmed by repeated tests employing 2 lots of antigen.

Influence of Methods for Antigen Preparation and Different Red Cells upon pH-dependency:

As mentioned previously, the antigen was extracted by either the AE or the SA method and all the tests were performed with goose cells. In order to ascertain whether the pH-dependency of HA pattern is an intrinsic property of each virus or influenced by certain other factors, influences of the method of antigen preparation and of the

Table 4. Comparison of pH-dependency in HA between G-1-Early and G-1-Late strains

pH	Antigen dilution 1 :										Optimum	Antigen dilution 1 :										Optimum
	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200		100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	
6.0	+	+	+	+	+	+	+	±	0	0	6.8	+	+	+	+	+	+	+	0	0	0	6.4
6.2	+	+	+	+	+	+	+	+	0*	0		+	+	+	+	+	+	+	(+)	0	0	
6.4	+	+	+	+	+	+	+	+	+	0		+	+	+	+	+	+	+	(+)	0*	0	
6.6	+	+	+	+	+	+	+	+	+	0*		+	+	+	+	+	+	+	±	0	0	
6.8	+	+	+	+	+	+	+	+	+	±		+	+	+	+	+	+	(+)	0*	0	0	
7.0	+	+	+	+	+	+	+	+	+	0*		+	+	+	+	+	+	(+)	0	0	0	

G-1-Early strain

AE antigen

Passage : AMBr (7) SMBr (3)

G-1-Late strain

AE antigen

Passage : AMBr (189) SMBr (1)

+ : Positive agglutination (+) : Partial agglutination ± : Trace agglutination

0* : Almost negative 0 : Negative Optimum : Highest optimum pH

Table 5. Relationship between passage levels in mice and pH-dependency of HA by 3 JEV strains

Strain Name	Passage Level	Antigen	HA titer	Highest Opt. pH
JaGAR 15460	SMBr (2)	S A	25600	6.8
	SMBr (2) AMBr (20) SMBr (1)	A E	12800	6.8
	SMBr (2) AMBr (40) SMBr (1)	A E	25600	6.8
	SMBr (2) AMBr (59) SMBr (1)	A E	3200	6.8
	SMBr (2) AMBr (65) SMBr (1)	A E	6400	6.6
	SMBr (2) AMBr (80) SMBr (1)	A E	6400	6.8
	SMBr (2) AMBr (100) SMBr (1)	A E	12800	6.6
	SMBr (2) AMBr (100) SMBr (1)	A E	12800	6.6
Nakayama-NIH	AMBr (##) SMBr (3)	A E	12800	6.4
	AMBr (##) SMBr (12)	A E	12800	6.4
I-58	AMBr (18) SMBr (1)	A E	6400	6.2
	AMBr (18) SMBr (16)	A E	3200	6.2

species of red cells were checked.

Several litters of suckling mice were infected with a 10^{-2} dilution of the stock lyophilized virus of I-58 or JaGAR#01 strain and the brains were harvested 3 days after inoculation. A batch of the infected suckling mouse brains was divided into 3 parts from which 1) crude alkaline aqueous, 2) AE and 3) SA antigens were prepared. They were tested simultaneously with goose and 1-day-chick cells. The results are illustrated in Table 7.

Although HA titers given by 1-day-chick red cells appeared to be uniformly lower than those by goose cells as reported earlier (Porterfield, 1957) and the HA at a relatively alkaline pH with crude antigen tended to be weak, the pH-dependency of each strain was not materially altered by the method of antigen preparation or by the species

Table 6. pH-dependency of the antigens prepared from single virus derived clone of each JEV strain in comparison with those of parental virus

Strain Name	Passage Level	Antigen	HA titer	Highest Opt. pH
JaGAr 15460	SMBr (2) AMBr (14) SMBr (1)	A E	6400	6.8
	SMBr (2) AMBr (15) AMBr LDP* (5) SMBr (1)	A E	3200	6.8
JaGAr #02	SMBr (7)	A E	3200	6.8
	CETC-SMBr** (2) SMBr (1)	A E	6400	6.8
I-58	AMBr (18) SMBr (1)	A E	6400	6.2
	AMBr (18) CETC-SMBr (3)** SMBr (2)	A E	6400	6.2
Nakayama-NIH	AMBr (##) SMBr (5)	A E	12800	6.4
	AMBr (##) SMBr (5) CETC-SMBr (3)** SMBr (4)	A E	6400	6.6
Nakayama-Yakken	AMBr (++) SMBr (1)	A E	12800	6.4
	AMBr (++) SMBr (1) CETC-SMBr (1)** SMBr (2)	A E	12800	6.4

* A limiting dilution passage through adult mouse brain.

** Plaque purification of the viruses in chick embryo tissue culture.

of red cells used. This finding constituted further evidence that the phenomenon of pH-dependency is due to an intrinsic property of each strain of JEV.

DISCUSSION

The present paper deals, for the first time, with the strain variation of JEV in pH-dependency of HA. Tests with more than 100 JEV strains have demonstrated that a certain number of strains differ in HA characteristics from fresh isolates. Strains Nakayama-NIH, Nakayama-Yakken, Kalinina, 2869, G-1-Late, H-58 and I-58 displayed similar HA characteristics as the laboratory-passed strains. The optimum pH for HA of those strains was 6.4 or lower. No fresh strain possessed the optimum pH at 6.4 or lower. Accordingly, the optimum pH for JEV HA lies between 6.6 and 7.0 with a strain isolated freshly by an intracerebral inoculation of suckling mice.

Comparatively acid optimum pH, characteristic to some laboratory-passed strains may have been caused by certain factors operative in successive passages. The adult mouse brain tissue may have selected such strains, although the experimental passage of JaGAr 15460 strain through adult mouse brains provides no evidence supporting the above hypothesis.

Investigation was made to find whether the HA characteristics furnish a good tool for the genetic study of JEV as a marker. This question should be answered by "yes" because 1) the HA characteristics did not distinguish goose cells from those of 1-day-chick, 2) the difference in the method of antigen preparation did not influence the HA characteristics, 3) the property remained unaltered during 100 adult mouse brain passages and 4) this was also true with cloned viruses.

An exception was encountered with Nakayama-NIH strain. Umclonized Nakayama-NIH strain consistently showed the optimum pH at 6.4, whereas the cloned strain at 6.6. Although the difference was merely 0.2, this was not likely to have been caused by technical errors, because of the high reproducibility of the result. By the plaque purification performed in exactly the same manner, I-58 strain failed to shift its optimum pH for HA. An explanation for these phenomena would be possible only by analysing

Table 7. Inability to influence the optimum pH in HA of two JEV strains by changing the methods of antigen preparation and cells

	pH	100*	12800*								100	12800										
AE antigen : Goose cell	6.0	+	+	+	+	+	(+)	0	0	0	0	+	+	+	+	+	+	+	±	0		
	6.2	+	+	+	+	+	+	±	0	0	0	0	+	+	+	+	+	+	+	(+)		
	6.4	+	+	+	+	+	+	(+)	0	0	0	0	+	+	+	+	+	(+)	±	0		
	6.6	+	+	+	+	+	+	+	±	0	0	0	+	+	+	(+)	±	0*	0	0	0	
	6.8	+	+	+	+	+	+	+	+	0	0	0	(+)	(+)	0*	0	0	0	0	0	0	
	7.0	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	
AE antigen : Chick cell	6.0	+	+	+	+	(+)	0	0	0	0	0	+	+	+	+	+	(+)	0	0	0	0	
	6.2	+	+	+	+	+	±	0	0	0	0	0	+	+	+	+	+	±	0	0	0	
	6.4	+	+	+	+	+	(+)	0	0	0	0	0	+	+	+	+	+	±	0	0	0	
	6.6	+	+	+	+	+	+	(+)	±	0	0	0	0	+	+	+	(+)	0	0	0	0	
	6.8	+	+	+	+	+	(+)	±	0	0	0	0	(+)	(+)	±	0	0	0	0	0	0	
	7.0	+	+	+	+	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	0	
SA antigen ; Goose cell	6.0	+	+	+	+	+	±	0	0	0	0	+	+	+	+	+	+	+	(+)	0*	0	
	6.2	+	+	+	+	+	(+)	±	0	0	0	+	+	+	+	+	+	+	+	0	0	
	6.4	+	+	+	+	+	+	+	0	0	0	+	+	+	+	+	+	+	(+)	0	0	
	6.6	+	+	+	+	+	+	+	±	0	0	+	+	+	+	+	(+)	±	0	0	0	
	6.8	+	+	+	+	+	+	+	+	±	0	+	+	+	+	(+)	0*	0	0	0	0	
	7.0	+	+	+	+	+	+	+	(+)	±	0	+	+	(+)	0*	0	0	0	0	0	0	
SA antigen : Chick cell	6.0	+	+	+	+	+	±	0	0	0	0	+	+	+	+	+	+	+	0*	0	0	0
	6.2	+	+	+	+	+	+	±	0	0	0	0	+	+	+	+	+	+	0*	0	0	0
	6.4	+	+	+	+	+	(+)	(+)	0	0	0	+	+	+	+	(+)	(+)	0*	0	0	0	0
	6.6	+	+	+	+	+	+	+	±	0	0	(+)	(+)	0	0	0	0	0	0	0	0	
	6.8	+	+	+	+	+	+	+	±	0	0	0*	0	0	0	0	0	0	0	0	0	
	7.0	+	+	+	+	+	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	
Crude antigen : Goose cell	6.0	+	+	+	+	(+)	±	0	0	0	0	+	+	+	+	+	+	±	0	0	0	
	6.2	+	+	+	+	+	(+)	0	0	0	0	+	+	+	+	+	+	+	0	0	0	
	6.4	+	+	+	+	+	±	0	0	0	0	+	+	+	+	+	+	+	(+)	0*	0	
	6.6	+	+	+	+	+	+	±	0	0	0	+	+	+	+	+	(+)	±	0	0	0	
	6.8	+	+	+	+	+	+	+	±	0	0	0*	0*	±	0	0	0	0	0	0	0	
	7.0	+	+	+	(+)	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Crude antigen : Chick cell	6.0	+	(+)	±	0	0	0	0	0	0	0	+	+	(+)	0*	0	0	0	0	0	0	
	6.2	+	+	+	±	0	0	0	0	0	0	+	+	+	+	±	0	0	0	0	0	
	6.4	+	+	+	+	±	0	0	0	0	0	+	+	(+)	±	0*	0	0	0	0	0	
	6.6	+	+	+	+	+	±	0	0	0	0	±	0	0	0	0	0	0	0	0	0	
	6.8	+	+	+	+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	7.0	+	+	+	(+)	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

JaGAr#01 strain

I-58 strain

+ : Positive agglutination (+) : Partial agglutination ± : Trace agglutination

0* : Almost negative 0 : Negative

* Indicates a dilution of antigen. The scale was adjusted for crude antigen because it was prepared into 10 %.

the population of certain strains by plaque selection on chick embryo cells. The shift in optimum pH during the processes of plaque purification of Nakayama-NIH strain in chick embryo cells would be attributable to selection from otherwise mixed virus particles having slightly alkaline optimum pH (6.6) for HA. If this assumption was correct, the ratio between these populations in the original Nakayama-NIH strain may have been maintained as far as the virus was passed through the mouse brain.

The foregoing results strongly indicate the existence of JEV particles differing from each other in their optimum pH for HA. The difference between both extremes, 6.2 and 7.0, is noticeable. It is most likely that the phenomenon results from a difference in the efficiency of adsorption of viral hemagglutinin onto red cells, but further investigation will be needed.

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