



# Seasonal prevalence of anti-Japanese encephalitis virus antibody in pigs in different regions of Taiwan

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Although the endemic season of Japanese encephalitis lasts from May through October, sporadic cases occur in other months of the year. To appreciate the real situation of Japanese encephalitis virus (JEV) activity throughout the year, a survey was conducted using hemagglutination-inhibition test to detect the presence of anti-JEV antibodies in pigs' sera, which were collected from 4 regions of Taiwan between January 2000 and January 2001. Results indicate that (1) JEV-infected pigs were found throughout the year; (2) the prevalence rates of JEV in the 4 regions in descending order were 70% in Miaoli, 59% in Hualien, 52% in Pingtung, and 49% in Nantou; (3) similar to that in 1990, the peak endemic months appeared 1 month after the date of 50% seroconversion (May 21, 2000); (4) infected pigs were present during the period from December through April as a reservoir for over-wintering of JEV; and (5) there was a suggestive evidence for the occurrence of sporadic cases of JEV during the December-April period. In conclusion, JEV propagates and is active indigenously in the winter as well as other seasons of the year.

**Key words:** Epidemiology, hemagglutination-inhibition test, Japanese encephalitis virus

Japanese encephalitis (JE) was first reported as "summer encephalitis" in Taiwan by Sakai [1] in 1935, indicating its occurrence in summer. Japanese encephalitis virus (JEV) was later isolated directly from fatal patients in 1938 [2] and 1958 [3], confirming its etiological role in JE. During 1955 to 1959, the successful isolation of JEV from mosquitoes [4] and the serial studies on the ecology of JE in Taiwan conducted by Wang *et al* [5] revealed the role of pigs as a main JEV-amplifying animal reservoir. Some puzzles such as the over-wintering mechanism of JEV, however, remain to be resolved. Because studies of seroprevalence in pigs were generally carried out during the period between April and July [6, 7], the whole picture of the seasonal prevalence of JE was not clear. As part of the research series on JE [6-8], this study was initiated to explain the existence of local JEV over-wintering and the occurrence of sporadic JE in winter season by collecting serum and examining the seasonal distribution of hemagglutination-inhibition (HI) antibody prevalence throughout a year.

## Materials and Methods

### Preparation of serum

Sera were collected from pigs raised natively for less than 6 months, with body weight less than 90 kg at the

time they were sacrificed at abattoirs in Miaoli, Hualien, Pingtung, and Nantou of Taiwan from January 2000 through January 2001. Sera were collected from 30 pigs 3 times per month at intervals of 10 days. All sera were refrigerated during shipping and stored at  $-20^{\circ}\text{C}$  in the laboratory. Serum of 0.1 mL was precipitated and extracted twice with 2 mL cold acetone. After drying in vacuum, the serum was reconstituted in 1 mL borate saline solution (pH 9) to give a 1:10 dilution of the original serum. Non-specific hemagglutinins were absorbing and removed with 0.1 mL packed male goose erythrocytes. The sera were finally inactivated at  $56^{\circ}\text{C}$  in water bath for 30 min. Reconstituted dilute sera were stabilized for at least 7 days at  $4^{\circ}\text{C}$ .

### Preparation of antigen

Sucrose-acetone-purified JEV (JaGAR#01 strain, isolated from *Culex tritaeniorhynchus* by National Institute of Health, Japan) prepared from infected suckling mouse brain was used as the stock antigen. The maximal hemagglutination titer usually reached 1:32768 to 1:65536. It was then sealed in glass ampoules and stored at  $-70^{\circ}\text{C}$ . The antigen (16 hemagglutination unit/0.05 mL) was prepared by diluting the stock antigen with 4% bovalbumin (Sigma Chemical, St. Louis, MO, US) in borate saline solution (pH 9).

### Hemagglutination-inhibition test

Hemagglutination-inhibition test (microplate) modified

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from the original method of Clarke [9] was adopted. Serial 2-fold dilution of acetone-extracted serum was prepared in 4% bovalbumin in borate saline solution (pH 9). An equal volume (8 units) of antigen was added to diluted serum 0.025 mL and kept at 4°C overnight. A volume of 0.05 mL of 0.33% goose erythrocyte suspension, prepared by diluting 8% goose erythrocyte suspension with virus adjusting diluent (pH 6.7), was added to each well and kept at 37°C in an incubator for 30 min. The reciprocal of highest serum dilution inhibiting hemagglutination was recorded as the HI titer. The lowest serum dilution tested was 1:10. An HI titer equal to or greater than 10 was considered positive [10].

## Results

Variations in anti-JEV seroprevalence rates in the pigs examined in the 4 regions throughout the year are shown in Fig. 1. In general, the prevalence rates can be roughly divided into 4 stages. In the first stage (late February to early May), the prevalence rates (0- <50%) were relatively lower than in the rest of year. In the second stage (mid-May to mid-June), the prevalence rate in Pingtung, which is situated within the torrid zone in southern Taiwan, exceeded 50% by May 21. This date was about 20 days earlier than that observed in Miaoli and Hualien, which are situated in the subtropical zone in northern and eastern locality, respectively, and was about 1 month earlier than that in Nantou in central Taiwan. Once the prevalence rates exceeded 50%, they

rose steeply to about 90% in all 4 regions within a short period (1 month) by June 20. In this third stage (mid-June to mid-August), the prevalence rates were kept at a high plateau (70%-100%) in all regions. In the fourth stage, which comprises 7 months from late August to mid-February the next year, the prevalence rates declined rapidly in general, came close to those of the first stage, and completed perfectly one cycle.

Different distributions of HI titers in the 4 regions were depicted in Tables 1 to 4. The prevalence rate was relatively higher in Miaoli (41%, 111/270) than in other regions (16%, 43/270 in Hualien; 24%, 73/300 in Pingtung; and 9%, 25/270 in Nantou) before May. After the 50% seroconversion point has been reached, it rose immediately to the peak and remained relatively high until the end of the year. Its mode of HI titers was 320, and 55% (427/777) of the pigs have HI titers  $\geq 320$ . The total prevalence rate was 70% (777/1110) in Miaoli, which was by far the highest among the 4 regions. The higher the prevalence rates, the higher the mode of HI titers.

The number of JEV-infected pigs in Hualien was especially small from January to May. It increased after the 50% seroconversion point to the peak at mid-June; like that of Miaoli, the maximal HI titers in Hualien ranged from 1280 to 2560. The 100% prevalence rate was maintained until early autumn only in Hualien among the 4 regions. The decline in prevalence in Hualien was more rapid than that of Miaoli, but was slower than those of Pingtung and Nantou. Although

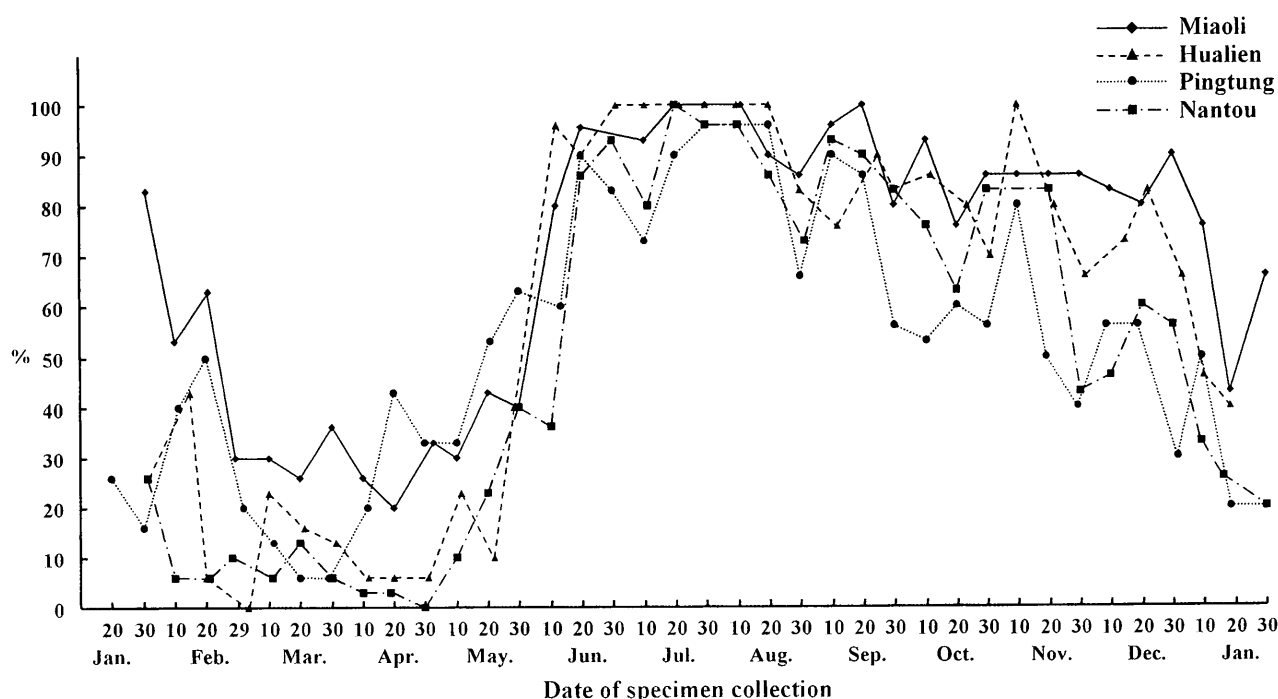


Fig. 1. Monthly hemagglutination-inhibition antibody seroconversion rates among adult pigs in Taiwan, January 2002 to January 2001.

**Table 1.** Distribution of hemagglutination-inhibition antibody titers among adult pigs in Miaoli region

Date mm/dd/yy	HI antibody titer <sup>a</sup>								HI antibody (+)		
	<10	10	20	40	80	160	320	640	1280	2560	%
01/31/00	5	-	-	7	5	9	4	-	-	-	83 <sup>b</sup>
02/10/00	14	2	1	-	3	2	5	2	1	-	53
02/21/00	11	-	2	2	6	6	3	-	-	-	63
02/29/00	21	-	2	1	1	1	2	2	-	-	30
03/10/00	21	-	2	-	-	3	2	1	1	-	30
03/20/00	22	1	-	2	-	-	3	1	1	-	26
03/30/00	19	-	-	-	3	2	6	-	-	-	36
04/10/00	22	-	2	-	3	2	-	1	-	-	26
04/20/00	24	-	-	1	2	3	-	-	-	-	20
05/02/00	20	-	1	-	3	-	2	4	-	-	33
05/10/00	21	2	-	-	-	-	1	6	-	-	30
05/20/00	17	-	-	-	4	4	2	-	3	-	43
05/30/00	18	1	1	1	2	1	5	-	1	-	40
06/11/00	6	-	4	1	-	1	7	7	4	-	80
06/20/00	1	-	-	-	2	-	5	7	15	-	96
06/30/00	2	-	1	-	1	4	6	9	7	-	93
07/10/00	2	-	-	1	1	4	13	7	1	1	93
07/20/00	0	1	-	-	-	3	4	17	5	-	100
07/30/00	0	-	1	1	1	12	9	6	-	-	100
08/11/00	0	-	-	3	1	1	14	10	1	-	100
08/20/00	3	-	-	-	-	6	9	7	2	3	90
08/30/00	4	-	-	1	-	11	6	6	2	-	86
09/10/00	1	-	1	2	4	10	11	1	-	-	96
09/20/00	0	-	-	1	9	7	6	5	2	-	100
09/30/00	6	-	-	5	2	4	9	4	-	-	80
10/10/00	2	-	1	2	3	4	10	4	1	3	93
10/20/00	7	-	2	3	6	5	-	4	3	-	76
10/30/00	4	-	-	1	5	12	3	4	1	-	86
11/10/00	4	-	-	1	6	3	6	7	3	-	86
11/20/00	4	1	1	2	1	5	8	6	2	-	86
11/30/00	4	-	-	3	3	6	10	3	1	-	86
12/10/00	5	-	-	-	2	8	4	7	4	-	83
12/20/00	6	-	-	-	4	8	3	5	4	-	80
12/30/00	3	-	-	2	3	9	1	7	2	3	90
01/10/01	7	-	-	6	4	4	4	3	1	1	76
01/18/01	17	-	-	5	2	3	2	1	-	-	43
01/30/01	10	-	-	5	4	2	5	2	2	-	66

Abbreviation: HI = hemagglutination-inhibition

<sup>a</sup>The highest serum dilution showing positive HI reaction.<sup>b</sup>83% = (30-5)/30.

Note: mode of HI titers = 320; total prevalence rate = 70%

infected pigs were not often found in Hualien before May, the mode of HI titers was 320, and 54% (346/641) of pigs had HI titers  $\geq 320$ . A similar phenomenon was also observed in Miaoli, and the total prevalence rate in Hualien (59%) even overtook that of Pingtung (52%).

The variation in the prevalence rates in Pingtung was prominently large. It reached the 50% seroconversion on May 21, and arrived at the peak more rapidly than other regions did. The prevalence rate also declined most rapidly from late August. The number of pigs with HI titer  $\geq 1280$  was small ( $n = 26$ ); only 134 pigs have HI titer  $\geq 640$  compared with 237 pigs of Miaoli. Although Pingtung is situated in the tropics, the mode of HI titers was 320 and the percentage of pigs with HI titer greater than or equal to the mode was 55% (326/596) of 596 (55%), which was similar to those of Miaoli (55%) and Hualien (54%), both situated in the subtropics. The total prevalence rate in Pingtung (52%) was lower than those of Miaoli (70%) and Hualien (59%).

The number of infected pigs in Nantou from January

**Table 2.** Distribution of hemagglutination-inhibition antibody titers among adult pigs in Hualien region

Date mm/dd/yy	HI antibody titer <sup>a</sup>								HI antibody (+)		
	<10	10	20	40	80	160	320	640	1280	2560	%
02/02/00	22	-	-	1	1	4	2	-	-	-	26 <sup>b</sup>
02/15/00	17	-	2	1	4	3	2	1	-	-	43
02/20/00	28	-	-	-	2	-	-	-	-	-	6
03/03/00	30	-	-	-	-	-	-	-	-	-	0
03/10/00	23	1	-	-	3	-	2	1	-	-	23
03/21/00	25	-	-	2	-	1	-	1	1	-	16
03/31/00	26	-	-	-	1	1	2	-	-	-	13
04/11/00	28	-	-	1	-	1	-	-	-	-	6
04/20/00	28	-	-	-	-	-	2	-	-	-	6
05/01/00	28	-	-	-	2	-	-	-	-	-	6
05/11/00	23	-	3	1	-	1	-	-	2	-	23
05/22/00	27	-	1	-	-	1	1	-	-	-	10
05/29/00	18	-	-	-	-	2	7	3	-	-	40
06/12/00	1	1	-	-	-	2	3	14	8	1	96
06/20/00	3	-	1	-	1	-	3	6	14	2	90
07/01/00	0	-	-	-	-	8	16	6	-	-	100
07/10/00	0	-	-	1	-	11	12	6	-	-	100
07/20/00	0	-	1	-	1	8	10	10	-	-	100
08/01/00	0	-	-	-	1	13	11	3	2	-	100
08/10/00	0	-	-	-	3	4	16	6	1	-	100
08/22/00	0	-	-	-	2	7	7	12	1	1	100
08/30/00	5	-	1	3	1	7	11	2	-	-	83
09/12/00	7	-	2	-	4	-	10	7	-	-	76
09/25/00	3	-	-	4	4	7	6	4	2	-	90
09/30/00	5	-	2	3	2	9	6	3	-	-	83
10/11/00	4	-	-	1	3	10	8	4	-	-	86
10/23/00	6	-	3	2	4	8	5	2	-	-	80
10/31/00	9	-	4	3	2	6	4	1	1	-	70
11/10/00	0	-	-	1	3	10	4	7	3	2	100
11/21/00	6	-	-	2	2	6	9	5	-	-	80
12/02/00	10	-	-	-	7	5	7	1	-	-	66
12/14/00	8	-	-	4	6	1	6	5	-	-	73
12/22/00	5	1	1	1	3	7	5	5	1	1	83
01/03/01	10	-	-	4	4	8	3	1	-	-	66
01/10/01	16	-	1	2	1	5	5	-	-	-	46
01/18/01	18	-	-	2	5	3	1	1	-	-	40

Abbreviation: HI = hemagglutination-inhibition

<sup>a</sup>The highest serum dilution showing positive HI reaction.<sup>b</sup>26% = (30-22)/30.

Note: mode of HI titers = 320; total prevalence rate = 59%

through early May was the lowest among the 4 regions, and the HI titers were also low. However, the prevalence rose to the 50% seroconversion on June 20 before reaching the peak. The variation in the prevalence in Nantou before early August bore a close resemblance to that of Hualien, and it showed a similar downward tendency to Pingtung after late August. The total prevalence rate (49%, 530/1080) and HI titers in Nantou were the lowest of all, with mode of HI titers being 160. Similar to Miaoli, the prevalence in Nantou drops as the mode of HI titers declines, and vice versa.

## Discussion

This study describes the seroepidemiological situation of JE in Taiwan based on the actual seroconversion rates in pigs rather than on reported human cases. The method of surveillance was employed for the following reasons. Pigs are known to be the main amplifying host of JEV, and could therefore be used as an indicator of the presence of JEV. The 100% coverage rate of serum

**Table 3.** Distribution of hemagglutination-inhibition antibody titers among adult pigs in Pingtung region

Date mm/dd/yy	HI antibody titer <sup>a</sup>									HI antibody (+) %	
	<10	10	20	40	80	160	320	640	1280		2560
01/20/00	22	-	-	1	1	2	3	1	-	-	26 <sup>b</sup>
01/30/00	25	-	-	4	1	-	-	-	-	-	16
02/11/00	18	-	-	2	3	-	5	2	-	-	40
02/20/00	15	-	-	-	1	6	4	4	-	-	50
03/02/00	24	-	1	2	-	2	1	-	-	-	20
03/11/00	26	-	1	1	-	-	2	-	-	-	13
03/20/00	28	-	-	-	-	1	1	-	-	-	6
03/29/00	28	-	-	1	-	-	-	-	1	-	6
04/11/00	24	-	-	-	1	1	3	1	-	-	20
04/20/00	17	1	-	-	1	2	8	1	-	-	43
04/30/00	20	-	-	1	-	4	3	2	-	-	33
05/10/00	20	-	-	-	-	1	3	4	2	-	33
05/21/00	14	-	-	-	2	8	4	2	-	-	53
05/30/00	11	-	1	-	1	5	7	3	2	-	63
06/13/00	12	-	-	-	3	-	6	6	3	-	60
06/20/00	3	-	-	1	-	2	4	10	9	1	90
06/30/00	5	-	-	1	3	9	4	7	1	-	83
07/10/00	8	-	-	1	3	3	12	3	-	-	73
07/20/00	3	-	-	1	-	7	9	10	-	-	90
07/30/00	1	-	2	-	2	10	11	4	-	-	96
08/10/00	1	1	-	-	2	7	11	8	-	-	96
08/20/00	1	-	1	2	2	6	9	8	1	-	96
08/30/00	10	-	1	2	2	4	6	4	1	-	66
09/10/00	3	-	-	5	-	5	13	4	-	-	90
09/20/00	4	-	-	2	6	3	11	4	-	-	86
09/29/00	13	-	-	1	4	2	7	3	-	-	56
10/11/00	14	-	-	1	5	2	7	1	-	-	53
10/21/00	12	-	-	-	1	8	5	4	-	-	60
10/30/00	13	-	-	3	2	6	6	-	-	-	56
11/10/00	6	-	2	2	2	7	8	2	1	-	80
11/18/00	15	-	1	-	3	5	3	3	-	-	50
11/29/00	18	-	2	-	3	3	2	2	-	-	40
12/08/00	13	-	-	4	1	6	3	-	2	1	56
12/18/00	13	-	1	4	-	5	5	2	-	-	56
01/02/01	21	-	-	2	5	2	1	-	-	-	30
01/09/01	15	-	-	3	2	5	3	1	1	-	50
01/18/01	24	-	-	3	-	2	-	1	-	-	20
01/28/01	24	-	-	-	2	1	2	1	-	-	20

Abbreviation: HI = hemagglutination-inhibition

<sup>a</sup>The highest serum dilution showing positive HI reaction.<sup>b</sup>26% = (30-22)/30.

Note: mode of HI titers = 320; total prevalence rate = 52%

collection for all dates and regions, would be representative of all. The selected 4 regions for serum collection are geographic representatives of the whole island. Based on these reasons, data obtained from pigs were better than those from reported human cases in investigating the epidemiology of JE.

The inclusion criteria to the pigs studied (eg, body weights <90 kg, age <6 months) and the location where they were raised were checked by a veterinarian and the slaughter-house chief, before sera were collected. Although the sources of sera were distributed in 4 different regions, the general tendencies of variations in their prevalence rates showed in general a similar trend throughout the year. On the other hand, the 4 curves were widely different from one another in terms of their prevalence rate. The prevalence rates in descending order were 70% in Miaoli, 59% in Hualien, 52% in Pingtung, and 49% Nantou. It was unexpected that Miaoli has the highest prevalence rate, and that Hualien

**Table 4.** Distribution of hemagglutination-inhibition antibody titers among adult pigs in Nantou region

Date mm/dd/yy	HI antibody titer <sup>a</sup>									HI antibody (+) %	
	<10	10	20	40	80	160	320	640	1280		2560
01/31/00	22	-	-	3	2	1	-	2	-	-	26 <sup>b</sup>
02/10/00	28	-	1	-	1	-	-	-	-	-	6
02/21/00	28	1	-	-	1	-	-	-	-	-	6
02/28/00	27	1	1	1	-	-	-	-	-	-	10
03/11/00	28	-	-	-	-	1	-	1	-	-	6
03/20/00	26	-	-	-	-	2	2	-	-	-	13
03/30/00	28	-	-	-	-	1	1	-	-	-	6
04/10/00	29	-	-	-	-	-	-	1	-	-	3
04/19/00	29	-	-	-	-	-	-	1	-	-	3
04/30/00	30	-	-	-	-	-	-	-	-	-	0
05/10/00	27	-	-	-	-	-	1	2	-	-	10
05/20/00	23	-	-	-	1	1	1	3	-	1	23
05/30/00	18	-	-	-	-	5	3	2	2	-	40
06/10/00	19	-	-	-	-	-	4	4	3	-	36
06/20/00	4	-	-	-	1	2	2	5	15	1	86
06/30/00	2	-	-	-	4	6	8	8	2	-	93
07/11/00	6	-	-	1	-	5	12	6	-	-	80
07/20/00	0	-	1	1	2	7	4	12	3	-	100
07/30/00	1	-	-	-	1	7	8	11	2	-	96
08/10/00	1	-	1	2	3	5	5	11	2	-	96
08/20/00	4	-	1	-	3	6	11	5	-	-	86
08/31/00	8	-	2	2	4	5	7	2	-	-	73
09/10/00	2	-	-	3	3	11	10	1	-	-	93
09/20/00	3	-	1	2	9	5	7	3	-	-	90
09/30/00	5	-	-	3	4	13	4	-	-	1	83
10/10/00	7	-	2	3	3	7	7	1	-	-	76
10/20/00	11	-	2	3	4	5	4	1	-	-	63
10/30/00	5	1	3	4	3	9	2	1	2	-	83
11/20/00	5	3	3	1	2	5	5	6	-	-	83
11/30/00	17	-	-	3	3	3	2	2	-	-	43
12/10/00	16	-	4	2	1	1	4	2	-	-	46
12/20/00	12	-	1	4	3	6	4	-	-	-	60
12/30/00	13	-	3	1	2	6	5	-	-	-	56
01/09/01	20	1	-	-	2	4	3	-	-	-	33
01/16/01	22	-	-	1	4	-	1	2	-	-	26
01/30/01	24	-	1	-	2	2	1	-	-	-	20

Abbreviation: HI = hemagglutination-inhibition

<sup>a</sup>The highest serum dilution showing positive HI reaction.<sup>b</sup>26% = (30-22)/30.

Note: mode of HI titers = 160; total prevalence rate = 49%

even surpassed Pingtung in prevalence. Results of this seroepidemiological surveillance may provide a practical basis for realizing the true status of JE epidemiology in Taiwan.

Although fluctuations exist, the date of 50% seroconversion tended to shift forward gradually—from July in the 1960s [11] to June in the 1970s [6], and to May in the 1980s [6], 1990 [7], 1997 [6], and 2000. Coupled with the shift in the dates of 50% seroconversion, the peak endemic months shifted forward by 1 month gradually—from July to September (peak in August) in 1960s [11] to June to August (peak in July) in the 1970s [6], and then to May to July (peak in June) in the 1980 [6], 1990 [7], 1996 [6], 1999 [12], and 2000 [13,14]. The dates of 50% seroconversion as well as the peak endemic months moved 1 month ahead of time. It is obvious that the dates of 50% seroconversion and peak endemic months in the 1980s were similar to those of the 1990s and 2000. It showed an apparent tendency of

stabilization in the past 2 decades, and will probably remain the same for the next decade.

Because the life span of virus-carrying mosquitoes was generally 1 to 2 months and almost all (70%-100%) pigs infected with JEV after the 50% seroconversion period bore anti-JEV antibody that induced viremia-free status, the number of JEV-free vector increased in great quantities. The peak endemic months appeared 1 month after the date of 50% seroconversion and extended 1 month before the appearance of JEV-free vector in massive volume, followed by remarkably diminished endemic.

From late August, living conditions such as temperature became unsuitable for the multiplication and activation of JEV, and the number of new cohort slowly decreased. The prevalence rate in pigs tended to decline until February next year, maintaining the lowest prevalence (0-<50%) until the end of April. It is therefore quite obvious that the low prevalence rate in pigs would provide virus-susceptible pigs to preserve JEV in a 2-way-like vector-pig interaction system, saving JEV from being interrupted and annihilated.

The relationships among the JEV, the vector, and pigs were so close that the existence of infected pigs in winter could indicate the presence of JEV and vector. The local over-wintering of JEV was apparently confirmed by data obtained in this study. An independent, natural surviving mechanism was developed to procreate the succeeding generation without depending on migratory birds in carrying JEV to Taiwan in spring. Because the life span of mosquitoes was only 1 to 2 months, it is suggested that virus-carrying mosquitoes were infected in winter from viremic pigs that were antibody-free. Moreover, the existence of pigs with high HI titers ( $\geq 1280$ ) in winter may indicate that they were recently infected [7,8,10] by JEV-carrying vector during the winter (from December to April). It is reasonable to predict that if there were activities of JEV-carrying vector, sporadic cases of JEV would occur. According to the criteria laid down by the National Institute of Health of Japan [10] and some other reports [6-8], there were cases that met the requirement of definite infection. Several patients were retrospectively sero-confirmed during routine specimen assay and their HI titers were as follows. In January 1987, one patient recorded HI titer of 640 on the 4th day after onset, 2560 on the 18th day, and 2560 on the 32nd day. In January 1989, HI titer of 320 was recorded in one patient on the 5th day, 1280 on the 19th day, and 320 on the 33rd day. In February 1991, one patient had HI titer of 40 on the 12th day, 640 on the 70th day, and 640 on the 87th day. In November 1987, HI titer in one patient reached 5120

on the 4th day and was 640 on the 30th day.

Review of the residential distribution of confirmed JE patients in Taiwan from 1966 through 1997 [6,7] showed that Miaoli was a more severe endemic area than the other regions. The Hualien region, though generally clean and far removed by mountains from the western and southern regions, was also endemic and would be difficult to escape the threat of JEV attacks. The more serious degree of endemic spread in these 2 regions suggested that the higher the prevalence rate, the more activity of JEV-carrying vectors.

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