行政院國家科學委員會補助專題研究計畫成果報告

# 台灣地區日本腦炎感染生態學與不同宿主的細胞之持續感染與交互作用

計畫類別：個別型計畫
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【註】 由於此生轉入其他研究室，原研究工作已轉由他人接任，致此晚交，敬請諒察。幸科學研究仍以「品質第一」及發表國際學刊為台大教授所應追求卓越的理想目標。

中 華 民 國 90 年 5 月 23 日

# 中文摘要

台灣地區的日本腦炎病毒（Japanese encephalitis virus）在初次感染人的中樞神經的吉兒癌細胞（Glioma cells），發現此細胞不但極易感染，且不如神經母細胞（neuroblastoma）在感染日本腦炎病毒後反易走向凋亡（apoptosis）。因此日本腦炎病毒是否可在吉兒癌神經細胞持續感染是一個值得探究的問題。本研究以日本腦炎疫苗株與分離自彰化的野生株感染吉兒癌細胞後，以免疫螢光抗體染色法發現兩病毒株可感染近達20%的此神經癌細胞，感染率相當高之外，且以感染指數為5的日本腦炎病毒續作對此癌細胞續代培養的感染，發現其仍可持續感染。是否此病毒在人的中樞神經系統細胞在造成持續感染後，在被感染者年紀變老時或遇其他感染時，是否易再被活化，仍是個謎。此持續感染之分子機轉為何，更是有趣。

此外進行日腦炎病毒在人致生抗體定性定量分析時，也發現：（1）其anti-E及中和抗體均隨著疫苗劑量增即增，（2）自然感染者在除含anti-E之外，另可呈現anti-NS, anti-preM兩抗體，（3）若以此anti-$NS_1$與anti-prM兩者去看，發現中和抗體效價在120以上的一些中樞神經病例即為日本腦炎患者。換言之，過去台灣以單一檢體HI效價大於160或急性與恢復期兩血清4倍抗體上升的準則來研判日本腦炎「確定」病例的實驗診斷法仍可能低估，即我們所知的流行病學形態及其因子仍有必要詳細評估。

由於本研究發現：（1）打第一劑疫苗前仍有不少日本腦炎感染者及（2）打第三劑疫苗後仍得日本腦炎之病例。再再顯示日本腦炎在台灣的感染幅度相當普遍，並未因打疫苗而消失無蹤。在未來台灣人口日漸年齡層老化趨勢，其公共衛生之威脅仍不容忽視，且不活性的日本腦炎鼠腦疫苗有待改進，必須加入非結構蛋白，以增其細胞及免疫記憶（immunologic memory）。

# Quantitative and Qualitative Differences in Antibody Kinetic Responses Induced by Japanese Encephalitis Vaccines Versus Viral Infection in Taiwan

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**Running Title:** Antibody Responses of Japanese Encephalitis Virus in Taiwan

**KEY WORDS:** Japanese encephalitis, immunization, antibody responses, natural infection, neutralization, vaccine failure

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# ABSTRACT

Japanese encephalitis (JE) virus is a member of mosquito-borne encephalitis virus with 20-46.2% chronic neurologic sequelae rate. To investigate quantitative and qualitative differences in antibody profiles induced by JE vaccine versus viral infection, their kinetic changes and associated important factors in Taiwan, a follow-up study recruited 31 confirmed and 64 probable JE cases in 1995-1996, 28 urban and suburban vaccinated babies in 1990-1995 in Taipei City and Taipei County and 37 vaccinated babies in 1990 and 1992 in Taipei City. Blood samples were collected to perform neutralization test against JEV Nakayama strain, western blot and radio-immunoprecipitation (RIP) analysis.

The geometric mean titer (GMT) of neutralization antibody (Nt Ab) among confirmed JE cases was statistically significant higher than vaccines (311.1 vs. 5.00, p=0.0001). In addition, seropositivity, mean band intensity, and qualitative analysis found that confirmed JE cases showed anti-NS1 and anti- preM antibodies, which were differentiable from probable JE cases and vaccinees, could be used in serosurvey to investigate areas of intensive JEV activities. By stratifying these two antibodies, those whose GMTs were higher than 120 should be classified as JE confirmed cases. Since more female confirmed JE cases occurred in Taiwan, gender difference also found that they manifested lower intensity of anti-E / anti-preM and lower GMT of anti-JEV Nt Ab at later acute stage but showed higher intensity of anti-NS1 and anti-NS1p at earlier convalescence stage than male confirmed JE cases.

The presence of anti-NS1, anti-E, anti-NS3 and Nt Ab before receiving the first dose of JE vaccine implied that JEV was very active in Taiwan. The levels of Nt Ab, which correlated very well with the intensity of anti-E, increased with dose response of vaccine but waned faster right before the 3rd and 4th doses in males signifying that antibody waning is a major drawback of inactivated mouse-brain JE vaccine.

We recommend that future JE vaccine in those JEV hyper-endemic and epidemic areas must elicit high levels of anti-NS1, anti-preM and anti-E antibodies before late-acute stage for better prevention of neurological symptoms.

# INTRODUCTION

Japanese encephalitis (JE), a member of mosquito borne virus, causes summer encephalitis generally, with a incidence rate ranging from 1/10000 to 1/100000 [Wu, 1999; Weekly Epidemiological Record, 1994] Case fatality rate and chronic neurologic sequelae rate of cases were about 20-40% [Halstead, 1981; Grossman, 1973] and 20-46.2%, respectively [Huy, 1994; NIPM Annual Report, 1994-1999]. The inactivated mouse brain JE vaccine produced by Nakayama strain was first developed in Japan in 1966 [Darwish, 1966]. Despite striking decrease of JE incidence rate from 2.02/10,000 in 1979 to 0.06/10,000 in 2000 in Taiwan since the mass immunization started in 1968, 167-426 suspected and 5-67 confirmed JE cases per year were documented [NIPM Annual Report, 1968-1998; Statistics of Communicable Diseases and Surveillance Report in Taiwan Area, 1998-1999; http://www.cdc.gov.tw]. The increasing incidence rate of JE in older than 40 year-old adult cases from 15.4 in 1994 to 46.15 % in 2000 plus JE cases with chronic sequelae have arisen urgent alert of family burden, social welfare concern and public health significance.

Age, immunization status, and ecological conditions are the four most important risk factors in acquiring JE viral infections [Vaughn, 1992; Uramni, 1985] The age groups of JE cases are different in various geographic areas in Asia. In fact, JE virus has usually attacked all ages when it is first introduced into virgin areas of the northern India, Nepal and Sri Lanka [Uramni, 1985]. However, 3-6 year-old children without complete immunization are more likely to be infected and might result in encephalitis in JEV highly endemic areas, whereas those 1-3 year old babies are at higher risk in most of other JE epidemic areas. In addition, countries with mass immunization program on children for several years have documented an increasing attack rate of JE in adults and elderly [Umenai, 1985; Wu, 1999].

Epidemiologic pattern of JE in Taiwan has been changed in the recent 40 years with the following five major features [NIPM Annual Report, 1968-1998; Statistics of Communicable Diseases and Surveillance Report in Taiwan Area, 1998-1999; http://www.cdc.gov.tw]. First, JE cases occurred year around rather than summer months. Second, the peak month of JE cases shifted one month earlier each decade since 1960s. Third, 15.4%, 18.5%, 19.05%, 83.33%, 27.27%, 29.17% and 46.15 % of JE confirmed cases were older than 40 years of age in 1994, 1995, 1996, 1997, 1998, 1999 and 2000, respectively. Fourth, more cases were reported in areas without pigs nor rice fields, implying that the virus is very active even in areas without past

documented maintenance animals, amplifying hosts and mosquitoes of *Culex tritaeniorhynchus* or *Culex annulus*. Fifth, about 4.76 - 15.38% of confirmed JE cases were immunized with Nakayama vaccine on schedule with at least two doses in 1994-2000 [Wu, 1999].

Six most commonly used methods to measure the effectiveness and duration of flavivirus antibodies are complement fixation test (CF), hemagglutination inhibition (HI) test, neutralization test, enzyme-linked immunosorbant assay (ELISA), western blot (WB) and radio-immunoprecipitation (RIP) [Lin, 1995]. CF test, which provides a clue for recent infection particularly in areas with several different flaviviruses, requires trained personnel and laborious procedures. HI test has been frequently applied to screened seropositive individuals in community-based seroepidemiologic investigation because its antibody persists for long periods of time. However, it has lower sensitivity and broader cross reactivity with other flaviviruses and is thus replaced by neutralization and ELISA testes in recent years [Clark, 1958]. Neutralization test, with the highest specificity and sensitivity, detects protective antibodies that neutralize infective and spreading virus [Russell, 1967; Smith, 1973; Mandel, 1984]. By contrast, ELISA test measures total antibodies [Skoura, 1999]. In addition to above quantitative methods, western blot (WB) and radio-immunoprecipitation (RIP) are suitable for detecting minor variation of antibodies against different viral proteins and the former reacts with linear epitopes whereas the latter recognizes conformational epitopes [Carcy, 1991]. Up to now, few papers have addressed the antibody profiles of different JE viral proteins together with levels of neutralization antibody and their kinetics after immunization versus natural infection in humans.

The specific aims of this study are: (1) to investigate quantitative and qualitative differences in antibody profiles induced by JE vaccine versus viral infection, (2) to compare antibodies against nonstructural and structural proteins of JE virus among patients at different days after onset as well as community residents living in different ecological settings, and (3) to find out factors affecting JEV antibody profiles for better prediction in the future.

# MATERIALS AND METHODS

## A. Research Design and Study Populations

A follow-up study of confirmed JE cases and vaccinees were performed to evaluate qualitative and quantitative differences in JE viral protein antibody profiles.

### 1. Confirmed JE Cases

We double blinded randomly selected 11 serum samples of 27 confirmed JE cases at a single time point in 1995 and another 10 human serum samples with negative neutralization antibody of JEV for pretest. The results of this pilot study found all those serum samples without JEV neutralization antibody showed no anti-JEV nonstructural protein antibody. Therefore, we further obtained another 20 paired serum samples out of total 21 confirmed JE cases in 1996 because one patient did not have enough blood at the second time point. In addition, two 1995 JE cases, who lived in Changhwa County were interviewed and followed-up after informed consent and agreement of family members to collect their serum samples at 62 and 69 days after onset of fever, respectively. Serum samples of three family members of one patient were also tested. In total, 53 serum samples of 31 confirmed JE cases in Taiwan were collected during 1993-1995.

### 2. Establishment of an Active JE Surveillance System

Because 4 confirmed JE cases were clustered in Changhua County, we established an active JE surveillance system in central part of Taiwan by collaborating with several neurologists in the major hospitals in central Taiwan since September, 1995. Suspected JE cases were defined as either persistent fever for three days or fever plus one of following symptoms, including severe headache while turning his/her head around, nausea, photophobia, meningeal signs, stupor, disorientation, coma, tremors, convulsions, spastic paralysis and other symptoms/signs of central nervous system (CNS). We finally collected one and 93 serum samples from 1-5 follow-up time points in 1995 and 1996, respectively from the Department of Neurology at the Changhua Christian Hospital. Totally, 93 serum samples from 64 suspected JE cases were tested.

### 3. JE Vaccinees:

To minimize possible natural infection of JE virus in most rural areas in Taiwan, we conducted a follow-up study from three study populations: A, B and C. (a) Population A selected 60 babies lived in 5 districts of Taipei City in 1993-1995, the

capital City of Taiwan. A total of 60 serum samples with each at 4 time points, including before the 1st, 2nd, 3rd doses and after the 3rd dose of JE vaccine Nakayama strain (n=30) and Beijing strain (n=30) were obtained from each vaccinee. (b) Population B randomly recruited 10 JE vaccine trial babies immunized at different time intervals for the 2nd dose were taken from the Department of Pediatrics at the National Taiwan University Hospital in Taipei City. (c) Population C involved 28 urban and suburban vaccinated babies in Taipei City and Taipei County and was followed-up from 1990-1995 right before their 4th dose of JE vaccine. A, B and C populations were followed for 2, 2 and 5 years, respectively. Taking out incomplete vaccination (n=26) and neutralization antibody seropositive individuals before the 1st dose (N=7), 105 serum samples in total with sufficient volume at 2-4 longitudinal time points collected from 65 remained babies were tested for qualitative antibodies by RIP and 105 for quantitative antibody assays. In other words, all samples from A and C populations were tested by western blot. For economic reason, we selected population A whose serum samples of 4 follow-ups were available or presence of JE–IgM seropositive or having clear antibody profile patterns plus B and C populations for reconfirmation by RIP.

## B. Laboratory Tests

### 1. Cell labeling and Radioimmunoprecipitation (RIP)

RIP procedures were modified from Lin et al [Lin, 1995]. Briefly, BHK-21 cells were cultured onto 6 well plates with a cell density of $0.8 \times 10^5$ cells/ml, 3 ml of cell media (RPMI + 10%FCS) were added in each well and then incubated at 37℃ with 5 % $CO_2$ overnight. The JE virus stock solution was diluted with RPMI + 2% FCS into multiplicity of infection (M.O.I.) = 10 and then infect the prepared BHK-21 cell. The prepared BHK-21 cells were washed with RPMI-$met^{(-)}$-$cys^{(-)}$ media twice and then starved with methionine/cysteine-free RPMI-1640 for 30 minutes. The starved BHK-21 cells were used to infect with JE virus Nakayama strain at M.O.I. of 10 in RPMI + 2% FCS. The infected cells were labeled with 50 microcurie of [$^{35}$S] Redivue (Amersham, England) per well for 6 hours at 37℃. The culture media were removed and washed with cold PBS twice. Cells in 6-well plate were placed on ice and 250 $\mu$l of lysis buffer(1% Nonide p-40, 50 mM Tri-HCl, 1mM EDTA, 7.5mM NaCl) containing inhibitors of phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin were added to each well. Cell lysates were collected and transferred into an eppendorf and centrifuged at 7000 rpm for 10 minutes at 4℃. The supernatant was aliquoted and stored at -70℃.

Tested samples or monoclonal antibodies against JE virus structural and nonstructural (NS) proteins were incubated with pre-aliquoted 20 $\mu$l of GammaBind Plus Sepharose (Pharmacia, USA) in each eppendorf for 1.5 hours at room temperature and then were mixed with the above centrifuged supernatant of [$^{35}$S] labelled JEV antigen prepared from BHK cells for 1.5 hr at room temperature. These immune complexes were washed with RIPA buffer (10 mM pH 7.5 Tris-HCl, 150mM NaCl, 5mM EDTA, 0.1% SDS, 1% Triton X-100 and 1% Sodium deoxycholate), added with sample buffer [50mM Tris-HCl (pH6.8), 2% SDS, 0.1% bronophenol blue, 10% glycerol and 0.9% $\beta$-mercaptenethanol] and boiled for 10 mins. All samples were run on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were then enhanced in 1M salicylate-glycerol and fluorographed at -70℃.

## 2. Plaque Reduction Neutralization Test (PRNT)

We determined the titers of neutralizing antibodies (Nt Ab) by the plaque reduction neutralization test [Russell and Nisalak, 1967]. Briefly, BHK-21 cell suspensions ($0.4 \times 10^5$ ml/well) were cultured onto a 24-well polystyrene plate (TPP, Switzerland) for 48 hours. The duplicated serum samples with 1:2 serial dilution and Nakayama strain of Japanese encephalitis virus were mixed. This mixtures were added onto the BHK-21 cell monolayers and then adsorbed at 37℃ for 1.5 hours. Then the infected or uninfected monolayers were covered with methylcellulose-MEM (Sigma, U.S.A). After 72 hours, the overlay of methylcellulose-MEM were taken away and stained the monolayer cells with 1% crystal violet-amido black mixture and the number of plaques were counted. The serotiters were read as the highest dilution of neutralizing antibody "seropositive" at cut-off point of 70% plaque reduction. Serotiter below 1:10 was read as "seronegative". Both two positive samples with Nt Ab serotiter $\geqq$ 1:1280 and 1:40~1:80 and one seronegative sample were used in each run with tested samples for quality control. Duplicated wells of each sample were tested and the mean of its PRNT titers was calculated.

## 3. Haemagglutination Inhibition Test

The HI test was followed by the Clark and Kaser's methods, 1958 [Clark, 1958]. Briefly, 25 µl of serum samples were added into the A, B and H lanes of 96 well U-bottom PS plates (Costar, U.S.A). Diluent (ie. 25 µl of 0.04% bovine albumin-borate saline (BABS), pH=9.0) were dispensed into lanes B-H. The serum mixtures were serially diluted from lane B to G and 25 µl of mixtures in lane G were removed. The haemagglutinin antigen (HA) derived form Nakayama strain of JEV was adjusted to 8 HA Units and 25 µl of them were added into lanes A-G. The wells

in lane H without JEV antigen were the "serum controls". The plates were shaken and reacted at 4℃ overnight. The HA were mixed with equal amount of BABS in another 96-well plate to serve as the "antigen control". Eighteen hours later, 50μl of 0.33% male goose fresh red blood cells (RBC) were added into lanes A-H. The mixed plates were incubated at 37℃ for one hour. The highest serum dilution showing the positive result (RBC clustered as a button) was determined as the serotiter of HI. Those wells showed RBC haemagglutination were seronegative. The same lot of HA was used for each test to increase internal reliability.

## C. Data Analysis

### 1. Determining the modified Geometric Mean Titer

The modified GMT (mGMT) of the $j^{th}$ group (such as different age groups) that human serum samples were tested were calculated as the following formula. In fact, the titer of the $i^{th}$ individual in each $j^{th}$ group was used for its neutralizing antibody titer directly ($x_{i=}$ $x_i$ ) into calculation of mGMT if the titer was between 10 to final endpoint. However, $x_i$ was assumed as 5 ($x_i$="5") if the antibody titer was less than 10. Besides, $x_i$ was assumed as $x_I$("$x_i$"= $x_i$) if the antibody titer was higher than $x_i$ ($\geqq x_i$), particularly when we screened large sample size and titrated to serotiter of 320 based on most of the serotiters of vaccinees are $\leqq$ 160 in the pilot study.

modified *Geometric Mean Titer* $(mGMT)_j = \log_{10}^{-1}\left(\frac{\sum_{i=1}^{n} n_i \log_{10} x_i}{N}\right)$

$x_i$=titer of the $i^{th}$ subgroup in each group

$i,j$=integer

$n_i$=number of the $i^{th}$ subgroup in each group

$N=\sum_{i=1}^{n} n_i$

### 2. Quality Control, Data Analysis and Statistical Methods

All tested samples were conducted in an approach of double blinded coding plus positive and negative controls simultaneously in each test. To facilitate data analysis of clinical JE cases, we divided clinical states as: (1) early-acute (0-3 days after onset

of fever), (2) late-acute (4-7 days after onset of fever), (3) convalescent stage (8-14 days after onset of fever), and (4) recovery stage (≧15 days after onset of fever).

To establish a more reasonable cut-off point for differentiating antibodies produced from natural infection versus vaccination, we pooled confirmed and probable JE cases for calculating the modified GMT (mGMT) by stratifying anti-NS1, and anit-preM status, two major qualitative differences in anti-JEV specific viral proteins.

Both univariate and multivariate statistical analyses were performed. The Logistic Regression Model was applied to assess the effect of ecological and vaccine factors within naturally infected versus immunized groups.

# RESULTS

## A. Quantitative Differences in JEV Antibody Responses among Immunization versus Natural Infection

### 1. Quantitative JEV Neutralization Antibodies Between Vaccinees and Naturally Infected Individuals

To establish a "spectrum of anti-JEV neutralization antibody mGMT", we tested both serum samples obtained from vaccinees and JE naturally infected individuals. The order of mGMT of anti-JEV neutralization antibody among immunized children (Fig. 1) from the lowest to the highest found that: (1) children before the second dose of JE vaccine ranked the lowest (8.05±5.00, n=11), (2) inapparently JE infected children before the 1st dose of JE vaccine (11.22±6.48, n=6) was the next, (3) the vaccinated children right before the third dose of JE vaccine showed slightly higher (11.49±7.47, n=5), (4) the vaccinated children right before the fourth dose of JE vaccine was still higher (13.63±16.36, n=28) than those before the 3rd dose, which had similar mGMT as JE probable cases without laboratory confirmation (13.88±6.72, n=55), (5) children at one month after the 2nd dose of JE vaccine (22.45 ± 14.27, n=6) was much higher than before the 2nd dose, and (6) vaccinated children at one month after the 3rd dose of JE vaccine had the highest GMT (74.37±35.43, n=19). The 3rd dose vaccine showed better booster anti-JEV neutralization antibody levels than after the 2nd dose. However, antibody waning was apparent at longer time intervals after vaccination no matter which dose. Therefore, the curve of neutralization antibody against JEV Nakayama strain is wave-like shape.

The mGMT of neutralization antibody against JEV Nakayama strain among confirmed JE cases (n=21) was statistically significant higher than vaccines (356.01 vs. 5.38, p=0.0001). The mGMT of unvaccinated JE cases was also significantly much lower than the JE confirmed cases with prior history of vaccination (265.78±7.64, n=28 vs.640.00±13.83, n=7, p=0.0269), the highest mGMT. From the above data, we found that the highest GMT of vaccinated children was 74.37 ±35.43 and about 64.62 % (42/65) of vaccinated children did not have anti-NS1. On the other hand, all those probable JE cases reported from physicians had similar neurologic symptoms/signs as JE (fever, drowsy, stiff neck, unconsciousness and Kerning sign ) and the second pair serum sample may not be available, it is important to figure out those people whose neutralization antibody above which level should belong to the

group of natural infection with JEV. To establish such a threshold level of anti-JEV neutralization antibody for naturally infected persons, we calculated the mGMTs for all those anti-NS1 seropositive JE cases as: (1) 125.17±2.54 (n=24) for pooled confirmed and probable cases, (2) 525.015±1.78 (n=14) for confirmed cases only, and (3) 12.12±1.79 (n=10) for probable cases only. Thus, those whose mGMT were higher than 120 should be classified into "JE confirmed cases", different from traditional quantitative definition of JE confirmed cases that was either based on serotiter over 320 for a single acute serum sample or 4-fold titer rise of HI or neutralization antibody by comparing convalescent versus acute serum samples.

Among epidemiologic factors, age and gender were strong risk factors in generating JEV antibodies. Confirmed male JE cases produced much lower overall GMT levels of neutralization antibody than female cases [283.16±7.66, n=17 vs. 337.08±9.15, n=22, p=0.3124]. Similar trend was found in 3-4 year old vaccinees because the overall GMT values in vaccinated boys were slightly lower than that of vaccinated girls [boys=71.27±12.47, n=12 vs. girls=80.00±22 77, n=7, p=0.44;].

## 2. Kinetics of Quantitative differences of Antibody Responses of Vaccination versus Natural Infection

### (a) Kinetics of mGMTs of Antibody Responses of Vaccination versus Natural Infection

#### (1) Vaccination:

Among those 65 follow-up vaccinees in total to study the kinetics of neutralization antibody, we found the mGMTs of anti-JEV neutralization antibodies among JE vaccines showed a wave-like curve with striking antibody waning after 1-year of the 2nd dose and 4-year after the 3rd dose. The mGMT were 5.59±5.68 (n=31) before the first dose, 5.95±5.00 (n=11) before the 2nd dose and then increased to 22.45±14.27 (n=6) at 1-month after the 2nd dose, then decreased to 11.49±7.47 (n=5) before the 3rd dose but became highly elevated to 74.37±35.43 (n=19) after the 3rd dose, and then strikingly decreased to 11.89±13.96 (n=28) before the 4th dose (i.e. one year after the 3rd dose). Therefore, neutralization antibody of inactivated mouse-brain JE vaccine waned very quickly but increased up to the mean value of around 75 when the 3rd booster dose was added. In general, the mGMT level increased about 3-4 times after the second dose and then elevated 3-4 times

magnitude again after the 3rd dose. Interesting findings related to gender difference found that males had slightly higher mGMT than females [males: 28.28 ± 13.63 (n=4) vs. females:14.14 ± 10.87 (n=2), p=0.76] only after primary vaccination of 2 doses. However, the level of neutralization antibody in males waned much faster than females at one year after the 2nd dose [males: 10.00 ±0.00 (n=1) vs. females: 11.89 ± 7.37(n=4)]. The difference in neutralization serotiter after the 3rd dose was even more because female kindergarten children became higher than male children at one month after the booster of the 3rd dose [males: 71.27 ± 12.47 (n=12) vs. females: 80.00 ± 22.77, p=0.44 (n= 7)] and also at right before the 4th dose in their grade one year [males: 9.60 ± 8.30 (n=17) vs. females: 16.56 ± 10.67 (n=11), p=0.09], although both groups had mGMT below 20 (Fig. 1).

Among those 65 follow-up vaccinees in total to study the kinetics of neutralization antibody, we found the mGMTs of anti-JEV neutralization antibodies among JE vaccines showed a wave-like curve with striking antibody waning after 1-year of the 2nd dose and 4-year after the 3rd dose. The mGMT were 5.59±5.68 (n=31) before the first dose, 5.95 ± 5.00 (n=11) before the 2nd dose and then increased to 22.45 ± 14.27 (n=6) at 1-month after the 2nd dose, then decreased to 11.49± 7.47 (n=5) before the 3rd dose but became highly elevated to 74.37 ± 35.43 (n=19) after the 3rd dose, and then strikingly decreased to 11.89 ± 13.96 (n=28) before the 4th dose (i.e. one year after the 3rd dose). Therefore, neutralization antibody of inactivated mouse-brain JE vaccine waned very quickly but increased up to the mean value of around 75 when the 3rd booster dose was added. In general, the mGMT level increased about 3-4 times after the second dose and then elevated 3-4 times magnitude again after the 3rd dose. Interesting findings related to gender difference found that males had slightly higher mGMT than females [males: 28.28 ± 13.63 (n=4) vs. females:14.14 ± 10.87 (n=2), p=0.76] only after primary vaccination of 2 doses. However, the level of neutralization antibody in males waned much faster than females at one year after the 2nd dose [males: 10.00 ±0.00 (n=1) vs. females: 11.89 ± 7.37(n=4)]. The difference in neutralization serotiter after the 3rd dose was even more because female kindergarten children became higher than male children at one month after the booster of the 3rd dose [males: 71.27 ± 12.47 (n=12) vs. females: 80.00 ± 22.77, p=0.44 (n= 7)] and also at right before the 4th dose in their grade one year [males: 9.60 ± 8.30 (n=17) vs. females: 16.56 ± 10.67 (n=11), p=0.09], although both groups had mGMT below 20 (Fig. 1).

## (2) Natural Infection:

To follow-up 21 confirmed and 64 probable JE cases, both neutralization (NT) and hemagglutination inhibition (HI) antibodies of confirmed cases were significantly higher than probable cases (NT: 311.1 ± 7.315, n=39, HI: 640.00 ± 8.77, n=39 ) and vaccinated children (NT: 11.53 ± 7.05, n=37, $p<0.001$). The kinetics in GMT of neutralization antibody of confirmed JE cases were 320.00 ± 5.00 (range: 320-320, n=4) in early-acute phase (0-3 days after onset of fever), and striking decrease to 84.76 ± 21.36 (range: 5-320, n=6) in late-acute phase (4-7 days after onset of fever), increasing again to 373.29 ± 10.21 (range: 160-5120, n=9) in convalescent phase (8-14 days after onset of fever) and striking increase to 435.45 ± 7.47 (range: 160-5120, n=18) in recovery phase (≧15 days after onset of fever) The neutralization antibody level along the infectious process increased almost four times, from 80 to 320. The levels of JEV neutralization antibody among confirmed JE cases at convalescent phase usually slightly lower than those at acute phase but became higher again at recovery phase. By contrast, the kinetics in mGMT of neutralization antibody of probable JE cases in early-acute, late-acute, convalescent and recovery stages remained quite low, with 12.60 ± 39.68 (range: 5-160, n=30), 20 ± 0.00, (range: 20, n=1), 5.00 ± 0.00 (range: 5-5, n=5) and 6.53 ± 6.22 (range: 5-10, n=13), respectively. (Figure 1).

Gender difference in the kinetics of anti-JEV neutralization antibody found that the its mGMT in females declined strikingly to 71.27 at late-acute stage while its mGMT in males was 100.79 even though their mGMTs in both genders were 320 at early-acute stage. Furthermore, the GMT of anti-JEV became increasing at both convalescent and recovery stage, much higher than males [convalescent stage: females = 422.24 vs. males = 320; recovery: females =485.03 vs. males =380.55].

## (b) Kinetics of Mean Band Intensity and seropositivity of Antibody Responses Against Different JE Viral Proteins Induced from Vaccination Versus Natural Infection

### (1). Vaccination

The kinetic curves of mean band intensity of anti-JEV antibody against different viral proteins among JE vaccinees were different by gender at 2-5 follow-up years [Fig.2]. The changes of four anti-JEV antibody profiles among vaccinees showed that: (1) anti-E was the most important antibody strongly correlated with dose responses

[mean intensigy: before the 1st dose = 0.61 (n= 33), one month after 2 dose = 1.73 (n= 41), one year after 2 dose = 1.13 (n= 32)]; (2) although majority of vaccinees (90.57 % and 94.34 % = 96/106 and 100/106) did not have anti-NS1 nor anti-NS1p, both antibodies waned very quickly in those anti-NS1 or anti-NS1p seropositive children at one month after 2 dose of vaccine; (3) more children showed anti-NS3 seropositive (15/106 = 14.15 %) than anti-NS1 or anti-NS1p seropositive but mean band intensity of anti-NS3 showed similar bell-shape pattern as anti-E; (4) fewest vaccinated children were anit-preM because only 4 out of 106 had anti-preM seropositive for all three time intervals.

Gender differences in mean band intensity of anti-E, anti-NS3 anti-NS3 and anti-NS1p were clearer. Although the patterns of mean band intensity of anti-NS3 and anti-E were almost the same among males versus females, male children had faster waning of anti-E and anti-NS3 at one year after 2 dose, [anti-E in males: before 1st = 0.59 (n=22), one month after 2 dose=1.81 (n=26), one year after 2 dose =0.95 (n=19) vs. anti-E in females: before 1st = 0.64 (n=11), one month after 2 dose=1.60 (n=15), one year after 2 dose = 1.38 (n=13); anti-NS3 in males: before 1st = 0.05 (n=22), one month after 2 dose=0.15 (n=26), one year after 2 dose =0.05 (n=19) vs. anti-E in females: before 1st = 0.18 (n=11), one month after 2 dose=0.47 (n=15), one year after 2 dose = 0.15 (n=13)). [Fig.2]. In addition, the band intensity of anti-NS1p remained seronegative before the 1st dose, seroconverted at one-month after 2 dose and then continuously increased over time in females but had decreasing trend in males. [Females: before 1st dose=0 (n=11), one month after 2 dose =0.13 (n=15), one year after2 dose =0.15 (n=13) vs. males: before 1st dose=0 (n=22), one month after 2 dose = 0.12 (n=26), one year after 2dose=0.05 (n=19)] [Fig. 2]. The slopes of mean band intensity of anti-NS3, anti-NS1p and anti-preM over time were much steeper in females than males. In particular, the slope of mean band intensity of anti-NS3 increased more steeply in females within one month of follow-up but its pattern reversed and began falling at one-year follow-up while males only slightly decreased. Except neutralizing antibody, anti-preM and anti-NS1p antibody, most of the trends of seropositivity rates of antibody profiles of JE vaccinees in different gender by follow-up stages were similar.

## (2) **Natural Infection**

The Mean band intensity of anti-JEV antibody against different viral proteins among confirmed JE cases varied at their different clinical stages. Firstly, the band intensity of anti-E apparently increased from acute to convalescence and then towards

recovery. Secondly, anti-NS3 was another antibody that had higher mean band instensity over 1.0 even at both early-acute (day 0 to 3) and late-acute (day 4 to 7) stages. Thirdly, anti-NS1, anti-NS1p and anti-preM antibodies remained seronegative at both early-acute and acute stages but only 55.0% (11/20) and 20.0% (4/20) of confirmed JE cases increased their band intensity during recovery stage, respectively. Furthermore, the majority of JE patients did not have these three antibodies highly elevated as anti-E or anit-NS3 at convalescent stage.

The antibody profiles against JEV were quite different among confirmed male versus female cases. Although the sample size of male is small, the band intensities of anti-E, anti-NS3, anti-NS1, anti-NS1p and anti-preM at early-acute and late-acute stages were almost the same in both genders. At convalescent stage, one male had high anti-NS3 but 3 female JE cases had elevated anti-E. By the time of recovery stage, more females decreased the band intensities of anti-E and anti-NS3 than males although the distribution data showed that female cases developed much higher anti-E band intensity at both convalescent and recovery phases than males.. On the other hand, more females increased the band intensities of anti-NS1, anti-NS1p and anti-preM. Therefore, the slopes of mean band intensity of anti-NS1 and anti-NS1p were higher in females than males. Male patients showed their anti-NS1 antibodies most at recovery phase, while most females elicited these antibody profiles starting at convalescence phase.

Although the seropositivity rate of anti-E remained constantly 100% among males and females at different disease progression stages, the kinetics of seropositivity rates of antibody against other JEV proteins in JE confirmed cases along the disease progression showed gender difference. The seropositivity rate of anti-NS3 in acute stage was 100% in both genders, but it declined faster to recovery stage in males than females at recovery stage. Both seropositivity rates of anti-NS1 and anti-NS1p antibodies increased earlier and higher in females than in males. Furthermore, the seropositivity rate of anti-preM remained 0% from clinical to recovery stages in females, while it increased continuously during different disease progression phases in males [Fig. 2].

It is very clear that JE confirmed cases showed more antibody responses pattern than vaccinees because 63.3% (19/30) of JE confirmed cases showed anti-NS1 antibody seropositive.

## B. Qualitative Differences in Antibody Responses Induced from Immunization versus Natural Infection

### 1. Competition of Anti-NS1 Antibody by Mouse Anti-NS1 Antibody Fab portion

To investigate whether human anti-NS1 and anti-NS1p were true antibodies generated from JE confirmed cases, we used mouse anti-NS1 antibody Fab portion to conduct competitive binding with JE viral NS1 protein. The results showed that the signal of anti-NS1 and anti-NS1p antibodies from serum samples of confirmed JE cases were greatly reduced to almost undetectable level. The intensity of anti-E antibody was also strikingly reduced after the competition with mouse antibody Fab portion. Thus, the two protein bands located at 46 and 51 kDa were true anti-NS1 and anti–NS1p antibodies in human serum samples [Fig. 3, Lane I, J]. Because the competition of binding of Fab protion of anti-NS1 and anti-NS1p antibodies largely reduced the band intensity, which also reduced the smear lead to the band of anti-E antibody looked thinner.

### 2. Vaccination

#### (a). Before the 1st Dose of JE Vaccine

Among 20 JEV neutralization antibody seronegative vaccinees' samples against JEV Nakayama strain, 6 of them (30%) who showed only anti-E seropositive band before first dose of JE vaccine did not have antibodies against JEV non-structural proteins (anti-NS1, anti-NS1p, and anti-NS3). Interestingly, the location of these anti-E bands before the first dose of vaccination were slightly lower than the anti-E bands generated after vaccination (Fig. 4, Lanes A-H). Moreover, 2 of these 6 anti-E seropositive babies were immunized with Nakayama strain (ID#05N14, ID#07N14, figure not shown) and the rest 4 were vaccinated with Beijing strain (ID#05B01, ID#05B05, ID#05B08, ID#05B10, figure not shown). Among these 6 babies, 5 of them (4 males, 1 female) lived in Chungjeng District of Taipei City without rice field nor pig farm and another one male baby (ID#05N14) lived in Wanhwa District of Taipei City where does not have pigs and rice fields either but was close to riverside with vegetable irritation. In other words, 14 of 20 (70%) were both qualitative and quantitative seronegative against any one of JE viral proteins and none of them had antibodies against JEV nonstructural proteins before the 1st dose of JE vaccine. Two babies, who immunized with 1 dose of JE Beijing vaccine and lived

in Chungjeng District, developed clinical symptoms (ID#05B10, ID#05B08), including fever, swelling of skin and rash, after vaccination, and one of them (ID#05B10), a 2-year-old male child was even hospitalized with severe symptoms and signs.

**(b). After the 1st Dose of JE Vaccine**

Among 22 and 15 of infants after receiving the first dose of JE Nakayama and Beijing vaccines, who were both anti-E seronegative and neutralization antibody seronegative before the 1st dose, had 36.36% (8/22) and 60% (9/15) anti-E antibody seroconversion, respectively. In addition, anti-NS1 antibody also appeared after the 1st dose of JE vaccine in 3 (ID#05B01, ID#05B05, ID#05B10) out of 6 babies who were anti-E seropositive before the 1st dose of JE vaccine. These three anti-NS1 seropositive babies were all males, immunized with Beijing vaccines only and lived in Chungjeng District of Taipei City. The intensity of anti-E showed the strongest after the 1st dose of Beijing vaccine in ID#05B10 who was hospitalized at one-month of follow-up after the 2nd dose of vaccination.

**(c). Dose Response of JE Vaccination**

The intensity of anti-E became higher after increasing the dose of Nakayama strain of JE vaccine. (Fig. 3. Lane # K-L, M-N, O-R, S-V, Fig. 4 Lane #A-B, C-D, E-F, G-H, I-J, K-L, M-N, O-P, Q-R, S-T, U-V, W-X). It manifested the highest at one month after booster of the 4th dose of JE vaccine. There was a striking dose relationship between the number of JE vaccine doses and the strength of anti-E antibody responses (Fig.1, Fig. 2, Fig. 3 Lane # K-L, M-N, O-R, S-V, Fig. 4 Lane #A-B, C-D, E-F,G-H, I-J, K-L, M-N, O-P, Q-R, S-T, U-V, W-X,)). The anti-E antibody was the predominant antibody response compared with antibodies against other JEV proteins after JE vaccination.

## 3. One Vaccine Failure Case Acquired Confirmed JE

One 12-year-old female child (ID#JE304, Fig. 3, Lane H-I), who received 3 doses of JE Nakayama vaccine but was infected with JEV, manifested with the symptoms of high fever, headache, vomiting, unconsciousness and blurred vision. Her anti-JEV antibody patterns included overwhelmed anti-E, anti-preM, anti-NS1, and anti-NS1p antibodies on the 9th day after onset (Fig.3, H lane H). Further follow-up the case, we found that she had all anti-E, anti-preM, anti-NS1, and anti-NS1p antibodies on day 62nd after onset of fever (Fig.3, H lane I) and her anti-E and

anti-preM were even much stronger than those of day 9th. Both bands of the anti-NS1 and anti-NS1p antibodies were quite light on day 9th but they became very sharp, clear and more dense on day 62nd. This JE Nakayma strain vaccinated but confirmed JE case demonstrated very clearly different antibody patterns with positive anti-preM, anti-NS1, and anti-NS1p antibodies (i.e. presence of these three antibodies), which were not found in vaccinated children.

## 4. Kinetics of Antibody Responses of Vaccination Versus Natural Infection

The earliest JE cases whose anti-NS1 seropositive on the 3rd day after onset were a 7 months-old boy and a 42 year-old man (ID#JE8500193A and JE8500298A, both without JE vaccination, figure not shown). Another two non-vaccinated 58 years-old man (ID#JE8500210A, figure not shown) and non-vaccinated 6 years-old girl (ID#JE8500120A, figure not shown) also appeared anti-NS1 seropositive but at later time on the 5th and 7th day after onset of fever. Dear Dr. King, It's very interesting that non-vaccinated persons showed anti-NS1 earlier than vaccinated which make sense because of anti-E Ab induced by vaccination will delayed virus entry and replication lead to less anti-NS1 antibody induced. The other two young adults with anti-NS1 seropositive were one 15year-old young boy (ID#JE8500286A, figure not shown) on 7th day after onset and another one 12-year-old girl (ID#JE304, Fig. 3, Lane H) who finished 3 doses of JE **Nakayama** vaccine but showed anti-NS1 seropositive on 9th day after onset. The longest persistent of anti-NS1 antibody was the 12 years-old girl (ID#JE304, Fig. 3, Lane H)) who finished 3 doses of JE vaccine and recovered quite well on 62nd days after onset. Threfore, anti-NS1 seropositive antibody of JEV started from the earliest day 3 till day 62 after the onset of fever.

The anti-NS1 and anti–NS1p did not always appear simultaneously. The intensities of anti-NS1 antibody in most JE cases were stronger than anti-NS1p antibody. The timing to present anti-NS1 seropositive was not the same as anti-NS1p either in two cases followed. For example, a 15-year-old boy (ID#JE8500286A, figure not shown) showed anti-NS1 (+) but anti-NS1p (-) antibody on the 7th days after onset and then became both anti-NS1 (+) and anti-NS1p (+) antibodies seropositive on the 55th days after onset. The other case was a 12-year-old girl whose anti-NS1 and anti–NS1p antibodies were quite light (band intensity read as level 1) at the convalescent stage on 9th day after onset but turned into much stronger (band intensity read as level 5) on the 62nd days after onset.

In summary, JE female cases showed higher, broader and earlier seroconverion of antibody responses (anti-preM, anti-NS1 and anti-NS1p antibodies) but earlier waning of anti-NS3 than those males. On the contrary, vaccinated girls showed continued increasing in anti-NS1p and levels of anti-JEV neutralization antibodies.

# DISCUSSION

This is the first paper to describe quantitative and qualitative anti-JEV antibody differences among naturally infected individuals and vaccinees. Quantitative levels of conventional neutralization and hemagglutinin inhibition antibodies can not only differentiate natural infection versus immunization but also present dose response increasing trend after vaccination. The appearance of anti-non-structural proteins (anti-NS1) and anti-preM antibodies in infected but unvaccinated individuals provides better insights about the qualitative differences of antibody profiles among different follow-up stages of confirmed cases or different doses of JE vaccine. In addition, humoral immunity induced by males varied from females in kinetics of antibody profiles against different JEV proteins among vaccinees and naturally infected persons as well.

Antibodies elicited from JEV infection are capable to neutralize extracellular virus. The kinetic study to follow confirmed JE cases by comparing both levels of neutralization antibody and various anti-JEV antibody profiles will be very helpful to understand important factors to prevent infection, neuroinvasiveness, neurologic severity, chronic sequelae and death.

Gender difference in neutralization antibody level and anti-preM, anti-NS1 anti-NS1p and anti-NS3 antibody profiles was observed in both immunization and infection. Among immmunized children, the striking difference was rapid waning of anti-NS3 in females and declined its intensity to 40% of one month after vaccination of 4 doses even just after one year. In the year of 1994, several middle aged female JE confirmed cases had severe neurologic symptoms/signs and some of them developed chronic sequelae. Therefore, comparing antibody profiles against these three proteins in both serum and cerebral spinal fluid (CSF) collected from males and females will clarify important humoral immunity factors to decrease case fatality and chronic sequelae rates of those encephalitis cases. Whether anti-NS1, anti-preM, anti-E and anti-NS3 antibody has influence from sexual hormone, which creates gender difference, needs further investigation by age-sepecfic seroepidemiologic cohort studies among young adults before and after puberty.

The modified GMT of boys (5.52) were slightly higher than the mGMT of girls (4.10) after the 2$^{nd}$ dose of JE vaccine, but the girls' mGMT (18.67) become much higher than the boys' before the 4$^{th}$ dose of vaccine (10). These results also are

consistent with our school-based follow-up survey that the immune responses of those children who were entering their adolescence were strike reversed than those lower grades students. We suggest that sex hormone might play a role in signaling the immune response, antigen presentation, memory or cell mediated immunity.

Since the appearance of anti-NS1 was strongly associated with natural infection of JEV, our detail calculation found that neutralization antibody of JEV above 120 should be naturally infected cases because our GMT values of pooled confirmed cases and probable case, confirmed cases only, and probable cases only were 125.17±2.54, 525.015±1.78, 12.12±1.79, respectively. On the other hand, the conventional criteria to judge confirmed JE cases required either neutralization titers were above 320 by single specimen or 4-fold serotiter rise or convalescent serum above 160 by HI antibody. Threfore, some JE naturally infected cases might be misclassified into JE probable case or negative ones.

The gender difference might imply the exposure to nature booster of female, occurred later and less frequently than male. Such a phenomenon could be a result of frequency of exposures, hormone, genetically immue response. The gender differences of antibody profiles and responses might be a metaphor of urgent needs in designing gender-difference vaccination schedule.

In summary, JE female cases showed higher, broader and earlier seroconverion of antibody responses (anti-preM, anti-NS1 and anti-NS1p antibodies) but earlier waning of anti-NS3 than those males. On the contrary, vaccinated girls showed continued increasing in anti-NS1p and levels of anti-JEV neutralization antibodies implied their later exposure to JEV.

Vaccination of mouse-brain prepared JE vaccine induced only anti-E antibody in those immunized infants while persons naturally infected with JEV showed two additional antibody profiles against NS1 and preM proteins. The same phenomenon were also observed in the immune responses of patients with Japanese encephalitis, U.S vaccinees and mouse animal models [Patarapotikul J et al, 1993; Falgout et al, 1991]. Although NS1 protein of JE may have other unknown function, most researchers suggested that the major function of NS1 protein would play an important role in viral replication [Muylaert, 1996], protection against following lethal challenge [Timofeev, 1998; Lin, 1998]. However, no one investigated at the differences between humoral immune responses induced by human vaccinee versus naturally infected with Japanese encephalitis virus in detail. Our results directly pointed out that the anti-NS1

and anti-preM antibodies were two major striking differences between clinical cases and vaccinees. In addition to anti-NS1 and anti-preM antibodies, the confirmed JE cases also showed strong anti-NS3 antibody, which were also found in those vaccinees. Future development of ELISA test against JEV specific B-cell epitopes on NS1 and preM will be very useful for seropidemiologic survey of natural infection of JEV in those special ecologic settings or identifying high risk populations for effective prevention.

Three major JE viral proteins, including NS1, preM and E, have been documented their important roles in protective immunity. Using synthesized recombinant dengue NS1 protein, Falgout and colleagues found that it protected mouse from death after lethal dose of JEV by i.c. challenge. The protective efficacy of anti-NS1 antibody was proven to be correlated to their ability to fix complement and then possible through complement-mediated lysis of JEV infected cells [Schlesinger et al., 1990]. Besides, recombinant preM and E also elicited higher levels of protective immunity [Konishi E, 1991]. The appearance of the anti-NS1 and anti-preM antibodies also insinuated that the possible pathogenesis mechanism of JEV. Our results had similar finding that a confirmed JE case (ID#JE304), a 12 year-old girl, who had been vaccinated with 3 doses of JE Nakayama vaccine, manifested with the symptoms of high fever, headache, vomiting, unconsciousness and blurred vision and recovered well and showed up anti-preM antibody quite early in the acute phase. On day 62$^{nd}$ after onset, she had all anti-E, anti-preM, anti-NS1, anti-NS1p and anti-NS3 antibodies even much stronger than those of day 9$^{th}$. This vaccinated but confirmed JE case demonstrated different antibody patterns with positive anti-preM, anti-NS1, and anti-NS1p antibodies, which were not seen by vaccinated children. In other words, both animal models and human confirmed JE case implied the importance of anti-NS1 and anti-preM antibodies against JEV infection [AbuBakar, 1997]. Therefore, future JE vaccine including E, NS1 and preM would be more protective against natural infection of JEV.

Because about 12.12% (4/33), 48.48% (16/33) and 9.09% (3/33) vaccinees were anti-NS1, anti-E and anti-NS3 antibody seropositive before the first dose of JE vaccine respectively, natural infection of JEV among people living in those countries with mass immunization of JE vaccine deserves further attention. Although all of these JEV seropositive children were living in Taipei City at that time but all of their residential areas were nearby the rural areas of Taipei, such as close to stream, river or farm. In fact, JEV infections in suburban areas have been documented in Viet Nam [].

Therefore, other ecologic factors ncluding domestic chickens, ducks, migrating birds, other animals and mosquitoes in those suburban areas deserve further epidemiologic investigation. After their first dose of vaccine, only 2 out of 37 showed symptoms/signs of adverse effect and one of them had been sent to the hospital. Among those who were anti-E antibody seropositive before the first dose of vaccine, 10 out of 16 were males. Such seropostive children before the first dose might due to either maternal residual antibody or natural infection. Most scientists suggest that the duration of maternal antibody prolongs for 6 months. Because our first dose of JE vaccine is scheduled at 15-month old children, it seems less likely to be maternal antibody. According to their residence nearby natural habitats of JEV, their mother would probably being naturally infected and thus their maternal antibody should persist longer than that from vaccinated mother. This longer persistence of maternal anti-NS1 might explain the rapid decline of anti-NS1 from those seropositive children before the 1st dose but became seronegaitve after the 2nd dose of vaccine. But due to the seropositivity and mean band intensity trend of anti-E, anti-NS1p and anti-NS3 antibodies showed a bell-shape curve during follow-up, the phenomenon of seropositive before vaccination would be most likely that those babies were naturally infected before their first dose of JE vaccination. Will these babies become vaccine failure ones need to be followed.

Ecological factor also plays an important role in JE infection and the humoral immune responses of vaccinees versus naturally infected indivdual. The modified GMT of vaccinees living in the urban (mGMT=3.1 after the 3rd dose in Daan) area were much lower than mGMT in the rural areas (mGMT=234.3 after the 3rd dose in Hsintien) in Taiwan. Hsintien located near the mountainous suburban area of Taipei and many residents feed pig in their house before facilitating an ecological niche for JE virus. Because the ecological conditions favoring animals in rural areas of Taiwan harboring JE virus, children in these area might be constantly boosted by natural infection and possibly they might aquire the infection even before they received the 1st dose of vaccine and produced higher neutralizing titers than those living in urban areas. In addition, the variation in quality of vaccine preservation (cold chain), evolution dynamics from wild type JE virus to vaccine strain, and the strain and the lot used in vaccine used would explain individual differences in neutralization antibody titer. The vaccine strain Daan area used were Nakayama; Tungkang and Lambay Islet both use Beijin-1 strain vaccines and Hsintien area uses either NBB (Nakayama strain for the first dose and Beijin-1 for the second and third) and BBB (Beijin-1 for all three doses). The virus stain we used for PRNT is Nakayama thus it is

less likely to be the original antigenic sin but rather constantly booster in the rural area and the preservation of vaccine would be the major factor.

Anti-NS1 of JE vaccinees showed antibody waning pattern during the follow-up process, which implies that the seropositivity before vaccination might come from maternal antibody or exposure to natural infection and become waning and the JE vaccination did not provide enough NS1 and NS1p antigen exposure to elicit sufficient amount for antigen processing and expression to elicit enough anti-NS1.

The modified GMT (mGMT) of vaccinees was increased accompanied with doses of vaccination. After the 4$^{h}$ dose of vaccination, the neutralizing antibody titers greatly boosted to 201.6 thus could provide enough protection against natural infection. According to our community survey, the geometric mean titer falls again to 26.14 which alarms us the importance of vaccination in those vaccinated adults and elderly (data not shown).

Our recommendations toward a better vaccine design is the combination of recombinant NS1, NS3 and preM protein in traditional inactivated mouse brain vaccine to induce more antibody profiles and increase immunogenicity. Adjust the dosage and route of vaccination to elicit longer immune memory and antibody duration. To set up a better gender-specific vaccination schedule for children and mass booster in those vaccinated young adults and elderly. To use multistrain vaccine against cross country infection with JE. Among those 65 follow-up vaccinees in total to study the kinetics of neutralization antibody, we found the mGMTs of anti-JEV neutralization antibodies among JE vaccines showed a wave-like curve with striking antibody waning after 1-year of the 2$^{nd}$ dose and 4-year after the 3$^{rd}$ dose. The mGMT were 5.59±5.68 (n=31) before the first dose, 5.95±5.00 (n=11) before the 2$^{nd}$ dose and then increased to 22.45 ± 14.27 (n=6) at 1-month after the 2$^{nd}$ dose, then decreased to 11.49 ± 7.47 (n=5) before the 3$^{rd}$ dose but became highly elevated to 74.37 ± 35.43 (n=19) after the 3$^{rd}$ dose, and then strikingly decreased to 11.89 ± 13.96 (n=28) before the 4th dose. Therefore, neutralization antibody of inactivated mouse-brain JE vaccine waned very quickly but increased up to the mean value of around 75 when the 3$^{rd}$ booster dose was added. In general, the mGMT level increased about 3-4 times after the second dose and then boostered 3-4 times magnitude after the 3$^{rd}$ dose. Interesting findings related to gender difference found that males had slightly higher mGMT than females [males: 28.28 ± 13.63 (n=4) vs. females: 14.14 ± 10.87 (n=2), p=0.76] after primary vaccination of 2 doses. However,

the level of neutralization antibody in males waned much faster than females at one year after the 2nd dose [males: 10.00±0.00 (n=1) vs. females: 11.89±7.37(n=4)]. Female kindergarden children became higher than male children at one month after the booster of the 3rd dose [males: 71.27±12.47 (n=12) vs. famales: 80.00±22.77, p=0.44 (n= 7)] and also at right before the 4th dose in their grade one year [males: 9.60±8.30 (n=17) vs. females: 15.56±10.67 (n=11), p=0.09], although both groups had mGMT below 20 (Fig. 2).

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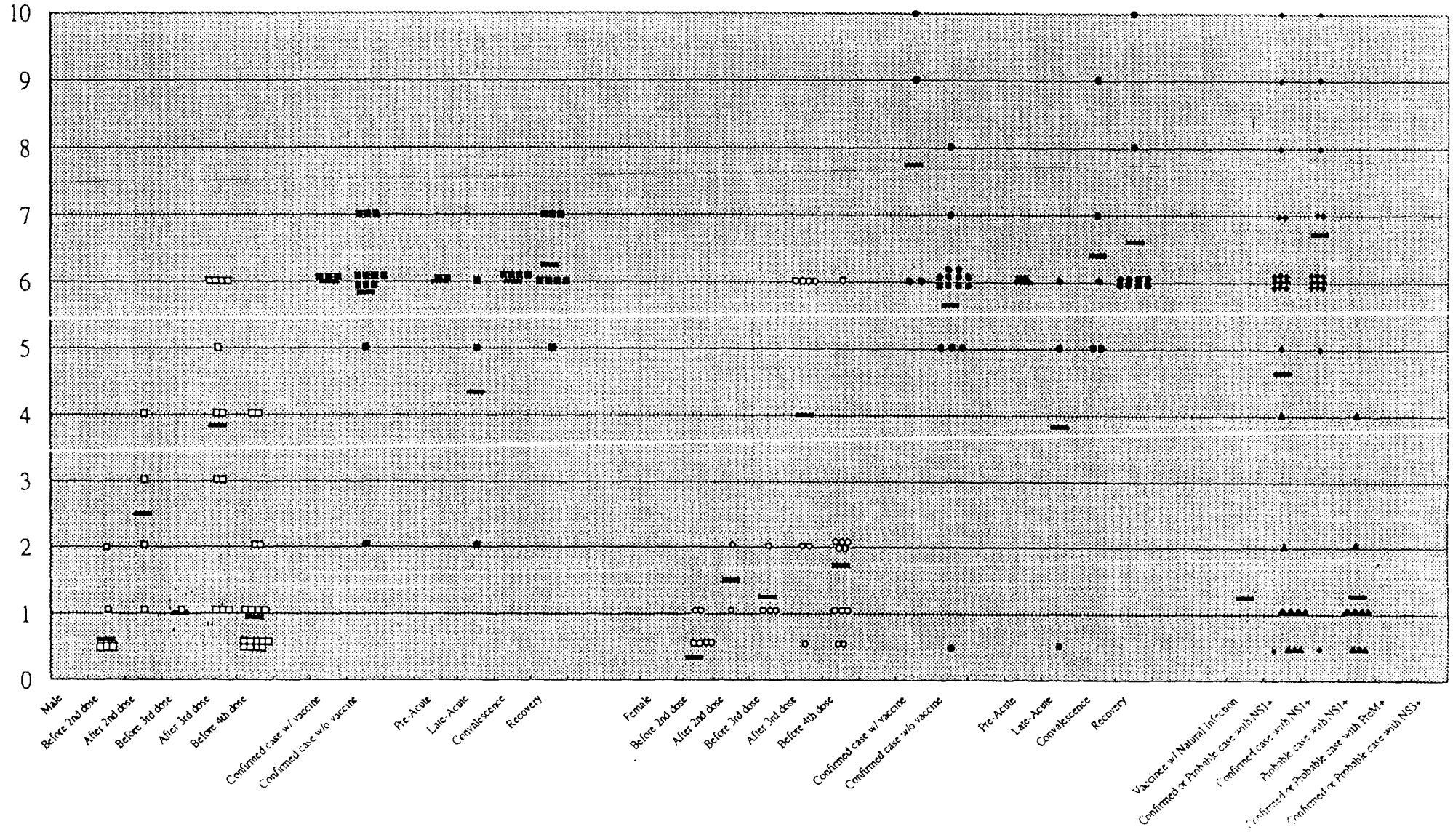
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7
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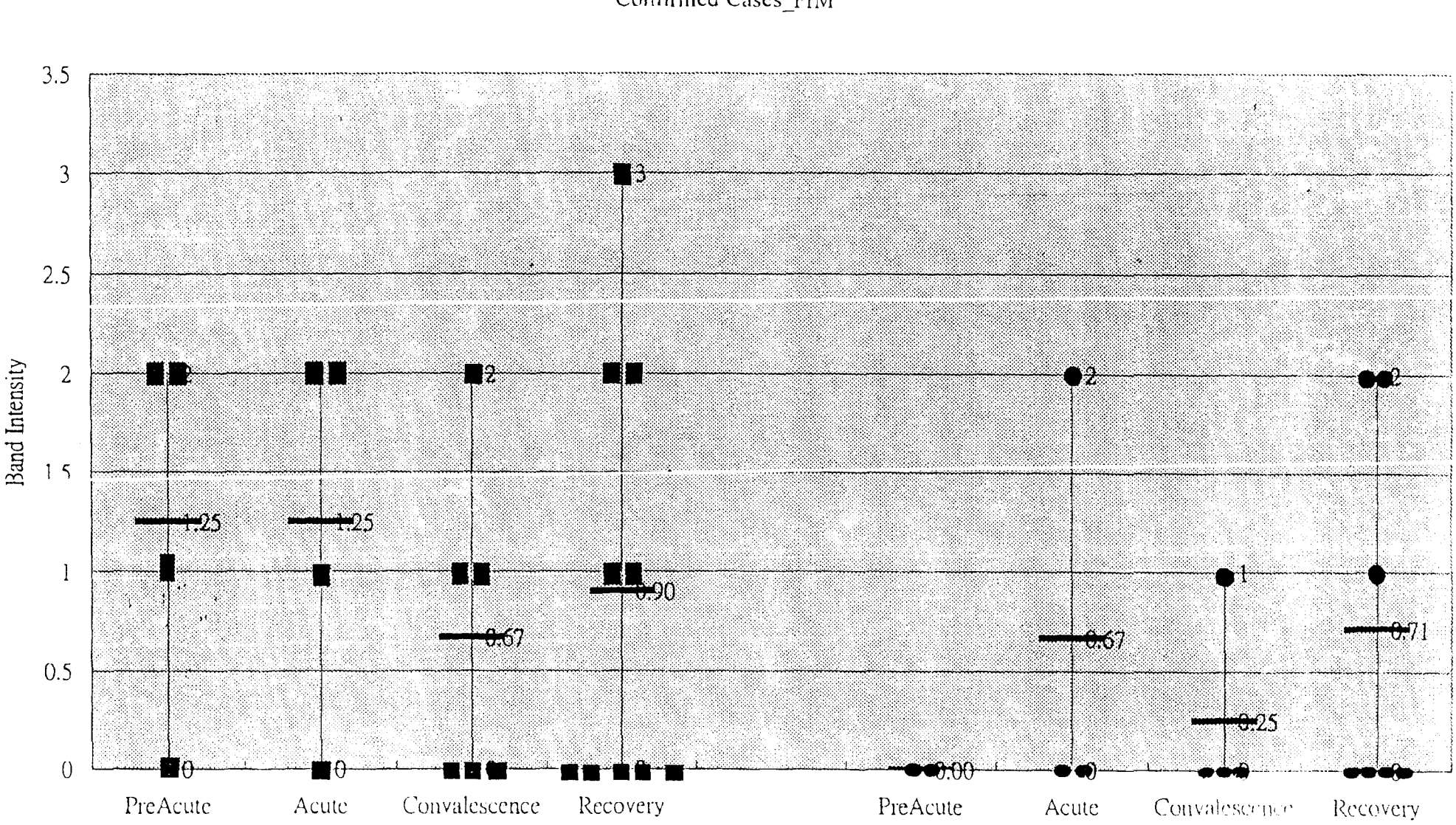
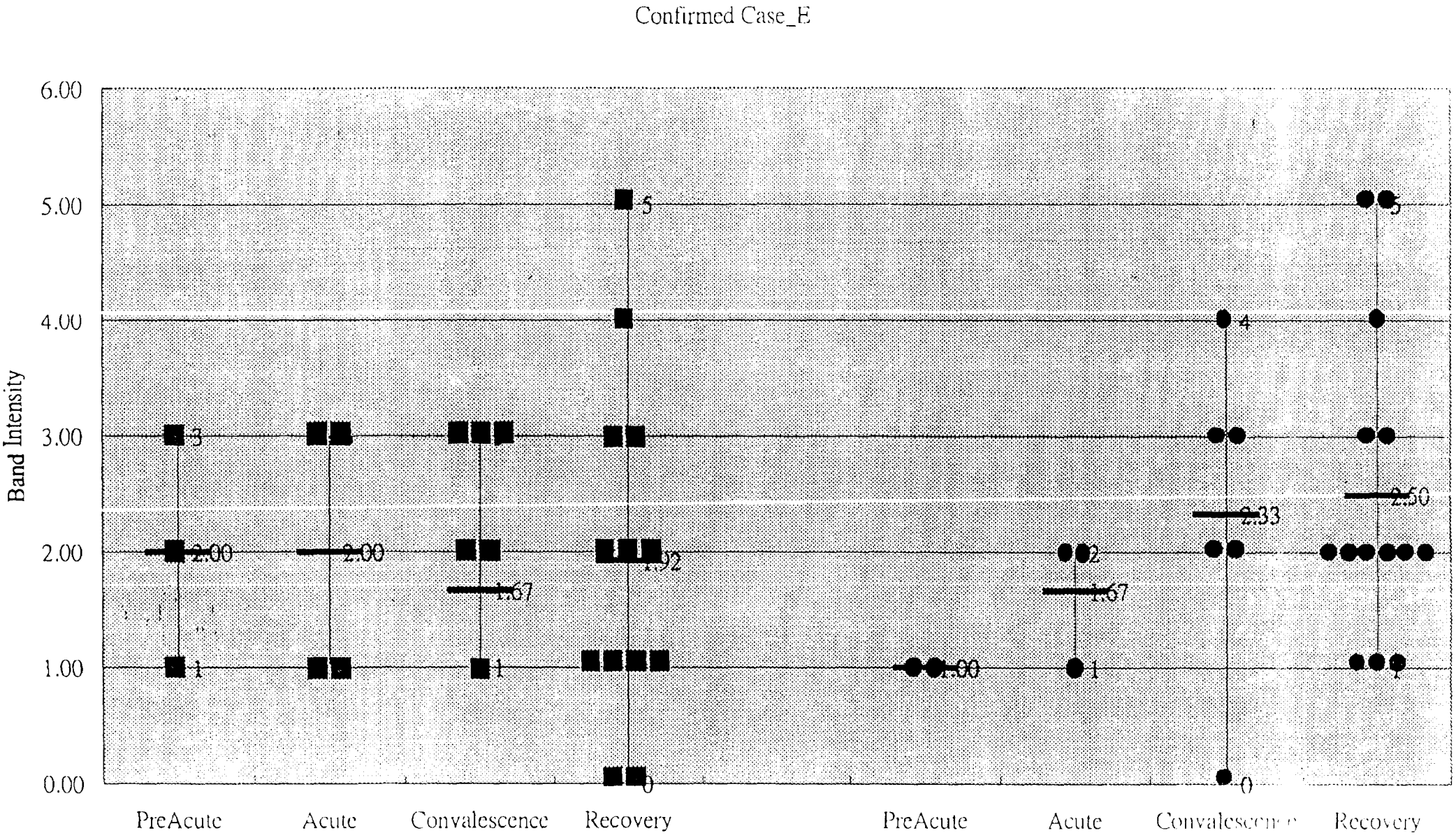
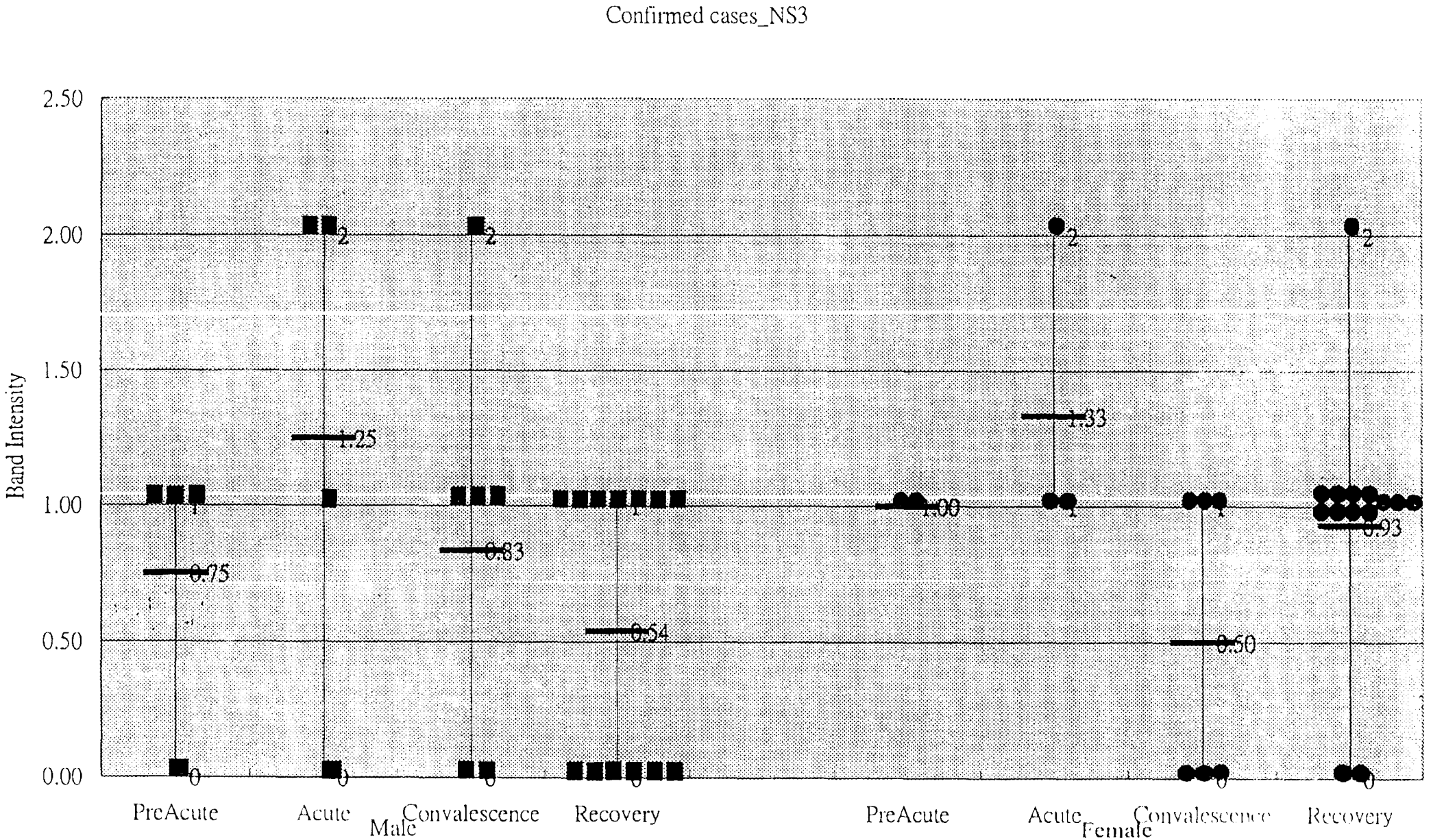
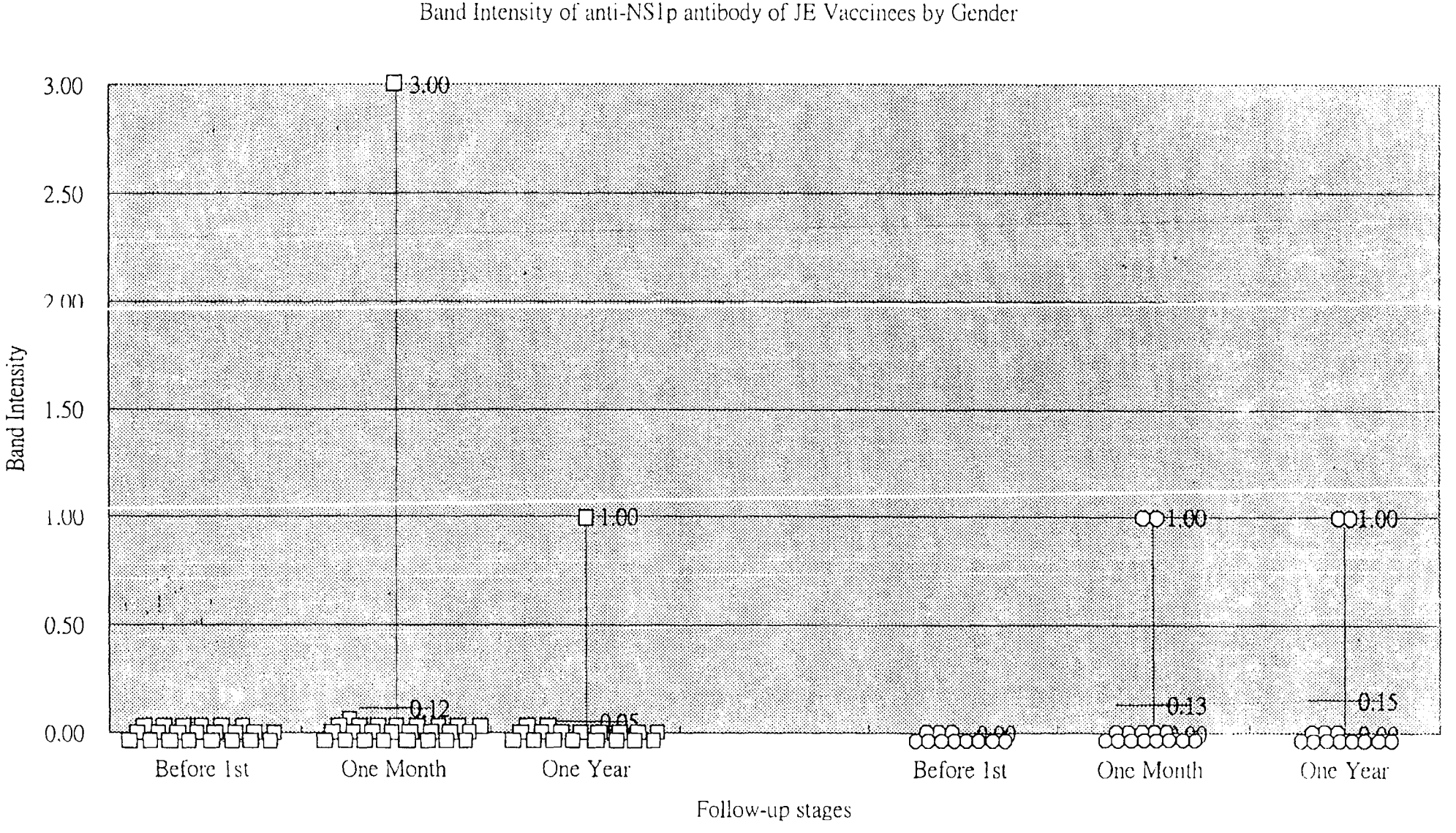
5
4
3
2
1
0
Male
Before 2nd dose
After 2nd dose
Before 3rd dose
After 3rd dose
Before 4th dose
Confirmed case w/ vaccine
Confirmed case w/o vaccine
Pre-Acute
Late-Acute
Convalescence
Recovery
Female
Before 2nd dose
After 2nd dose
Before 3rd dose
After 3rd dose
Before 4th dose
Confirmed case w/ vaccine
Confirmed case w/o vaccine
Pre-Acute
Late-Acute
Convalescence
Recovery
Vaccine w/ Natural Infection
Confirmed or Probable case with NS1+
Confirmed case with NS1+
Probable case with NS1+
Confirmed or Probable case with PreM+
Confirmed or Probable case with NS1+


Confirmed Cases_PrM
Band Intensity
3.5
3
2.5
2
1.5
1
0.5
0
1.25
1.25
0.67
0.90
0.00
0.67
0.25
0.71
PreAcute
Acute
Convalescence
Recovery
PreAcute
Acute
Convalescence
Recovery


Confirmed Case_E
Band Intensity
6.00
5.00
4.00
3.00
2.00
1.00
0.00
PreAcute
Acute
Convalescence
Recovery
PreAcute
Acute
Convalescence
Recovery
2.00
2.00
1.67
1.92
1.00
1.67
2.33
2.50


Confirmed cases_NS3
Band Intensity
2.50
2.00
1.50
1.00
0.50
0.00
2
2
2
2
1.25
1.33
1
1
1
1
1.00
0.93
0.83
0.75
0.54
0.50
0
0
0
0
0
0
PreAcute
Acute
Convalescence
Recovery
Male
PreAcute
Acute
Convalescence
Recovery
Female


Band Intensity of anti-NS1p antibody of JE Vaccinees by Gender
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Band Intensity
3.00
1.00
1.00
1.00
0.12
0.05
0.00
0.13
0.15
0.00
Before 1st
One Month
One Year
Before 1st
One Month
One Year
Follow-up stages

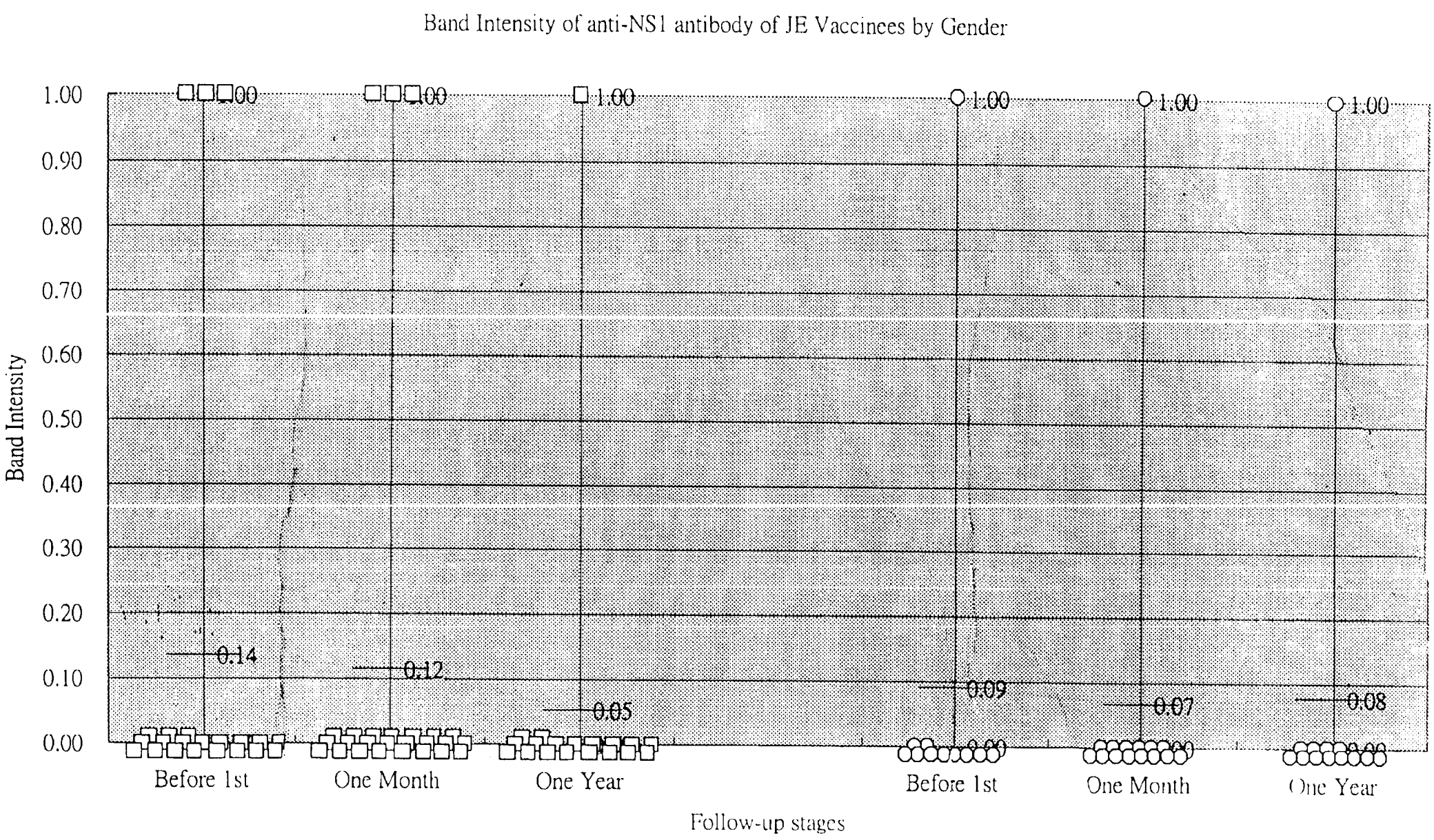

Band Intensity of anti-NS1 antibody of JE Vaccinees by Gender
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Band Intensity
1.00
1.00
1.00
1.00
1.00
1.00
0.14
0.12
0.05
0.09
0.07
0.08
0.00
0.00
0.00
Before 1st
One Month
One Year
Before 1st
One Month
One Year
Follow-up stages

Fig. 3 RIP Results of Japanese Encephalitis Confirmed Cases, IgM(+) and Babys Before and After Vaccination, Taiwan

A Anti-prM MAb
B Anti-E MAb
C Anti-NS1 MAb
D Anti-NS3 MAb
E Marker
F Anti-(prM, E, NS1, NS3) MAbs
G Anti-(prM, E, NS1, NS3) MAbs + C' Fab Fract
H NIPM JE (+) Case, day 9 (ID#JE304)
I JE (+) Case #1, day 62 (ID#JE304)
J JE (+) Case #1 + C', d62 Fab Fract (ID#JE304)
K JE Vac Baby #1 A Bef. 1st* ( ID#JEVAC鄭奕彰A )
L JE Vac Baby #1 B Aft. 2nd** ( ID#JEVAC鄭奕漳彰B )
M JE Vac Baby #2 A Bef. 1st* ( ID#JEVAC蘇秀惇A )
N JE Vac Baby #2 B Aft. 2nd** ( ID#JEVAC蘇秀婷B )
O JE Vac Baby #3 A Bef. 1st* ( ID#05N05A )
P JE Vac Baby #3 B Bef. 2nc* ( ID#05N05B )
Q JE Vac Baby #3 C Bef. 3rd* ( ID#05N05C )
R JE Vac Baby #3 D Aft. 3rd* ( ID#05N05D )
S JE Vac Baby #4 A Bef. 1st* ( ID#05N10A )
T JE Vac Baby #4 B Bef. 2nd* ( ID#05N10B )
U E Vac Baby #4 C Bef. 3rd* ( ID#05N10C )
V JE Vac Baby #4 D Aft. 3rd* ( ID#05N10D )
W JE IgM(+) children from Changhua Area
X JE IgM(+) children from Changhua Area
Y JE Nt Ab, IgM(-) adult

Fig.23 RIP Results of Japanese Encephalitis Confirmed Cases, IgM(+) and Babys Before and After Vaccination, Taiwan

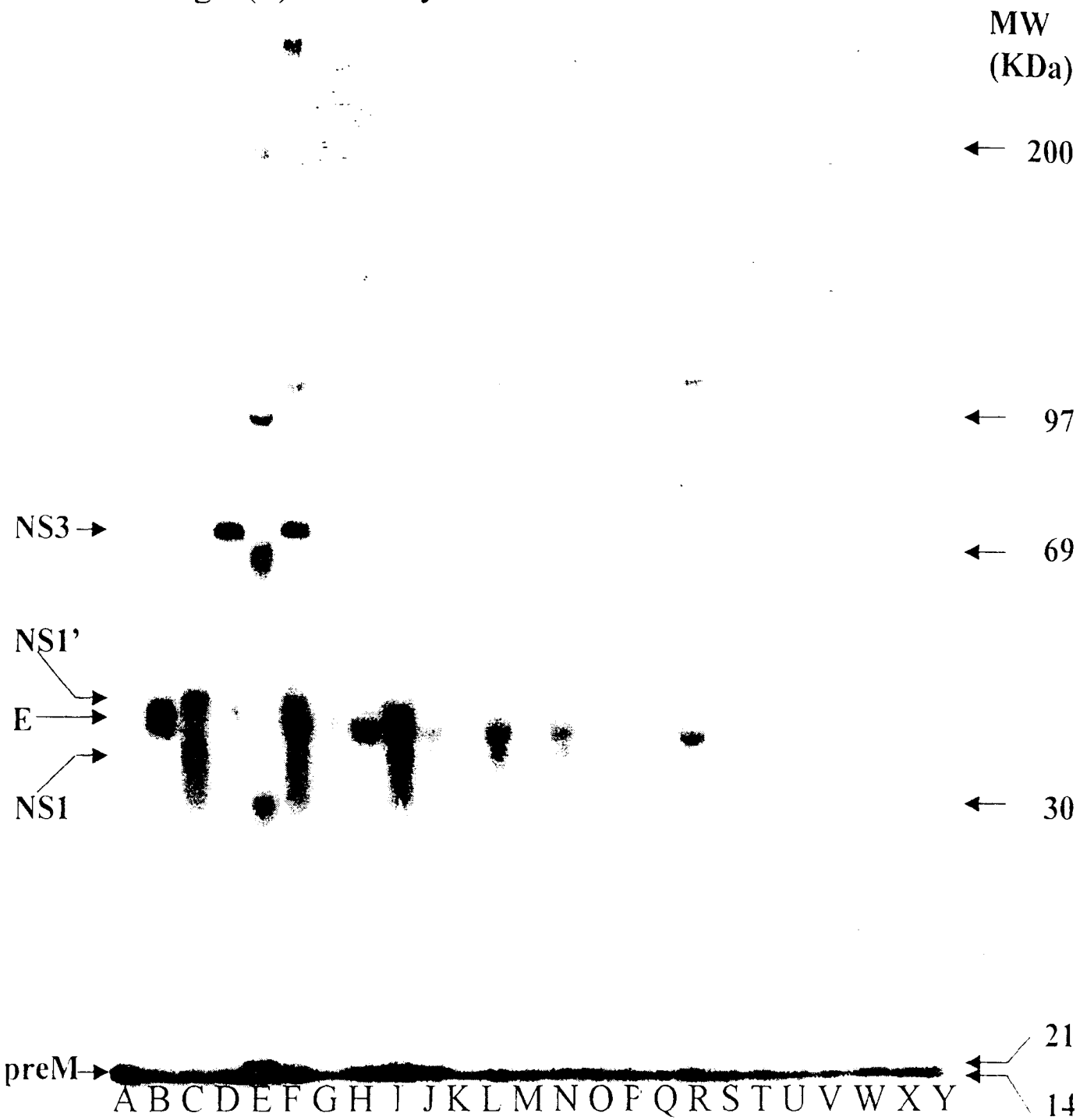


Fig.5 RIP Results of Japanese Encephalitis Confirmed Cases v.s. Babys Before and After Vaccination, Taiwan, 1995

A JE Vac Baby #1 A Bef. 1st** ( ID# JEVAC李光中A )
B JE Vac Baby #1 B Aft. 2nc** ( ID# JEVAC李光中B )
C JE Vac Baby #2 A Bef. 1st** ( ID# JEVAC羅儀萱A )
D JE Vac Baby #2 B Aft. 2nc** ( ID# JEVAC羅儀萱B )
E JE Vac Baby #3 A Bef. 1st** ( ID# JEVAC蘇豐穎A )
F JE Vac Baby #3 B Aft. 2nc** ( ID# JEVAC蘇豐穎B )
G JE Vac Baby #4 A Bef. 1st** ( ID# JEVAC楊菁蓓A )
H JE Vac Baby #4 B Aft. 2nc** ( ID# JEVAC楊菁蓓B )
I JE Vac Baby #5 A Bef. 1st** ( ID# 07N16A )
J JE Vac Baby #5 B Bef. 2nd* ( ID#07N16B )
K JE Vac Baby #5 C Bef. 3rd* ( ID#07N16C )
L JE Vac Baby #5 D Aft. 3rd* ( ID#07N 6D )
M JE Vac Baby #6 A Bef. 1st** ( ID#07N 10A )
N JE Vac Baby #6 B Bef. 2nd* ( ID#07N16B )
O JE Vac Baby #6 C Bef. 3rd* ( ID#07N 6C )
P JE Vac Baby #6 D Aft. 3rd* ( ID#07N 6D )
Q JE Vac Baby #7 A Bef. 1st** ( ID# JEVAC洪千惠A )
R JE Vac Baby #7 B Aft. 2nd** ( ID# JEVAC洪千惠B )
S JE Vac Baby #8 A Bef. 1st** ( ID# JEVAC傅伯威A )
T JE Vac Baby #8 B Aft. 2nd** ( ID# JEVAC傅伯威A )
U JE Vac Baby #9 A Bef. 1st** ( ID# JEVAC魏玲佳A )
V JE Vac Baby #9 B Aft. 2nd** ( ID# JEVAC魏玲佳B )
W JE Vac Baby #10 A Bef. 1st** ( ID# JEVAC黃光男A )
X JE Vac Baby #10 B Aft. 2nd** ( ID# JEVAC黃光男B )
Y Marker

Fig.4 RIP Results of Japanese Encephalitis Confirmed Cases v.s. Babys Before and After Vaccination, Taiwan, 1995