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The effect of vaccination with a live attenuated strain of Japanese encephalitis virus on stillbirths in swine in Taiwan

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Since an excellent candidate strain (M) for live virus vaccination of swine against Japanese encephalitis was developed, a number of large vaccination programmes have been implemented in Japan with the aim of controlling Japanese encephalitis epidemics in man by reducing the population size of nonimmune swine. Encouraging results have been obtained but no studies have been made on the benefits of live-virus vaccination to the industry. In order to determine the effect of vaccination upon the number of stillbirths occurring in the Japanese encephalitis post-emergence season, a total of 74 vaccinated, and subsequently mated, gilts at a large breeding farm in subtropical Taiwan were subjected to follow-up observations in comparison with a control group. The total incidence of litter stillbirths in the vaccinated group (1/74) was significantly lower than that in the control group (21/68). Over 92% of the newborn piglets from the vaccinated gilts were healthy, while 31.6–54.1% of the newborn piglets in the control groups were stillborn. The litter size of vaccinated gilts was larger than that of the control gilts. The vaccine was shown by tests in 22 swine to have an adequate degree of safety. Four weeks after vaccination with either $10^{5.6}$ or $10^{8.0}$ smicLD_{50} of virus, all the swine developed an adequate level of antibody. The results indicate that live virus vaccination could benefit the industry in addition to achieving the main aim of controlling Japanese encephalitis viraemia in swine.

It is generally agreed that nonimmune swine were the principal source of virus infecting vector mosquitos shortly before epidemics of Japanese encephalitis in man in Japan (Buescher & Scherer, 1959) and Taiwan (Wang et al., 1962; Hurlbut, 1963a). Buescher & Scherer (1959) designated such a virus donor as the amplifying host, then suggested that it might be possible to control the disease by vaccinating swine. In Japan (Expert Committee for Prevention of Swine Stillbirth, 1968), the immunization of swine with a killed vaccine was attempted with the aim of protecting swine against stillbirths in autumn, for which Japanese encephalitis virus infections were thought to be responsible. In Taiwan, on the other hand, vaccination of swine with killed virus was attempted in order to control the viraemia in swine caused by infection with wild Japanese encephalitis viruses (Hurlbut, 1964). In contrast to the equine immunization programme, which had been remarkably successful in Japan (Hoshi & Ito, 1951), the rate of successful vaccinations with killed virus in swine was not considered to be sufficiently high (Expert Committee for Prevention of Swine Stillbirth, 1968).

Inoue (1964) reported the isolation of an attenuated Japanese encephalitis virus strain that still retains the property of multiplication after peripheral inoculation in mice. Subsequently, Inoue and his colleagues reported (Kodama et al., 1968) that the strain multiplies in swine without causing a detect-

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able degree of viraemia or systemic disease. The antibody conversion observed in animals vaccinated with the attenuated strain was more pronounced and uniform than that produced by killed virus vaccine. These results suggested that strain M could be used to control swine viraemias resulting from infections with wild Japanese encephalitis viruses in the field.

The first field trial of strain M was carried out in 1967 on the small offshore island of Iki, west of Japan. The aim was to determine whether the elimination of susceptible swine by M-vaccination affects the ordinary transmission cycle and leads to a reduced incidence of Japanese encephalitis in man (Takahashi et al., 1968). Similar trials have been undertaken in a number of study areas in western Japan (Tsuchiya et al., 1970). None of the trials demonstrated a clear-cut difference in human morbidity between vaccinated and control areas, mainly on account of the unforeseen fall in the magnitude of Japanese encephalitis epidemics throughout the country in recent years. However, there was a consensus of opinion among investigators that vaccination of the swine population in a pre-emergence month prevents the massive infection of the population with wild Japanese encephalitis viruses that precedes human epidemics. The low level of swine viraemia resulting from vaccination has been reported to lead to a low density of infected mosquitos. An important point that has not been studied in detail is how the vaccination of swine with strain M benefits the pig-raising industry; this is of particular importance in Taiwan since the island possesses the highest swine density in East Asia. The production of swine in Taiwan is very large; 3 634 206 pigs were slaughtered in 1969 and the Government of Taiwan hopes to raise this number to 6 600 000 by 1977. On the other hand, considerable economic loss resulting from swine stillbirths has been reported by veterinary surgeons of the Taiwan Sugar Corporation, the island's largest supplier of swine. According to the statistics for 1968, the incidence of stillborn piglets from a group of young sows (gilts) mated in the months of March, April, and May were 37.5%, 42.6%, and 52.6%, respectively, whereas those for gilts mated in June and July were as low as 7.4% and 2.6%, respectively. Although little virological evidence has been provided, the seasonal occurrence and clinical picture of the stillbirths suggested a Japanese encephalitis virus etiology.

The small field trial described in this report was undertaken to determine the effectiveness of M-vaccination on the autumn stillbirth rate in a swine colony on a large breeding farm in subtropical Taiwan, and to confirm the safety of the vaccine.

MATERIALS AND METHODS

Study site

The Farm Animal Breeding Station of the Taiwan Sugar Corporation where the trial was carried out occupies 154.55 ha in Chunan Township of Miaoli County, 8 km south-west of Hsinchu City. The farm is surrounded by a vast area of wet rice paddies. The annual production of pigs in 1970 reached 35 031 head. The seasonal monitoring of swine antibody against Japanese encephalitis virus has been carried out on this farm since 1968 and the results indicate that massive infection occurs some time between the end of June and the beginning of July each year.

Vaccine

Attenuated, live strain M vaccine was supplied on two occasions by one of the authors (Y.K.I.). Both lyophilized lots, 69-1 and 70-1, were produced by the Biken Laboratories, Inc., Uji-shi, Japan, according to the original method of Inoue (1964), employing pig kidney cell culture. The lots passed the internal assay by the Biken Laboratories for bacteriological sterility, adventitious viruses, attenuation, and potency.

Swine

From a number of lines kept on the farm, Landrace hybrids were chosen in view of their increasing popularity in Taiwan. Only female animals were vaccinated. Except for the pregnant sows used to test the safety of the vaccine, all the experimental animals were 7–8 months old when they were inoculated and they were mated shortly after the vaccination.

In each year of the experiment, healthy female Landrace hybrid swine 6–7 months old were carefully selected early in March. The allocation of the animals to vaccinated and control groups was made after a preliminary test to confirm the absence of maternal antibody. Efforts were made to keep the two groups comparable. The animals in each group were identified by ear markings but all the routine observations and most of the bleedings were made without reference to the vaccination history of the animals in order to minimize the chance that bias could occur.

Cell culture for virus titration

A Y-15 line of pig kidney stable (PS) cells was selected. Routine transfers were carried out every

5 days at the Provincial Taiwan Serum and Vaccine (TSV) Laboratory with 1×10^5 cells per ml of growth medium which consisted of LA-Earle (45 parts), 199 (45 parts), and Difco calf serum (10 parts). For maintenance, LA-Earle medium supplemented with 2% calf serum was employed.

Haemagglutinating antigen

A batch of lyophilized sucrose–acetone-extracted antigen (Clarke & Casals, 1958) prepared from suckling mouse brain infected with JaGAr 01 strain virus was used. The antigen was employed in a dilution of about 1 : 1 000 at pH 6.8 to yield 8 units in haemagglutination-inhibition (HI) tests.

Titration of vaccine virus

Either PS cell culture for TCD_{50} determinations, or suckling mice inoculated intracerebrally for smic-LD_{50}s were used. In the first-year vaccination, the titration was carried out in duplicate at the TSV laboratory and the Institute for Virus Research. Since both titrations produced similar results, the potency of the vaccine in the second year was expressed in terms of the results obtained in the Institute for Virus Research only.

Test for antibody conversion

Swine sera to be tested were treated with acetone [1] in three stages, then absorbed with goose erythrocytes to remove the non-specific haemagglutinin-inhibitor and the spontaneous agglutinin against goose erythrocytes, respectively. All sera were inactivated at 56°C for 30 minutes before being tested. The HI test was performed according to the method of Clarke & Casals (1958).

Swine inoculation

In the first year (1969), the vaccine dose was $10^{5.6}$ smicLD_{50}/3ml or $10^{6.0}TCD_{50}$/3 ml while in the second year (1970) it was $10^{8.0}$smicLD_{50}/2 ml. Inoculations were given on 12 and 26 April in the first year, and on 13 April and 6 May in the second year. In both years, the final serum specimens were collected more than 1 month before the onset of massive infection of the swine on this farm with Japanese encephalitis virus.

Each year, the viraemia check was first made on animals kept in a mosquito-proof barn. A larger number of swine in open sheds were inoculated only after the viraemia check had shown negative results. All the inoculations were made subcutaneously behind the ear. The vaccinated and control gilts were kept together after vaccination and mating in order to avoid bias.

Viraemia check

In the first year, 10 gilts were bled daily after inoculation until the eighth day; the 10 gilts were also bled 4 hours after inoculation in the second year. Sera were separated within 2 hours at Chunan and were then transported to the TSV laboratory in a container cooled with dry-ice. After storage for a short period at −60°C, 0.02 ml of each serum was inoculated intracerebrally into a litter of 3–4-day-old mice. The inoculated mice were observed for 10 days.

Clinical observation of swine

Each year, 10 inoculated and 5 control gilts were observed clinically for a 3-week period starting 1 week prior to the date of inoculation. Body temperatures were read twice a day.

Follow-up observations on the occurrence of stillbirths

For the follow-up, 25 vaccinated and 21 control swine were available for observation in the first year, and 99 vaccinated and 85 control swine in the second year. However, because of unsuccessful matings before 1 July when the natural transmission of Japanese encephalitis virus became imminent on the farm, there was a substantial number of drop-outs. The numbers finally followed up to the end of October each year were: 12 vaccinated and 11 control in 1969, 62 vaccinated and 57 control in 1970.

Safety test for pregnant swine

Two young females born after the Japanese encephalitis season in 1969 were shown to be antibody-free in April 1970 and were then mated. Five weeks after the mating, 2 ml of concentrated ($\times 10$) vaccine estimated to contain $10^{9.0}$ smicLD_{50} of strain M virus were inoculated subcutaneously. A viraemia check was made each day until the sixth day. On the 16th day the animals were killed and autopsied, blood, placentas, and embryos being removed and subjected to virus isolation tests and histopathological examination or HI tests.

RESULTS

Clinical response of the vaccinated swine

Transient fever ranging from 40.2° to 41.2°C was observed during the period between the third and sixth days in 4 of the 10 animals inoculated with

[1] Guaranteed reagent (GR) grade; Japanese Industrial Standard K-8034.

$10^{5.6}$ smicLD$_{50}$ of virus. One of the 10 animals inoculated with $10^{8.0}$ smicLD$_{50}$ of virus developed a fever of over 40°C, which lasted 1 day only, on the third day of inoculation. No control animal developed fever in either of the years. Despite the fever, the animals' appetite was not impaired and the general condition of their health remained normal.

Viraemia check

Regardless of the size of the inoculum, all serum specimens collected at daily, or even at shorter, intervals following each vaccination were found to be negative.

Conversion of HI antibody

Although HI tests were repeated from time to time, the results given in Tables 1 and 2 are derived from a single simultaneous test in order to validate the comparison between different doses of the live vaccine virus. Table 1 illustrates the antibody conversion during the 4-week period shown by each of 5 gilts given $10^{5.6}$ smicLD$_{50}$ of virus in comparison with the 6 control gilts. It can be seen that no control animal converted antibody but all the vaccinated animals developed antibody with titres ranging from 1 : 10 to 1 : 40. A slight increase in antibody titre occurred between the two post-vaccination serum samples in 4 of the 5 gilts. In the 1970 trial, when a larger inoculum was employed, there was again 100% conversion with a higher rise in titre. The mean antibody titres were 1 : 53 and 1 : 80 against the vaccine

Table 1. Swine HI antibody titres before and after vaccination with $10^{5.6}$ smic LD$_{50}$ of strain (M) virus and in control animals

Animal	HI antibody titre		
	A [a]	I [b]	J [c]
vaccinated			
21	<10	40	40
24	<10	40	80
27	<10	20	40
28	<10	20	80
33	<10	10	40
control			
23	<10	—[d]	<10
25	<10	—[d]	<10
26	10	—[d]	<10
30	<10	—[d]	<10
32	<10	—[d]	<10
35	<10	—[d]	<10

[a] Pre-vaccination samples (1 April 1969).
[b] 2 weeks post-vaccination samples (26 April 1969).
[c] 4 weeks post-vaccination samples (10 May 1969).
[d] Test not carried out.

Table 2. Swine HI antibody titres before and after vaccination with $10^{8.0}$ smic LD$_{50}$ of strain (M) virus

Animal	HI antibody titre	
	A [a]	H [b]
vaccinated		
1	<10	80
4	<10	40
5	<10	40
7	<10	80
8	<10	80
10	<10	40
11	<10	160
12	<10	160
14	<10	80
15	<10	160
control		
2	<10	20
3	<10	<10
6	<10	<10
9	<10	<10
13	10	10

[a] Pre-vaccination samples (9 April 1970).
[b] 4 weeks post-vaccination samples (11 May 1970).

Table 3. Conversion rates of HI antibody and mean titres 4 weeks after vaccination for the two dosage rates of the vaccine

	Virus dose (smicLD^{50})	
	$10^{5.6}$	$10^{8.0}$
conversion rate of HI antibody (%)	5/5 (100)	10/10 (100)
mean HI antibody titre	1 : 53	1 : 80

virus doses of $10^{5.6}$ and $10^{8.0}$ smicLD_{50}, respectively, (see Table 3). On the other hand, the antibody titre in one control gilt was negative on 9 April 1970 but it subsequently rose to 1 : 20 on 11 May.

Incidence of stillbirths among vaccinated and control gilts; follow-up observations

Tables 4 and 7 show that the incidence of autumn stillbirths on the Chunan farm, particularly total litter stillbirths, is greatly reduced by vaccination with live virus. The incidence of total litter stillbirths in 1969 and 1970 in vaccinated animals was 0/12 and 1/62, respectively, while the control groups showed an incidence 30% or more each year. The smaller difference between the vaccinated and control groups in respect of normal farrowing for 1970 compared with 1969 can be attributed to the negligible difference in the incidence of partial litter stillbirths.

The fraction of healthy piglets among all piglets born in each group of gilts was 99.2% in the first year and 92.9% in the second year, while the loss of piglets was 54.1% and 31.6% in the control groups (Tables 5 and 8). It can be seen in Tables 6 and 9 that the vaccination group not only yielded a larger number of healthy piglets but the average litter size was 2–3 head larger than in the controls. The average duration of pregnancy was 2–5 days shorter in the vaccinated group than in the control group.

Table 4. Comparison of still birth incidence between vaccinated and control groups, 1969

	Vaccinated [a]	Control
number of animals pregnant before 1 July	12	11
farrowing normal litter	11 (91.7 %)	4 (36.4 %)
partial litter stillbirths	1 (8.3 %)	3 (27.3 %)
total litter stillbirths	0	4 (36.3 %)

[a] Vaccine dose = $10^{5.6}$ smic LD_{50}.

Table 5. Comparison of numbers of healthy piglets born to vaccinated and control animals, 1969

Number of piglets born	Vaccinated [a]	Control
healthy	118 (99.2 %)	34 (45.9 %)
still or alive but malformed	1 (0.8 %)	40 (54.1 %)
total	119	74

[a] Virus dose = $10^{5.6}$ smic LD_{50}.

Table 6. Comparison of litter size and duration of pregnancy in vaccinated and control groups, 1969

	Vaccinated [a]	Control
average litter size	9.91	6.93
average number of healthy piglets per litter	9.83	3.09
average length of pregnancy (days)	113.3	118.3

[a] Vaccine dose = $10^{5.6}$ smic LD_{50}.

Table 7. Comparison between incidence of stillbirths in vaccinated and control groups, 1970

	Vaccinated [a]	Control
number of animals pregnant before 1 July	62	57
farrowing normal litter	48 (77.4 %)	28 (49.1 %)
partial litter stillbirths	13 (21.0 %)	12 (21.1 %)
total litter stillbirths	1 (1.6 %)	17 (29.8 %)

[a] Vaccine dose = $10^{8.0}$ smic LD_{50}.

Table 8. Comparison of numbers of healthy piglets born to vaccinated and control animals, 1970

Number of piglets born	Vaccinated [a]	Control
healthy	522 (92.9 %)	299 (68.4 %)
still or alive but malformed	40 (7.1 %)	138 (31.6 %)
total	562	437

[a] Vaccine dose = $10^{8.0}$ smic LD_{50}

Safety of strain M virus in early pregnancy

Since pregnancy of mammals is generally accompanied by an appreciable degree of metabolic change that could modify the process of virus infection, it

Table 9. Comparison of litter size and duration of pregnancy in vaccinated and control groups, 1970

	Vaccinated [a]	Control
average litter size	9.06	7.67
average number of healthy piglets per litter	8.42	5.25
average length of pregnancy (days)	114.6	117.1

[a] Vaccine dose = $10^{8.0}$ smic LD_{50}.

was felt necessary to make sure that the vaccine virus inoculated into gilts in early pregnancy would not cause either viraemia or infection of placental or embryonic tissues that could in turn result in abnormal farrowing.

The results of the viraemia check, which was carried out with the sera collected daily from the two gilts, were completely negative; despite the large amount of vaccine virus given to the animals, they did not show any abnormal clinical response. At autopsy on on the 16th day of inoculation, one of the animals was found not to be pregnant. No virus could be recovered from blood, placenta, and embryos of the other animal, and histopathological examination of these specimens failed to reveal any pathological condition. The HI antibody titre of the pregnant gilt was found to have risen to 1 : 160.

DISCUSSION

Hurlbut (1963b, 1964) treated swine with a single dose of an inactivated vaccine and observed that in 1 out of 3 animals with maternal immunity the titre of neutralizing antibody was clearly elevated. In Japan, the Expert Committee for Prevention of Swine Stillbirth (1968) concluded that approximately 90% of the swine developed neutralizing antibody only after three 10-ml doses of an inactivated mouse-brain vaccine, the tissue concentration of which was 10%. In view of these reports, this study appears to confirm that, as reported by Kodama et al. (1968) strain M vaccine is more effective than inactivated vaccines in inducing antibody formation. Slight differences in the present trial arose from the use of a particular hybrid line of swine at a large breeding farm in subtropical Taiwan where the ecological features of Japanese encephalitis virus vary somewhat from those in Japan. In this trial, antibody with titres higher than 1 : 50 was produced in 100% of the vaccinated animals after a single inoculation of $10^{5.6}$–$10^{8.0}$ smicLD_{50} of vaccine virus. The implications of the apparent antibody conversion in one of the control animals in the second year are not yet clear, but the conversion has no connexion with the massive infection of the Taiwan Sugar Corporation swine that occurred during mid-July of that year. The observed high level of antibody production following M-vaccination suggests the possibility of completely eliminating viraemic swine from a population, provided that the timing of the vaccination is arranged to minimize unsuccessful vaccination resulting from maternal immunity.

The absence of viraemia following the inoculation of swine with a large dose of vaccine is an essential requirement. The earlier report of Kodama et al. (1968) excluded viraemia by employing 10 colostrum-deprived piglets inoculated with various doses of the strain M virus. Although no colostrum-deprived animals were used in the present trial, the high success rate strongly suggests that none of the 20 7–8-month-old animals possessed neutralizing antibody at the time of inoculation. Consequently, no viraemia was found in those animals. Viraemia was also ruled out in 2 gilts inoculated with $10^{9.0}$ smicLD_{50} of strain M virus, while the gilt in early pregnancy inoculated with $10^{9.0}$ smicLD_{50} of strain M virus showed no evidence of intrauterine growth of virus but developed a high HI titre. These results are considered to provide further assurance of the safety of strain M virus for vaccinating swine against Japanese encephalitis.

Despite many previous achievements in vaccination of swine with live attenuated Japanese encephalitis virus, no published information is available at present about the effectiveness of vaccination against swine stillbirth. This study shows, for the first time, that a single dose of strain M vaccine could almost completely eliminate stillbirths induced by Japanese encephalitis virus. The results for the first year of the trial support this view. The slightly less significant results in the second year can be interpreted as indicating the occurrence of causes other than Japanese encephalitis virus infection stillbirths in that year, taking into consideration the high vaccination success rate. For the same reason, a dose-related response, such as that observed for the antibody level, was not found in respect of the incidence of stillbirths.

Under the circumstance in which swine are the main amplifying host for Japanese encephalitis virus, reducing the population size of nonimmune swine by vaccination is a very radical approach to the

successful control of Japanese encephalitis. A difficulty exists, however, in obtaining the close co-operation of public health personnel in both medical and veterinary fields. Such cooperation would be necessary in order to obtain a high rate of vaccination coverage for swine. The excellent protection of gilts in their first pregnancy against stillbirths resulting from Japanese encephalitis virus infections by the extremely simple vaccination method described in this report should greatly facilitate such cooperation. The economic implications of using live vaccine are readily calculated for a large breeding farm. To extend the vaccination programme to meat animals that are raised on a small scale by the majority of agrarian households in this part of the world, a combined inoculation of attenuated Japanese encephalitis virus and the swine cholera vaccine that was developed by Sazawa et al. (1969) might be useful.

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RÉSUMÉ

EFFET DE LA VACCINATION PAR UNE SOUCHE VIVANTE ATTÉNUÉE DU VIRUS DE L'ENCÉPHALITE JAPONAISE SUR LA MORTINATALITÉ CHEZ LES PORCS, À TAÏWAN

On envisage depuis un certain temps de lutter contre les épidémies d'encéphalite japonaise (JE) en Asie orientale en agissant sur les réservoirs de virus. Depuis qu'on sait que le porc est un des principaux hôtes de l'infection et qu'on dispose d'une souche de virus (souche M) qui se prête à la préparation d'un vaccin vivant atténué, on a effectué plusieurs tentatives en vue de réduire le taux d'infection des vecteurs par la vaccination massive de la population porcine durant la période pré-épidémique. Des résultats encourageants ont été obtenus.

Afin d'évaluer l'effet de la vaccination sur le taux de la mortinatalité — considérée comme une conséquence de l'infection par le virus JE — chez le porc, un essai limité a eu lieu dans un élevage de Taïwan. Avant d'être accouplées, 74 jeunes truies ont été vaccinées et suivies en 1969-1970 en même temps qu'un groupe témoin de 68 truies non vaccinées. L'incidence des portées mort-nées a été de 1 pour 74 femelles pleines chez les animaux vaccinés et de 21 pour 68 chez les non-vaccinés. Au cours des deux années, les truies vaccinées ont donné naissance à 99,2 et 92,9% de porcelets bien constitués, alors que chez les animaux non vaccinés il y a eu respectivement 54,1 et 31,6% de porcelets mort-nés ou porteurs de malformations. En outre, dans le groupe vacciné, on a enregistré des portées comptant 2 à 3 têtes de plus que dans le groupe témoin.

La souche vivante atténuée a fait la preuve de son innocuité. La recherche d'une virémie dans le sérum d'un certain nombre d'animaux vaccinés a donné constamment des résultats entièrement négatifs. On a constaté l'apparition d'anticorps IH anti-virus JE chez tous les animaux immunisés. Après 4 semaines, les titres moyens étaient de 1: 53 et 1: 80 suivant l'importance de la dose vaccinale.

L'excellente protection conférée par la vaccination par le virus vivant atténué contre l'encéphalite japonaise et la mortinatalité qu'elle entraîne chez le porc justifie son application à une proportion aussi élevée que possible de la population porcine. Indépendamment de son rôle dans la réduction du réservoir de virus, cette pratique présente un intérêt économique considérable.

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