EXPERIMENTAL STUDIES ON THE SUSCEPTIBILITY OF DOMESTIC PIGS TO WEST NILE VIRUS FOLLOWED BY JAPANESE ENCEPHALITIS VIRUS INFECTION AND VICE VERSA

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Summary. – A study on the susceptibility of domestic pigs to West Nile virus (WNV) and Japanese encephalitis virus (JEV) infection was carried out. One batch of pigs was inoculated with WNV followed by JEV and another batch was inoculated vice versa. The first batch developed low level of viraemia and haemagglutination-inhibition (HI) antibodies to both viruses. There was a booster effect on the already existing WNV antibodies after challenging with JEV. In the second batch the animals developed high level of JE viraemia but did not develop WN viraemia. They developed HI antibodies to both JEV and WNV with low booster effect of WNV infection on JEV antibodies. Fresh batches of pigs were infected through bite of WNV- and JEV-infected *Culex vishnui* mosquitoes. WNV-infected pigs did not show viraemia, whereas JEV-infected ones developed JE viraemia. The study indicated that pigs were poor hosts for WNV but good ones for JEV. However, WNV antibodies reduced the level of JE viraemia and JEV infection boosted the already existing WNV antibodies.

Key words: West Nile virus; Japanese encephalitis virus; domestic pigs; viraemia; antibodies

Introduction

WNV is widely distributed throughout Africa, the Middle East, parts of Europe and former Soviet Union, India and Indonesia and is believed to be maintained in nature in a cycle involving certain bird species and mosquitoes (Work *et al.*, 1955; Taylor *et al.*, 1956; Monath, 1990). In India, though there is no report of WNV epidemics, it has been isolated from humans, bats and several species of mosquitoes (Rodrigues *et al.*, 1980; George *et al.*, 1984). Several species of birds and mammals have been shown to possess WNV antibodies (Carey *et al.*, 1968; Rodrigues *et al.*, 1981). Another mosquito-borne viral disease, which is similar in distribution to WN fever is one of the major disease of public health importance in India and in several south-east Asian countries (Buescher and Scherer, 1959; Rosen, 1986). Domestic pigs are considered to be the main source of JEV infection for the mosquitoes which transmit the disease to man (Carey *et al.*, 1969; Okumo *et al.*, 1973; Fukumi *et al.*, 1975). In India a number of JE epidemics have been reported from different parts of the country (Rodrigues *et al.*, 1984). JEV has been isolated from pigs and several species of mosquitoes (Geevarghese *et al.*, 1987*a*; Dhanda *et al.*, 1989; Mourya *et al.*, 1989).

Recent isolation of WNV from serum of a domestic pig along with several seroconversions to WNV while monitoring JEV activities in Kolar district, Karnataka state, have necessitated further studies on the role of pigs in the natural cycle of WNV (Geevarghese *et al.*, 1987*b* and unpublished data). In order to understand the involvement of pigs in the natural infection with WNV and its effect on JE, experimental viraemia studies were carried out in pigs with both WNV and JEV. In the present paper we report the results of these studies.

Materials and Methods

Experimental animals. About 10 – 20 day-old local breed pigs, obtained from Kolar and Mysore districts in Karnataka state were used. Pigs found negative for antibodies against JEV and WNV by HI test were used in the experiments. Throughout the studies the animals were held under mosquito-proof conditions.

Viruses. WNV strain 804990, originally isolated from a pool of 17F *Culex vishnui* mosquitoes collected from Kolar area in 1980

and passaged 8 to 9 times through infant mouse brain was used. JEV strain 897795 used was originally isolated from a pig serum collected at Kolar and underwent 11 to 12 passages through infant mouse brain.

Mosquitoes. Culex vishnui were obtained from the laboratory colony maintained at the Kolar/Mysore field units of the Institute. The mosquitoes were infected with WNV and JEV by the method of Rosen and Gubler (1974). The inoculated mosquitoes were incubated for 8 – 10 days before feeding on the experimental pigs. All experimental mosquitoes were held at 28 ± 1 °C and 80 – 85% relative humidity.

Infection of pigs by virus inoculation. One batch consisting of four pigs was initially inoculated intramuscularly (im) with $10^{6.7}$ LD_{50} of WNV. On day 84 p.i. the same pigs were challenged subcutaneously (sc) with $10^{5.1}$ LD_{50} of JEV. Another batch consisting of four pigs was initially inoculated sc with 10^{4} LD_{50} of JEV. These pigs were challenged im with $10^{4.2}$ LD_{50} of WNV on day 61 p.i.

Infection of pigs by mosquito bite. Culex vishnui were inoculated separately with WNV and JEV and were incubated 9 to 13 days before feeding on two fresh batches of pigs. One batch consisting of three pigs was infected by two feedings of 45 WNV-infected mosquitoes on each pig with an interval of three days between the mosquito feeds. Similarly, another batch consisting of three pigs was infected by two feedings of 30 JEV-inoculated mosquitoes on each pig with an interval of three days.

Viraemia and antibody detection. To study viraemia, the pigs were bled every day up to day 10 p.i. after inoculation with WNV and JEV, and then on different days p.i. the antibodies were assayed.

Detection of viraemia in pigs was carried out by intracerebral (ic) inoculation of the sera into Swiss suckling infant mice. The inoculated mice were observed for sickness or death every day. Virus titers were calculated according to Reed and Muench (1938). Assay of HI antibodies was performed according to the method of Clarke and Casals (1958) modified by Sever (1962) for microtiter plates. HI titers were expressed as reciprocals of serum dilutions. In the infected mosquitoes, the detection of viral antigen was carried out by the indirect fluorescent antibody (IFA) technique as described by Ilkal *et al.* (1984).

Table 1. Viraemia due to WNV infection followed by JEV infection

Pig No.	WN viraemia (virus titer)	Duration (days p.i.)	JE viraemia (virus titer)	Duration (days p.i.)
1	10^{1}	1 – 2	undetectable	0
3	undetectable	0	$\leq 10^{0.5}$**	1 – 3
4	$\leq 10^{0.6}$*	1 – 4	$\leq 10^{0.5}$**	1 – 3
5	$10^{0.3} - 10^{0.5}$	1 – 2	**	1

Virus titers expressed in LD_{50}/0.02 ml.
*Mouse mortality ratio for undiluted serum was 2/6.
**Mouse mortality ratio for undiluted serum was 3/7.

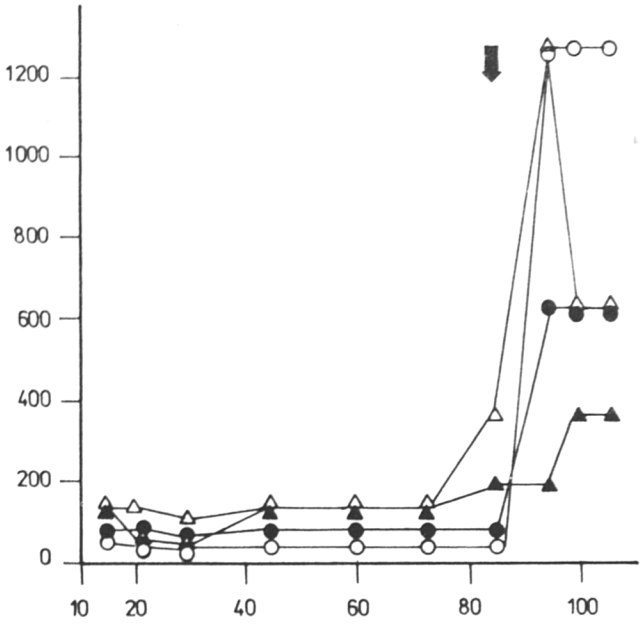

Fig. 1
WNV HI antibodies response in pigs inoculated with WNV followed by JEV
Abscissa: days p.i.; ordinate: titers of antibodies. Arrow indicates the day of challenge. Pig. No. 1 (●), No. 3 (○), No. 4 (△), No. 5 (▲).

Results

WNV infection followed by JEV challenge by inoculation

Out of four pigs inoculated with WNV, pigs No. 1, 4, 5 developed low level of WNV viraemia on days 1 – 4 p.i., whereas viraemia could not be detected in pig No. 3 (Table 1). All the four pigs developed HI antibodies to WNV with titers 20 – 320. Pigs No. 1, 4, 5 showed WN HI antibody titers 80, 320, and 160, respectively on day 84 p.i., the day on which these pigs were challenged with JEV. These were the highest WN HI antibody titers observed. The pig. No. 3 showed the highest HI antibody titer (40) on day 15 p.i.

After challenge with JEV, pigs No. 3, 4, 5 developed low level of JE viraemia on days 1 – 3 and pig. No. 1 did not develop any JE viraemia (Table 1). All the four pigs developed HI antibodies to JEV with titers 20 – 320. Pigs No. 1, 3, 4 and 5 showed JEV HI antibody titers 320, 320 ,80 and 40, respectively on day 21 post JEV infection (i.e. day 105 post WNV infection). These were the highest HI antibody titers observed. After challenge with JEV there was a rise in WNV antibody titers up to ≥ 1280 indicating a booster effect of JEV infection on the already

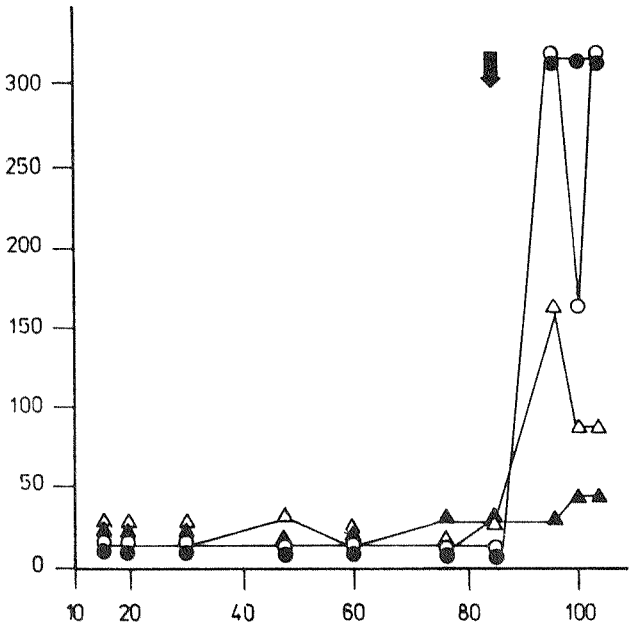

Fig. 2
JEV HI antibodies response in pigs inoculated with WNV followed by JEV
Pig. No. 1 (●), No. 3 (○), No. 4 (△), No. 5 (▲).
For the rest of legend see Fig. 1.

existing WNV antibodies. Pigs. No. 3 and 4 showed WNV HI antibody titer ≥ 1280, the remaining two pigs No. 1 and 5 showed titers 640 and 320, respectively (Figs. 1, 2).

JEV infection followed by WNV challenge by inoculation

All the four pigs No. 15, 16, 17, 18 inoculated with JEV developed JE viraemia titer ranging from $10^{0.2}$ to $10^{4.1}$ LD_{50}/0.02 ml (Table 2). In pigs No. 15, 16, 18 the viraemia period lasted 1 – 4 days p.i. The pig. No. 17 developed viraemia as late as on day 6 p.i., and continued up to day 7 p.i. The highest viraemia titer $10^{4.1}$ LD_{50}/0.02 ml was obtained in pig. No. 15 on day 3 p.i. All the four pigs developed HI antibodies to JEV with titers 10 – 320. JEV HI antibody titer 320 was obtained in pig, No. 17 which died on day 47 p.i. before challenging with WNV. Autopsy was carried out and no virus was isolated from the brain specimen. Probably the animal died due to worm infection. Pigs No. 15, 16, 18 showed JEV HI antibody titers 80, 20 and 80, rspectively on day of challenge with WNV.

On day 61 post JEV infection, the three surviving pigs. No. 15, 16, 18 were challenged with WNV. None of the pigs developed WN viraemia (Table 2). They developed WNV HI antibodies with titers 20 – 80.

All the three pigs showed WNV HI antibody titer 80 on day 21 post WNV infection (i.e. day 82 post JEV infection). There was a slight increase in JEV antibody titer indicating a low booster effect of WNV infection on JEV-infected animals (Figs. 3, 4).

WNV infection through bite of infected mosquitoes

Three pigs No. 8, 9, 11 were infected through the bite of WNV-infected *Cx. vishnui*. 45 mosquitoes were fed on each pig twice with an interval of 3 days. The number of engorged mosquitoes in both the feeds varied from 7 to 21. The fed mosquitoes were tested by IFA technique for WNV antigen. Where more than 10 mosquitoes were engorged, only 10 of them were tested. All the mosquitoes tested were found positive. No WN viraemia was found in any of the pigs when tested up to day 10 after first feed. All three pigs developed WNV HI antibodies. Pig No. 9 developed WNV antibodies from day 7 and pigs No. 8 and 11 from day 10 after the first feed. The WNV HI antibody titers ranged from 10 to 40.

JEV infection through bite of infected mosquitoes

Three pigs No. 24, 25, 26 were infected through the bite of JEV-infected *Cx. vishnui*. 30 – 35 mosquitoes were fed on each pig twice with an interval of 3 days. The number of engorged mosquitoes in both the feeds varied from 11 to 18. Ten fed mosquitoes were tested by IFA technique for JEV antigen and all of them were found positive. All the pigs

Table 2. Viraemia due to JEV infection followed by WNV infection

Pig No.	JE viraemia (virus titer)	Duration (days p.i.)	WN viraemia (virus titer)	Duration (days p.i.)
15	$10^{0.2} - 10^{4.1}$	1 – 4	undetectable	0
16	$10^{0.7} - 10^{3.0}$	1 – 3	undetectable	0
17*	$10^{0.3} - 10^{0.6}$	6 – 7	ND	0
18	$10^{0.8} - 10^{3.0}$	1 – 3	undetectable	0

Virus titer expressed in LD_{50}/0.02 ml.
*Died on day 47 post JEV infection before challenging with WNV.
ND – not done.

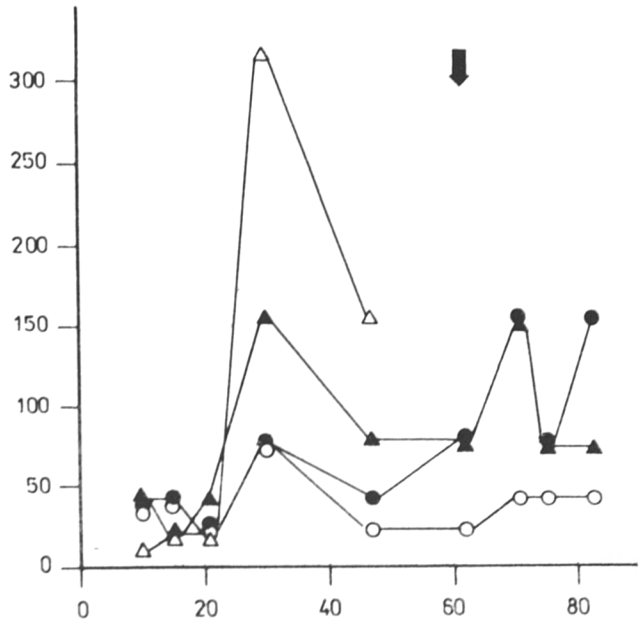

Fig. 3
JEV HI antibodies response in pigs inoculated with JEV followed by WNV
Pig. No. 15 (●), No. 16 (○), No. 17 (△), No. 18 (▲).
For the rest of legend see Fig. 1.

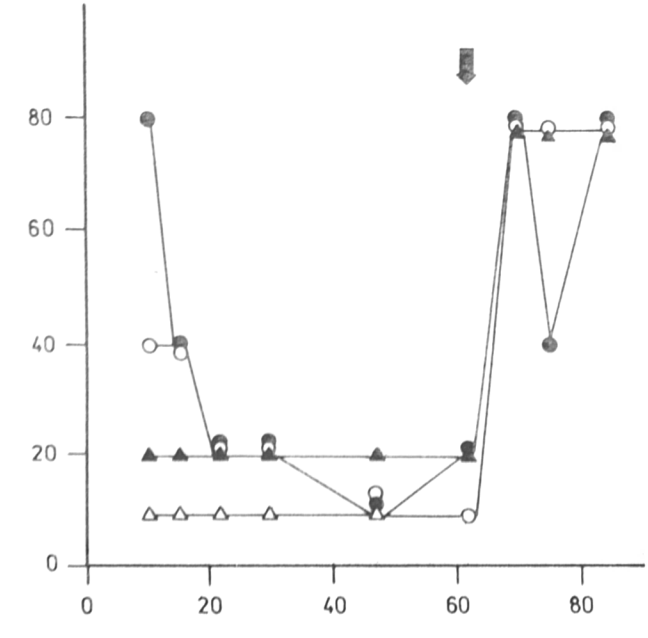

Fig. 4
WNV HI antibodies response in pigs inoculated with JEV followed by WNV
Pig. No. 15 (●), No. 16 (○), No. 17 (△), No. 18 (▲).
For the rest of legend see Fig. 1.

developed JE viraemia, titers ranging from $10^{0.5}$ to $10^{4.2}$ mouse LD_{50}/0.02 ml. Pig No. 24 developed viraemia 1 – 4 days p.i. whereas pigs No. 25 and 26 developed viraemia 1 – 5 days p.i.. All the three pigs developed JEV HI antibodies from days 7 after the first feed to the last day of bleeding, i.e. day 48 p.i. The HI antibody titers ranged between 10 to 40.

Discussion

Earlier studies carried out on cross-protection between WNV and JEV in bonnet macaque (*Macaca radiata*) by Goverdhan *et al.* (1992) have indicated that WNV immunization redusec the severity of the disease due to JEV whereas JEV immunization protects the animals against WNV. The present study showed that the pigs having WNV antibodies developed low level of JE viraemia when challenged with JEV, and the pigs having JEV antibodies did not develop WN viraemia when challenged with WNV.

Antibodies to WNV or JEV or both have been reported to be prevalent in a number of bird species and some vertebrates including pigs. (Carey *et al.*, 1968; Rodrigues *et al.*, 1981). During a study conducted at Kolar, Karnataka state for monitoring JEV and WNV activities in nature, out of 389 pigs studied 10 seroconverted solely to WNV infection and 28 to both WNV and JEV infections (Geevarghese *et al.*, 1987*b*). In the present study all the pigs developed antibodies to WNV and JEV, and at the same time the JEV infection had booster effect on already existing WNV antibodies with titers up to 1 280. This could be one of the reasons for getting high titer of WNV antibodies in pigs during JEV epidemic.

In nature, animals are subjected to multiple bites of mosquitoes. While studying bitting cycle of mosquitoes on bovine bait, Reuben (1971) has observed that on average 1 050 and 694 mosquito species of *Culex*, which are suspected to be vectors of JEV and WNV, bite a bullock and a buffalo, respectively in one night. In nature, pigs are exposed to mosquito bites and may get multiple infective bites containing both WNV and JEV which are similar in distribution and could occur in nature simultaneously. The high proportion of pigs with WN or JE antibodies in nature can therefore be explained as being due to multiple infection with WNV or JEV through mosquito bites. In our earlier serological studies on seven sentinel pigs under natural conditions, we observed that the pigs initially serocenverted either to WNV or JEV, then showed an increase in the antibody titers during subsequent epidemic or interepidemic seasons probably due to reinfection with WNV or JEV or both by multiple bites of infected mosquitoes (unpublished data).

Pigs are the most important amplifiers of JEV and constitute a major source of infection for mosquitoes (Carey *et*

al., 1969; Okumo *et al.,* 1973; Fukumi *et al.,* 1975). As far as the WNV is concerned a number of bird species has been shown to circulate the virus sufficient enough to infect the vector mosquitoes feeding on them (Work *et al.,* 1955; Taylor *et al.,* 1956). In the present experiments we found that the pigs developed high level of JE viraemia but were poor hosts for WNV infection. However, extensive studies are required to find out their role in the natural cycle of WNV.

References

Buescher, E.L., and Scherer, W.F. (1959): Ecological studies on Japanese encephalitis virus in Japan IX. Epidemiologic correlations and conclusions. *Am. J. trop. Med. Hyg.* **8**, 719–722.

Carey, D.E., Reuben, R., Myers, R.M., and George, S. (1968): Japanese encephalitis studies in Vellore, South India. Part IV. Search for virological and serological evidence of infection in animals other than man. *Indian J. med. Res.* **59**, 1340–1352.

Carey, D.E., Reuben, R., and Myers, R.M. (1969): Japanese encephalitis studies in Vellore, South India. Part V. Experimental infection and transmission. *Indian J. med. Res.* **57**, 282–289.

Clarke, D.H., and Casals, J. (1958): Technique for haemagglutination and haemagglutination inhibition with arthropod borne viruses. *Am. J. trop. Med. Hyg.* **7**, 561.

Dhanda, V., Mourya, D.T., Mishra, A.C., Ilkal, M.A., Pant, U., George Jacob, P., and Bhat, H.R. (1989): Japanese encephalitis infection in mosquitoes reared from field collected immatures and wild caught males. *Am. J. trop. Med. Hyg.* **41**, 732–736.

Fukumi, H., Hoyashi, K., Mifune, K., Schichijo, A., and Matsuo, S. (1975): Ecology of Japanese encephalitis virus in Japan. I. Mosquito and pig infection with the virus in relation to human incidences. *trop. Med.* **17**, 97–110.

Geevarghese, G., George, S., Bhat, H.R., Prasanna, Y., and Pavri, K.M. (1987*a*): Isolation of Japanese encephalitis virus from domestic sentinel pig from Kolar district in Karnataka. *Indian J. med. Res.* **86**, 273–275.

Geevarghese, G., Shaikh, B.H., George Jacob, P., Bhat, H.R., and Pavri, K.M. (1987*b*): Domestic pigs as sentinels to monitor the activity of Japanese encephalitis and West Nile viruses in Kolar district, Karnataka. *Indian J. med. Res.* **86**, 413–418.

George, S., Gouri-Devi, M., Rao, J.R., Prasad, S.R., and Pavri, K.M. (1984): Isolation of West Nile virus from the brains of children who died of encephalitis. *Bull. Wrld. Hlth. Org.* **62**, 879–888.

Goverdhan, M.K., Kulkarni, A.B., Gupta, A.K., Tupe, C.D., and Rodrigues, J.J. (1992): Two-way cross-protection between West Nile and Japanese encephalitis viruses in *Bonnet macaques. Acta virol.* **36**, 277–283.

Ilkal, M.A., Dhanda, V., Rodrigues, J.J., Mohan Rao, C.V.R., and Mourya, D. (1984): Xenodiagnosis of laboratory acquired dengue infection by mosquito inoculation and immunofluorescence. *Indian J. med. Res.* **79**, 587–590.

Monath, T.P. (1990): Flaviviruses. In Bennard, N. Fields and David M. Knipe (Ed): *Fields Virology.* Vol. 1, 2nd Ed., Raven Press, New York, pp. 763–814.

Mourya, D.T., Ilkal, M.A., Mishra, A.C., George Jacob, P., Pant, U., Romanujum, S., Mavale, M.S., Bhat, H.R., and Dhanda, V. (1989): Isolation of Japanese encephalitis virus from mosquitoes collected in Karnataka State, India from 1985 to 1987. *Tran. roy. Soc. trop. Med. Hyg.* **83**, 550–552.

Okumo, T., Mitcheu, C.J., Chen, P.S., Wang, J.S., and Lin, S.Y. (1973): Seasonal infection of *Culex* mosquitoes and swine with Japanese encephalitis virus. *Bull. Wrld. Hlth. Org.* **49**, 347–352.

Reed, C.J., and Muench, H. (1938): A simple method of estimating fifty percent end point. *Am. J. Hyg.* **27**, 493.

Reuben, R. (1971): Studies on the mosquitoes of North Arcot district Madras State, India. Part 2. Biting cycles and behaviour on human and bovine baits at two villages. *J. Med. Entomol.* **8,** 127–134.

Rodrigues, F.M. (1984): Epidemiology of Japanese encephalitis in India: A brief overview. *Proceedings of the National Conference on Japanese Encephalitis.* New Delhi, pp. 1–9.

Rodrigues, F.M., Guttikar, S.N., and Pinto, B.D. (1981): Prevalence of antibodies to Japanese encephalitis and West Nile viruses among birds in the Krishna-Godavari delta, Andhra Pradesh, India. *Trans. roy. Soc. trop. Med. Hyg.* **75**, 258–262.

Rodrigues, F.M., Bright Singh, P., Dandawate, C.N., Soman, R.S., Guttikar, S.N., and Kaul, H.N. (1980): Isolation of Japanese encephalitis and West Nile viruses from mosquitoes collected in Andhra Pradesh. *Indian J. Parasitol.* **4**, 149–153.

Rosen, L. (1986): The natural history of Japanese encephalitis. *Ann. Rev. Microbiol.* **40**, 395–414.

Rosen, L., and Gubler, D.J. (1974): The use of mosquitoes to detect and propagate dengue viruses. *Am. J. trop. Med. Hyg.* **23**, 1153–1160.

Sever, J.L. (1962): Application of microtechnique to viral serological investigations. *J. Imm.* **88**, 320–329.

Taylor, R.M., Work, T.H., Hurlbut, H.S., and Rizk, F. (1956): A study of the ecology of West Nile virus in Egypt. *Am. J. trop. Med. Hyg.* **5**, 579–620.

Work, T.H., Hurlbut, H.S., and Taylor, R.M. (1955): Indigenous wild birds of the Nile delta as potential West Nile virus circulating reservoirs. *Am. J. trop. Med. Hyg.* **4**, 872–888.