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ISOLATION OF WEST NILE VIRUS FROM *CULEX FATIGANS* MOSQUITOES FROM WESTERN INDIA.

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INTRODUCTION.

WEST Nile virus is known to be responsible for mild, non-fatal cases of dengue-like illness in humans, and the occurrence of abortive cases is not infrequent (Rivers and Horsfall, 1959). Banker (1952) was the first to detect West Nile antibodies in the sera of people residing in Bombay, a finding which was amply confirmed by Smithburn, Kerr and Gatne (1954). The latter group of workers found neutralizing antibodies to West Nile to be prevalent in Peninsular India, nearly 25 per cent of the human population under study being immune to this virus. They came to the conclusion that, even considering the serological overlap of Casals' group B arthropod-borne (arbo) viruses, West Nile infection in humans would stand second in prevalence, only to the dengue type I infection.

Several strains of West Nile virus were isolated during the period 1955-1957, from *'Culex vishnui'* and *Culex fatigans* in North Arcot District, Madras State (VRC unpublished data). Varma (1960), employing one of these strains (Tamilnad) has successfully transmitted it from chick to chick by the bites of *Culex fatigans* and *Aedes albopictus*.

Epidemiological investigations at Manjri† in Poona district, Maharashtra State, have revealed hæmagglutination-inhibiting (HI) antibodies for Japanese encephalitis (JE)-West Nile (WN) complex of viruses, not only in humans but also in a number of bird and rodent sera collected from that area. These studies also showed a large number of animals with HI antibodies for Chittoor virus, belonging to Bunyamwera group (VRC unpublished data). A project has been undertaken to isolate viruses from mosquitoes, birds, rodents and whenever possible, from humans and large domestic animals in this area.

The purpose of the present communication is to report the isolation of West Nile virus from *Culex fatigans* mosquitoes collected from Manjri.

MATERIALS AND METHODS.

Two different methods were used to collect mosquitoes :

1. *Resting collections.*—Mosquitoes, resting inside the stables of horses were caught with plastic aspirator tubes and blown into 6" × 6" mosquito-cages.

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† Manjri is an irrigated area 7 miles from Poona city. A description of the area as well as the prevalent mosquito fauna have been dealt with by Rao and Rajagopalan (1957).

2. *Animal baited trap collection.*—A stable trap designed after Bates (1949), with a young cow calf as bait was used. The calf was placed in the stable trap at dusk and removed in the morning. After the removal of the calf, mosquitoes were caught in the same manner as the resting mosquitoes.

As both these collections yielded mainly fed mosquitoes, they were held in the laboratory at 30°C. and 80 to 85 per cent relative humidity for 24 to 48 hours for digestion of blood. The mosquitoes were then identified after immobilization with ether. Similar species from both types of collections were usually pooled and stored at −50°C. before inoculation.

Each pool of mosquitoes was ground in a mortar with 2·0 ml. of 0·75 per cent bovalbumin in phosphate saline (BAPS), pH 7·2, containing 1,000 units of penicillin and 1·0 mg. of streptomycin per ml. The suspensions were centrifuged at 2,000 r.p.m. for ten minutes and the supernatant fluid was inoculated intracerebrally (ic) into two litters of 2-3 day old mice and into a group of six 3-4 week old mice. An aliquot was stored at —50°C. Passages were made from the brains of sick mice.

Preliminary serological identifications of virus isolates were made employing crude 10 per cent suspensions of infected mouse brains as antigens in a complement fixation (CF) test performed according to the method described earlier (Pavri *et al.*, 1962). Preparation of antigens and hyperimmune sera followed the methods and procedures described by Clarke and Casals (1958). The neutralization test was carried out in 2-3 day old infant mice using intraperitoneal route. Sera were not inactivated, and the serum virus mixtures were incubated at 37°C. for one hour. Each infant mouse received 0·03 ml. of the mixture. Titres were calculated according to the method of Reed and Muench. Agar gel precipitin tests were carried out according to the technique of Clarke (1962).

Nakayama strain of JE virus at passage level 50+ and E 101 strain of West Nile virus at a passage level 30+ were employed for specific identification of the isolates.

Results.

Table I gives a summary of the mosquito pools tested for virus isolation. The mosquitoes were collected between March 31, 1964 and August 31, 1964.

Out of 55 pools of *Culex fatigans* mosquitoes tested for virus, West Nile virus was isolated from two pools : one (VRC specimen No. 643009), consisting of 48 females collected on June 30th and July 7th from stables of horses, was stored for 20 days before inoculation ; the other pool (VRC specimen No. 643658-2), consisting of 50 females, collected on August 14th from stables of horses and calf baited trap, was stored for approximately two months before inoculation. The details of isolation and identification of the two isolates are given below :

VRC No. 643009.—Of the 16 infant mice inoculated with the mosquito suspension, 12 showed signs of illness by the 6th or 7th day. A passage of the brain from one of these was made and brains of three sick mice were harvested

TABLE I.

Mosquito pools tested for virus, during the period March 1964, to August 1964, collected from Manjri, Poona.

Name of species	Number of pools.	Number of mosquitoes.
Aedes (stegomyia) vittatus ...	8	22
Aedes (stegomyia) albopictus ...	2	6
Aedes (Christophersiomyia) thomsoni ..	1	1
Aedes (aedimorphus) vexans ...	4	31
Culex (culex) fatigans ...	55	2,419
Culex (culex) gelidus ...	6	58
Culex (culex) vishnui ...	13	555
Culex (culex) bitaeniorphychus ...	16	621
Anopheles subpictus ...	15	178
Anopheles vagus ...	2	4
Anopheles stephensi ...	6	105
Anopheles culicifacies ...	4	72
Anopheles theobaldi ...	2	46
Anopheles tessellatus ...	10	458
Anopheles annularis .	1	7
Anopheles karwari ...	1	1
Anopheles jamesi ...	1	1
Total ...	147	4,585

and stored at —50°C. The rest of the sick mice died by the 8th day. The incubation period was reduced to 3 days from the second passage onwards, i.e. all the mice became sick on the third day and died by the fourth day. No sickness was observed in the adult mice inoculated with the mosquito suspension.

Re-isolation was attempted 11 days after the first isolation. A slightly sick mouse, passed on the third day into 16 infant mice, produced illness in one mouse only ; the remaining mice stayed healthy throughout the observation period of 21 days. The virus seemed to be well adapted to mice from the third passage.

VRC No. 643658-2.—All the 16 inoculated infant mice were sick on the 6th day. A passage made from two, and four mouse brains was stored at —50°C. The remaining ten mice died by the 7th or 8th day. Of the six adult mice inoculated with the mosquito suspension, three became sick and died within 7 to 10 days. On the second passage of the brain of sick infant mouse, all the inoculated infant mice became ill by the third day.

Re-isolation was attempted approximately 15 days after the first isolation. Five white leghorn one-day old chicks were inoculated with 0·05 ml. of the stored mosquito suspension intramuscularly. The chicks were bled on the third day and the pooled plasma titrated in infant mice intracerebrally, yielded a titre of 1,000,000 $L.D_{50}$ per 0·02 ml.

In CF tests, crude 10 per cent suspensions of the first infant mouse passage of both the strains, reacted with the hyperimmune sera for JE and WN viruses only. There was no reaction with sera for dengue and Kyasanur Forest disease (KFD) viruses belonging to group B. Checkerboard CF tests were performed using acetone

ether extracted antigens and the respective hyperimmune sera employing mouse brains at the second or third passage level. Results presented in Table II indicate both the strains VRC Nos. 643009 and 643658-2 to be identical to West Nile virus.

TABLE II.

Results of checkerboard CF test of the isolates 643009 and 643658-2 with JE and WN antigens.

Antigens.	HYPERIMMUNE SERA :			
	JE.	WN.	643009	643658-2
JE ...	256/<128*	64/64	16/32	16/32
WN ...	32/64	>256/>128	64/>128	128/>128
643009 ...	16/16	>256/64	64/32	128/64
643658-2 ...	16/16	>256/64	64/64	128/64

* Numerator — The highest titre of the serum.
Denominator — The highest titre of the antigen.

Similar results were obtained in neutralization test, which was carried out employing serial ten-fold dilutions of 643658-2, WN and JE viruses and their homologous hyperimmune sera. The 643658-2 and the WN sera neutralized both, 643658-2 and WN viruses completely : approximately, 10,000,000 (643658-2) and >100,000 (WN) infant mouse LD_{50} per 0·03 ml. The JE serum, which neutralized over 10,000,000 infant mouse LD_{50} per 0·03 ml. of the homologous virus, was able to neutralize only 1,000 LD_{50} of both 643658-2 and WN viruses.

Further confirmation of the identity was obtained by the agar gel-precipitin test, wherein the precipitin reaction was observed only with WN virus and not with JE.

DISCUSSION.

The finding of West Nile virus in *Culex fatigans*, an ubiquitous species of mosquito known to bite humans frequently, brings out the need for investigations on the role of this virus in the production of human illness in Western India. Continued studies on the natural history of the virus are also indicated.

SUMMARY.

West Nile virus has been isolated from two pools of *Culex fatigans* mosquitoes collected from Manjri, in Poona district, Maharashtra.

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