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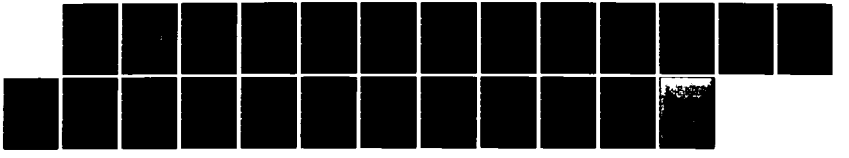

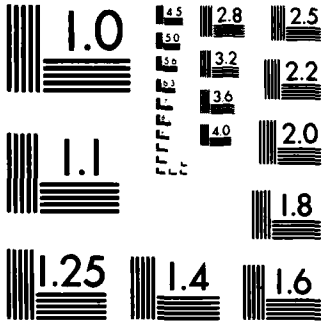

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Pathogenesis of dengue vaccine viruses in mosquitoes

First Annual Report

Barry J. Beaty, Ph.D.
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January 1, 1980

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An in vitro assay was developed to detect transmission of Dengue-2 parent and vaccine virus strains by vector mosquitoes. After intrathoracic inoculation, transmission of both viruses was detected at 7 and 14 days incubation. Rates of transmission were similar.

A mosquito inoculation-headsquash-immunofluorescence technique was found to be more sensitive than Aedes albopictus cells(C6/36) for the isolation of dengue virus in the in vitro transmission assay. Flavivirus viremias in suckling mice proved relatively noninfectious for Aedes aegypti.

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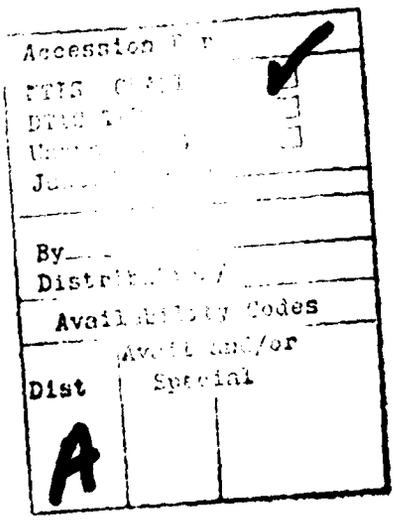
LLC-MK_2 cells were adapted to a medium containing calf serum in order to conserve fetal calf serum. Cells were maintained and grown in calf serum; fetal calf serum was used only in the overlay medium used in the plague assay test.

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



Summary

Initial studies were conducted to develop and to assess the efficacy of an *in vitro* transmission assay for the detection of vector transmission of the parent PR159 and the S-1 vaccine dengue-2 viruses. Intrathoracically-infected mosquitoes were assayed for transmission after 7 and 14 days extrinsic incubation.

Studies were also conducted to compare the ability of the two virus strains to replicate in *Aedes aegypti* as well as in C6/36 cells. The parent strain replicated to a greater titer in mosquitoes. The C6/36 cells were much less sensitive than a mosquito inoculation technique for isolation of both virus strains.

LLC-MK_2 cells were adapted to a medium containing calf serum in order to conserve fetal calf serum (FCS). Cells were maintained and grown in calf serum; FCS was used only in the overlay medium for the plaque assay test.

Flavivirus viremias in suckling mice proved relatively non-infectious for *Aedes aegypti*.

Table of Contents

I. Statement of the problem

The purpose of this research project is to determine if dengue parental and candidate vaccine viruses differ in their respective abilities to infect, to replicate in, and to be transmitted by Aedes aegypti and Aedes albopictus mosquitoes. Attenuated candidate vaccines and parental strains of dengue-2 and dengue-3, and dengue-4 viruses will be compared in their vector-virus interactions.

The second, and related, objective of this research project is to determine if attenuated vaccine strains revert to virulence after mosquito passage. Should a live dengue vaccine be capable of infecting and subsequently be transmitted by mosquitoes to a new vertebrate and should the vaccine revert to virulence as a consequence of mosquito passage, then a natural infection cycle could be initiated.

The rationale for this project is that the temperature sensitive (ts) vaccine strains of the dengue viruses which are attenuated for man will also be modified in one or more paramaters of vector-virus interactions. The hypotheses are 1) that the vaccine strains will be less capable than parental strains of vector infection, 2) that vaccine strains will differ from parent strains in their mode of development, 3) that the vaccine strains will be less efficiently transmitted than parent strains, and 4) that the small plaque ts-mutant populations in the vaccines will remain stable upon passage in vector mosquitoes.

II. Background

Dengue is of great tactical significance to the military because large numbers of troops can become incapacitated in a short period of time. Attenuated dengue vaccines have been developed at WRAIR.

The dengue-2 S-1 vaccine and PR 159 parent strains are the subject of this project report. The S-1 vaccine was derived from the serum of patient PR 159 of Puerto Rico (Eckels, et al., 1976). The virus was passaged 6 times in Lederle certified African green monkey kidney cells. Passage 6 is designated the parent strain and S-1 represents the progeny of a small plague derived from the parent strain. The S-1 clone is ts, titers 340 times higher in LLC-MK_3 cells than in mice, does not produce viremia in rhesus monkeys, produces barely detectable viremia in chimps, and a low-titered viremia in man. Only 1 of 150 Aedes aegypti mosquitoes fed on volunteers became infected, but it did not transmit the virus after 21 days extrinsic incubation.

Ideally a vaccine should not produce viremia, but if it does, it is reasonable to expect that the vaccine strain will infect mosquitoes poorly and will be inefficiently transmitted. This was demonstrated with the 17D yellow fever vaccine (Roubaud et al., 1937; Whitman, 1939), French neurotropic yellow fever vaccine (Davis et al., 1932; Roubaud and Stefanopoulo, 1933; Pettier et al., 1939), mouse-adapted dengue type 1 (Sabin, 1948); and African green monkey kidney-adapted dengue type 2 (Prince, 1973). Sabin (1948) showed that attenuated dengue, passed through mosquitoes, did not revert to pathogenicity for man.

Thus even if the vaccine did develop sufficient viremia to infect vectors, there would be little likelihood that the virus would be transmitted and that it would revert to virulence.

III. Approach

The working hypothesis was made that the ts candidate vaccine viruses and the parental wild-type viruses would behave differently in vector mosquitoes. To test this hypothesis the efficiency of per os infection of each parental and vaccine candidate strain was to be determined in dose response studies. Sequential 10-fold dilutions of the virus preparations were to be used to infect groups of a minimum of 10 sibling mosquitoes per dilution. Such studies would also provide information about the optimal infective dose for the transmission and pathogenesis studies; doses much greater than the threshold could obscure differences in infectivity between the vaccine and parental viruses.

Mosquitoes for studies to determine infection rates, extrinsic incubation periods, and rates of per os transmission were to be infected via engorgement through an animal membrane on known titer blood-virus mixtures. In the event of low infection rates, several animal models were to be investigated.

Vector-virus interactions were to be further investigated using IF techniques to localize antigen in situ in organ dissections and cryostat sections of infected mosquitoes. The sites of restriction of replication (if restriction exists) of the vaccine strains would be defined by the comparative IF studies of antigen development in organs of mosquitoes.

The combination of transmission and comparative pathogenesis studies and the determination of dose-response curves should be adequate to reveal differences in vector-virus interactions between parental and vaccine viruses.

In order to implement the proposed research, it was necessary in this first granting period to adapt methodologies used in yellow fever research (especially the in vitro transmission assay) to the dengue studies. The in vitro assay as originally conceived (Beaty and Aitken, 1979) called for the contents of the feeding capillaries to be inoculated into recipient mosquitoes, a laborious procedure. Since the C6/36 clone of the Aedes albopictus cell line had been shown to be useful for dengue isolation (Tesh, 1979), studies were conducted to compared the sensitivity of the cells and mosquito inoculation for dengue virus isolation. It was also necessary to implement the LLC-MK_2 plaque assay technique for the titration of the S-1 clone and other attenuated dengue viruses.

Vector competence studies and especially dose-response studies are greatly facilitated by the use of artificial blood meals. Unlike a viremic host, a known titer of virus can be presented to the mosquitoes and sequential dilutions of virus can be prepared for dose-response studies. Unfortunately, it is necessary to prepare blood virus mixtures with extremely high titers in order to obtain the same mosquito infection rate as would be obtained if the mosquitoes fed on a viremic host with a much lower titered viremia. This is indicative that an unnatural infection

route is being utilized by the virus which may not be pertinent to field circumstances. Studies were conducted with other types of blood meal preparations and viremic hosts to find a more satisfactory infection mechanism. The isolation of dengue and yellow fever virus from leukocytes (Wheelock and Edelman, 1969; Halstead et al., 1977; Marchette and Halstead, 1978) suggests that one or more of the white cells may function to promote midgut infection. Since mechanical defibrination of blood specimens would disrupte the leukocytes, studies were conducted using chemical defibrination.

IV. Materials and methods

A. Viruses:

Stock viruses for both the parental and S-1 vaccine strains of dengue-2 virus were prepared in both LLC-MK_2 cells and mosquitoes. The original infected human serum (PR-159) was the source of the parental virus. The experimental vaccine (Lot #4, Jan. 1976, WRAIR) was the seed for the vaccine stocks. To prepare the tissue culture stock pools, monolayers of LLC-MK_2 cells were inoculated with the respective seed virus. On day 6 post inoculation, fluids were harvested, centrifuged, and the supernatant was aliquoted and frozen. To prepare the mosquito pool virus stocks, Aedes aegypti mosquitoes were inoculated intrathoracically with approximately 0.0006 ml of the respective virus seed. After 21 days incubation, mosquitoes were titrated in 10% FCS-PBS (0.1 ml/mosquito). After centrifugation, the supernatant was aliquoted and stored frozen.

The yellow fever-Haemagogus virus was originally isolated from a pool of H. janthinomys mosquitoes collected in Brazil. It had been passed 4 times in Aedes aegypti mosquitoes.

B. Mosquitoes:

Strains of Aedes aegypti mosquitoes used in these studies were from dengue endemic areas. They were the:

Kampala strain - Uganda -F_{11}
Santo Domino strain - Caribbean - F_{17}

The mosquitoes were maintained at 27°C, 65 to 75% RH in screened ice cream cartons, and provided 3% sugar water ad libitum.

C. Conjugate:

The anti-dengue-2 conjugate was prepared by hyperimmunization of mice (Brandt et al., 1967). Immunoglobulins were precipitated from the ascitic fluids with $(NH_4)_2SO_4$ and conjugated with fluorescein isothiocyanate (Spendlove, 1966; Hebert et al., 1972). Conjugated antibodies were purified by Sephadex G-50 column chromatography. The conjugate titered 1:32 and was used at 1:16.

D. Virus assay:

Titrations - Two methods were used. In the first, sequential dilutions of materials were intrathoracically inoculated into groups of recipient Aedes aegypti mosquitoes (10 per dilution). After 10-14 days the heads of these recipients were squashed and processed by immunofluorescence (IF) (Kuberski and Rosen, 1977). Materials were also titrated in 10-fold dilutions by plaque assay in LLC-MK_2 cells by the method of Halstead et al. (1977).

Antigen detection - IF was used to localize viral antigen in situ in organ dissections and cryostat sections of mosquitoes (Beaty and Thompson, 1976) and in head and abdominal squash preparations (Kuberski and Rosen, 1977).

E. Per os transmission assay:

Transmission experiments were carried out by the technique of Beaty and Aitken (1979) in which mosquito saliva was collected in capillary tubes containing a measured amount of diluent. The saliva was then assayed in either Aedes albopictus (C6/36) cells or in Aedes aegypti by intrathoracic inoculation.

F. Chamber slides:

Mosquito cells (Singh's Aedes albopictus, clone C6/36) were used in conjunction with inoculated mosquitoes to test for dengue-2 transmission using the in vitro method.

Cells were grown in a medium consisting of basal medium Eagle (BME) prepared with Hanks' balanced salt solution supplemented with 10% tryptose phosphate broth, 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 mg/ml). Cell monolayers were split, diluted to 1 x 10^5 cells/ml and seeded into 8 chamber Lab-Tek® slides 24 to 48 hr prior to use.

The mosquito "meals" were expressed into 0.2 ml growth medium in micro Eppendorf® centrifuge tubes and spun at 15,000 x g for 1-3 minutes to remove fungal and bacterial contaminants. The supernatant fluid (0.1 ml) was aspirated and inoculated into a single well of a Lab-Tek® slide. At the end of the adsorption period (1 hr at room temperature) 0.2 ml of maintenance media (as above but with 3% fetal calf serum) was added per chamber and the slides incubated for 7-10 days at 28°C in a humidified box. At the end of the incubation period the slides were examined for viral antigen by the IF technique.

G. In vitro transmission of dengue viruses:

To assess the efficacy of the in vitro technique for detection of parental and vaccine virus transmission, Aedes aegypti (Santo Domingo) were intrathoracically infected with approximately 100 MID_{50} of each virus strain. At 7 and 14 days, extrinsic , cubated 7°C) mosquitoes were assayed for transmission using the in vitro te. nique (Aitken,

1979; Beaty and Aitken, 1979). Capillaries were charged with 0.015 ml of a 10% FCS-3% sucrose-10^2 M ATP solution. Wings and the anterior 4 legs of the mosquito were removed, and, grasping the mosquito by the remaining legs with forceps, the proboscis was inserted into the capillary. Mosquitoes were allowed to engorge for 1 hour before they were removed, following which head and abdomen were severed and squashed on slides. The smears were stained with the anti-dengue conjugate and examined for the presence of viral antigen using a Leitz-Wetzlar microscope with an HBO Osram 200W mercury vapor bulb and a KPO 490-K510 filter system.

Capillary meals were either 1) inoculated promptly into mosquitoes, 2) inoculated into C6/36 mosquito tissue culture, or 3) a portion of the meal was inoculated into both systems. After 10-14 days incubation, recipient mosquitoes were processed by the headsquash-IF procedure as related previously. Tissue culture preparations were processed at 7 or 10 days post inoculation. Slides were washed, fixed in acetone, and examined by IF for the presence of viral antigen.

H. Dose-response studies to determine the efficiency of infection:

Parental and vaccine viruses, prepared in either the LLC-MK_2 tissue culture or by mosquito inoculation, were serially diluted (10-fold) in sucrose-defibrinated rabbit blood. The blood-virus mixture was placed on pledgets, or in membrane feeders, and the mosquitoes were allowed to feed for one hour. After 21 days extrinsic incubation, the mosquitoes were examined for the presence of viral antigen using the head and abdomen squash-IF technique.

I. Comparison of C6/36 cell line and mosquito inoculation for virus isolation:

As before mentioned, in the in vitro transmission studies some of the feeding capillary contents were inoculated into tissue culture, some into mosquitoes, and some of the contents were split and inoculated into both systems. Isolation rates of the two systems were compared.

The sensitivity of the two flavivirus isolation systems was also compared in parallel titrations of yellow fever virus (Haemagogus) in C6/36 tissue culture and mosquitoes.

J. Development of LLC-MK2 plaque assay using calf serum instead of fetal calf serum (FCS):

Recent shortages of FCS jeopardize tissue culture applications which required FCS as a medium constituent. The LLC-MK_2 plaque assay for the attenuated vaccine strains requires 20% FCS (Eckels et al., 1976). The LLC-MK_2 cells at YARU were passed 6 times using calf serum instead of FCS. These cells were used to titrate the seed viruses obtained from WRAIR as well as stock virus pools prepared at Yale in LLC-MK_2 cells.

K. Alternate blood meal sources:

Defibrinated blood-virus preparations are known to be much less efficient than a viremic host in mediating midgut infection. The artificial meal must be several logs higher in titer in order to obtain the same infection rate. Studies were conducted to assess other blood sources and blood preparations. These included: 1) the use of blood from vertebrates other than rabbits, 2) the use of chemically defibrinated instead of mechanically-defibrinated blood, and 3) the use of viremic suckling mice or guinea pigs.

The Haemagogus virus strain of yellow fever was used in these studies instead of dengue viruses. This was done in order to facilitate assay of per os transmission inasmuch as the yellow fever virus kills baby mice and unadapted dengue virus fails to do so.

The effect of the blood source on the efficiency of mosquito infection was investigated using blood from 5 vertebrate species: rabbit, guinea pig, chicken, and monkey or human. The stock virus was diluted in identical amounts of defibrinated blood from each species. Pledget feeding was used to infect the mosquitoes.

Since mechanical defibrination could physically disrupt leukocytes, chemical defibrination of the blood meals was also investigated. Blood samples were treated with heparin or diluted in Alsever's solution (Tables 4 and 6).

Mosquitoes were incubated 11-20 days prior to viral assay using the headsquash-IF technique.

Since guinea pigs had been reported to circulate yellow fever virus in the blood, they were investigated as a possible viremia source (Sawyer and Frobisher, 1932). Animals were inoculated IP with $10^{8.5}$ $SMICLD_{50}$ and groups of 50 mosquitoes were permitted to feed on the animals on days 2 through 7. The blood was also titrated on days 2, 4, and 6.

One final alternate source investigated was the use of viremic suckling mice. Many arboviruses, including yellow fever, do cause a viremia in suckling mouse, although adults do not become viremic. Suckling mice were infected with yellow fever virus, and on days 3, 4, 5, 6 post infection, Aedes aegypti mosquitoes were permitted to feed. Fourteen days later, the mosquitoes were processed using the headsquash-IF procedure.

V. Results

A. In vitro transmission

Both the PR-159 parent and the S-1 vaccine strain replicated in Aedes aegypti after intrathoracic inoculation. By 14 days incubation, viral antigen was detectable in 100% of the inoculated specimens (Table 1). Both heads and abdomens were squashed and examined by IF. There was only one specimen that contained viral antigen in the midgut that did

not contain antigen in the head. There did seem to be a difference in the amount of antigen in the headsquash preparations. Those mosquitoes infected with the parent strain averaged a rating of 2.5 on a scale of 1 to 4. Those infected with the vaccine strain averaged 1.9. Titration studies are currently in progress to determine the growth curves of each virus in the vector mosquitoes. Stock virus pools of the parent and vaccine viruses titered approximately $10^{8.7}$ and $10^{7.2}$ MID_{50} per ml respectively. These pools were prepared identically, suggesting that parent virus replicates better.

Both viruses were transmitted by the parenterally-infected mosquitoes on the 7th day of incubation (Table 2). For the parent strain there was no increase in transmission rate after an additional 7 days incubation. There was an apparent increase in the transmission rate for the vaccine virus after an additional 7 days, suggesting a longer developmental time in the vector for the vaccine virus. Larger numbers are needed to assess the statistical significance.

B. Efficiency of per os infection studies:

The titers of the blood-virus preparations used are shown in Table 3. The titers were uniformly high, but the meals were almost noninfectious for the mosquitoes. Only one mosquito became infected, and only at the lowest dilution of the parent PR-159 mosquito pool preparation. No mosquitoes became infected that engorged on either of the vaccine virus preparations.

C. Comparison of Aedes albopictus C6/36 cell line and mosquito inoculation:

Both the parent and vaccine viruses grew well in the C6/36 cells, however, the cells were not as sensitive as mosquito inoculation. Although approximately 50% (14/26) of the mosquitoes in the in vitro study transmitted, as detected by mosquito inoculation of the capillary contents (Table 1), no transmissions (0/33) were detected using the C6/36 cells.

The C6/36 cells also proved less sensitive than the live mosquito in parallel titrations of a yellow fever virus stock. In the cells, the stock titered $10^{4.5}$ $\log_{10}$ $TCID_{50}$ per ml. In the mosquitoes, the stock titered $10^{6.02}$ $\log_{10}$ MID_{50} per ml.

D. Titration of viruses in LLC-MK2 cells:

The PR-159 parent and S-1 vaccine seeds obtained from WRAIR titered 1.4×10^3 and 1.1×10^4 pfu/ml respectively in LLC-MK_2 cells grown in medium containing FCS. Stock virus pools prepared in LLC-MK_2 cells at YARU titered 1.8×10^5 and 6.8×10^4 pfu/ml respectively for the parent and vaccine strains. FCS was only used in the overlay in the latter titrations.

E. Alternate blood meal sources:

The infection rates obtained after feeding on heparinized blood prepartions are shown in Table 4; those obtained with mechanically-defibrinated blood are shown in Table 5; and those obtained with mechanically-defibrinated and/or Alsever's-treated samples, the primate blood seemed to be the most efficient in obtaining midgut infection. Virus titers were essentially equal for all blood preparations and all treatment regimens. Infection rates were low, but in the chemically-treated preparations, the guinea pig blood seemed superior with infection rates of 67% and 29%. However, none of the mosquitoes that fed upon the mechanically-defibrinated guinea pig blood became infected.

Guinea pigs did not develop a viremia after I.P. inoculation with yellow fever virus and no feeding mosquitoes became infected.

Suckling mice did develop a viremia approximately 4 days post inoculation. The titer averaged 4 logs (Table 7) on days 4, 5, and 6. Nonetheless, only two engorging mosquitoes became infected, and only one of these developed a disseminated infection (Table 8).

VI. Discussion

The in vitro technique appears to be readily applicable to the detection of per os transmission of both the PR-159 parental and S-1 vaccine strains of dengue-2 virus. Since unpassaged dengue viruses and especially the vaccine viruses cause little or no observable morbidity or mortality in commonly used laboratory animals., the in vitro technique will greatly facilitate the proposed transmission studies.

It must be emphasized, however, that the approximately equivalent transmission rates of the parent and vaccine viruses are probably due entirely to the fact that the mosquitoes were infected parenterally with extremely large quantities of virus. The study was conducted to determine if the in vitro assay was capable of detecting transmission of both viruses, and would not be expected to indicate differences between the two.

Development of a suitable blood source which will result in high mosquito infection rates at low titers remains a problem. Chemically-defibrinated guinea pig blood appears to facilitate midgut infection. Unfortunately there was a substantial overall mortality in the mosquitoes before the extrinsic incubation period was finished. Approximately 50 mosquitoes were permitted to engorge in each regimen. This study will be repeated, but the blood-virus preparations will be presented to the mosquitoes in membrane feeding devices as well as in pledgets. If macrophages do somehow mediate midgut infection, pledget feeding may be inappropriate because the monocyte-macrophages may adhere to the fiber in the pledget.

Currently, studies are being conducted to determine if high-titered viremias can be achieved by direct injection of virus into the blood stream of mice. Coleman and McLean, 1973 were able to develop high-titered dengue viremias for a short period of time using such a technique.

Advantages of such a system include the ability to create a viremia of known titer and to create a series of sequential dilutions for the efficiency of infection studies.

Plaque assay of dengue viruses in LLC-MK_2 cells grown in a medium containing calf serum instead of FCS is possible. Although FCS was still used in the overlay, there is a substantial savings. A disadvantage is that growth of the cells is somewhat slower than that obtained using FCS, and splitting times are prolonged. Now it is necessary to determine if the presence of FCS in the overlay instead of calf serum increases the sensitivity of the plaque assay.

Conversely, the C6/36 cells proved not to be a substitute for mosquito-inoculation for isolation of dengue viruses. Not one transmission isolation was made in tissue culture, whereas 50% of the mosquito transmission attempts assayed using the mosquito inoculation technique were successful. Nor was the tissue culture as sensitive in the titration of another flavivirus, yellow fever. Although the volume of the inoculum was 166 times greater in the tissue culture, the titer as detected in the mosquito was over 1 log greater. The intact mosquito is obviously much more sensitive than the mosquito tissue culture.

Finally, these studies, conducted in the first months of the grant, do not permit any final conclusions concerning differential vector-virus interactions using parent and vaccine dengue 2 viruses. The parent strain replicated to a greater titer in the mosquitoes produced more viral antigen, and yield the only midgut infection. These facts suggest that the vaccine strain of virus might be deficient in vector-virus interactions as compared to the parent strain. These leads will be rigorously investigated in the 6 months remaining in this granting period and in subsequent granting periods.

VII. Conclusions:

1. The <u>in vitro</u> transmission technique is satisfactory for assay of parental and dengue virus transmission, thereby greatly facilitating the proposed comparative transmission studies.

2. The direct immunofluorescent assay has proven to be a rapid reliable, and inexpensive technique for the detection of both parent and vaccine viral antigen in mosquito tissues and in C6/36 cells.

3. The mosquito C6/36 cells were substantially less sensitive than mosquito inoculation for flavivirus isolation. All isolation attempts in future studies will be done by mosquito inoculation.

4. A satisfactory plaque assay for the dengue-2 parent and vaccine viruses can be obtained when calf serum is substituted for FCS in the medium.

Table 1

Detection by IF of dengue viral antigen in parenterally-infected Aedes aegypti

Days Incubation	Virus: Parent PR-159 Head		Parent PR-159 Abdomen		S-1 Vaccine Head		S-1 Vaccine Abdomen	
7	8/12[a]	67%	8/12	67%	5/12	42%	6/12	50%
14	17/17	100%	17/17	100%	16/16	100%	16/16	100%

[a] Numerator - number containing detectable viral antigen
Denominator - number examined

Table 2

Transmission of dengue virus by parenterally-infected Aedes aegypti

Days Incubation	Virus: Parent PR-159		S-1 Vaccine	
7	4/7[a]	57%	1/3	33%
14	4/8	50%	5/8	63%

[a] Numerator - number transmitting virus
Denominator - number of headsquash IF-positive mosquitoes imbibing a detectable amount of the meal

Table 3

Titers of blood-virus preparations used in the efficiency of infection studies

Virus	Virus source	Pre-feed titer[a]	Post-feed titer
Parent PR-159	LLC-MK_2 cells	8.3	8.9
	Aedes aegypti (SDO) pool	7.5	7.2
S-1 Vaccine	LLC-MK_2 cells	7.7	7.3
	Aedes aegypti (SDO) pool	6.9	6.7

[a] Log_{10} mosquito infective $dose_{50}$ per ml.

Table 4

Yellow fever virus infection rates obtained with heparinized Vertebrate bloods

	Blood Source							
	Rabbit		Guinea pig		Chicken		Monkey	
Aedes aegypti strain								
Santo Domingo	3/20[a]	15%	4/6	67%	1/19	5%	5/15	33%
Kampala	0/17	0%	0/12	0%	2/24	8%	1/8	13%
Post-feeding titration (MID_{50}/ml)	6.0		6.2		6.1		5.7	

[a] Numerator - number infected
Denominator - number examined

Table 5

Yellow fever virus infection rates in mechanically-defibrinated invertebrate bloods

	Rabbit		Guinea pig		Chicken		Human	
Infection rate	1/5	20%	0/8	0%	0/6	0%	2/15	13%
Post-feeding titration (MID_{50}/ml)	5.7		6.1		6.0		6.2	

Table 6

Yellow fever infection rates in Aedes aegypti mosquitoes fed upon mechanically-defibrinated and Alsever's-treated guinea pig blood

Dilution	Defibrinated		Alsever's		Alsever's-defibrinated	
2×10^{-1}	0/6	0%	5/17	29%	0/8	0%
1×10^{-1}	0/2	0%	0/13	0%	0/11	0%
Post-feeding titration (MID_{50}/ml)	6.0		6.3		6.0	

Table 7

Viremia titers in suckling mice inoculated with yellow fever virus[a]

Days post-inoculation	Log_{10} $TCID_{50}$/ml
1	0
2	0
3	0
4	4.5[b]
5	3.5
6	4.0

[a] Mice were inoculated i.c.

[b] Three mice were bled per day.

Table 3

Aedes aegypti infection rates obtained by feeding on yellow fever viremic baby mice

Days post inoculation	Viremia[a]	Days extrinsic incubation	Mosquito IF infection rate[b] Heads	Thoraces	Abdomen
4	3.75	16	0/10	0/10	0/10
4	3.75	19	0/2	0/2	1/2
5	2.50	15	0/10	0/10	0/10
5	2.50	18	0/30	0/30	0/30
6	Not detected	14	1/2	1/2	1/2

[a] Log_{10} $TCID_{50}$/ml

[b] Number IF postive/number tested

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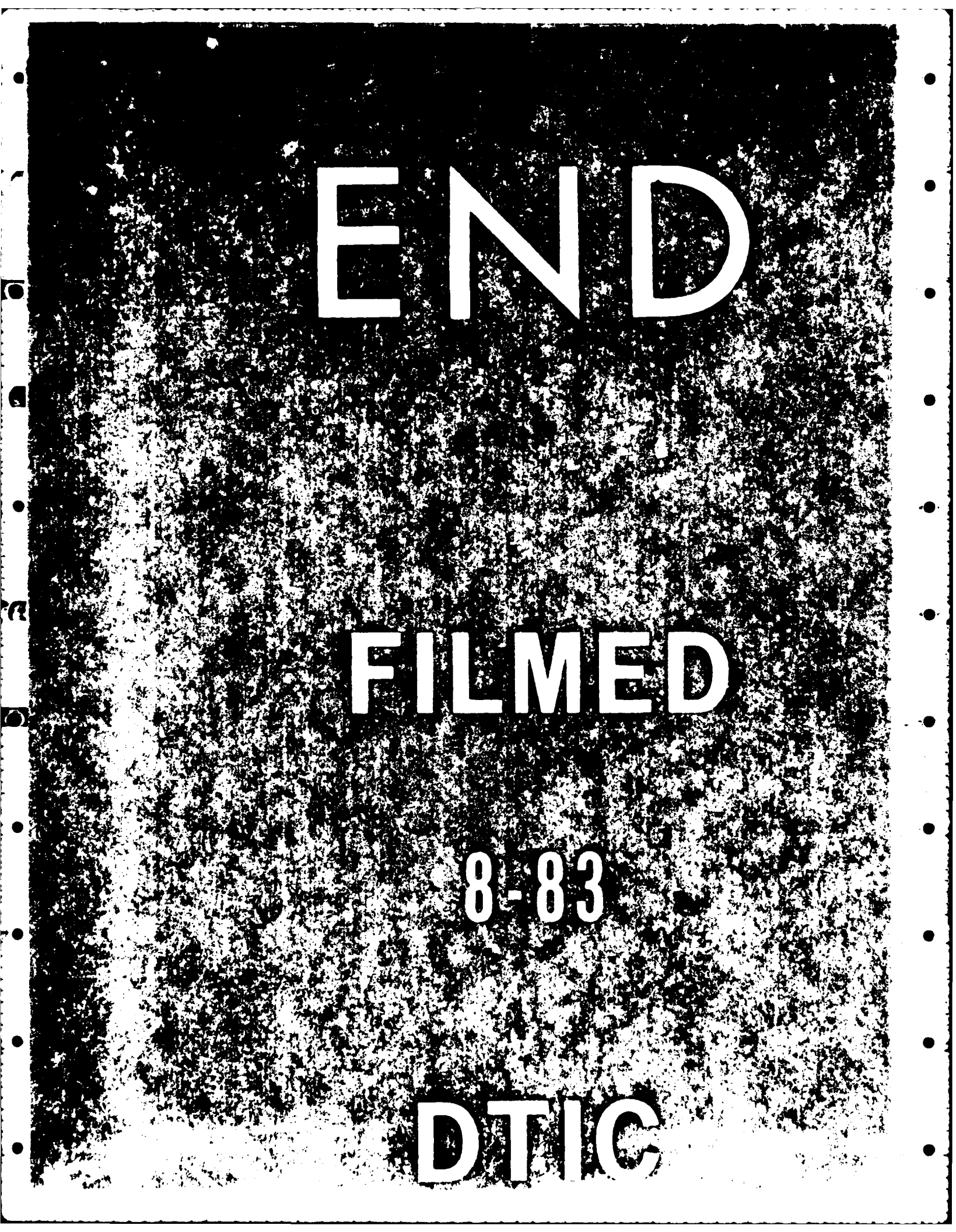
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