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# EXPERIMENTAL TRANSMISSION OF YELLOW FEVER BY CENTRAL AMERICAN SPECIES OF *HAEMAGOGUS* AND *SABETHES CHLOROPTERUS*

PEDRO GALINDO, ENID DE RODANICHE and HAROLD TRAPIDO

# EXPERIMENTAL TRANSMISSION OF YELLOW FEVER BY CENTRAL AMERICAN SPECIES OF *HAEMAGOGUS* AND *SABETHES CHLOROPTERUS*

PEDRO GALINDO, ENID DE RODANICHE AND HAROLD TRAPIDO[1]

*Gorgas Memorial Laboratory, Panama, R. P.*

The invasion of Middle America by sylvan yellow fever in the years since its appearance in central Panama in 1948 has raised the problem of natural vectors in this region other than those known to transmit the disease in South America. The experiments reported here were intended to throw light on this problem. Studies on the forest mosquito fauna have shown that the known South American vectors with ranges which extend into Middle America (*Haemagogus spegazzinii falco* and *Aedes leucocelaenus* ssp.) have been either uncommon, rare or absent in certain of the places where yellow fever has recently occurred (Trapido and Galindo, 1955, and Galindo and Trapido, 1957a). The South American *Haemagogus* mentioned above is replaced in Middle America by other species. Most widespread is *Haemagogus equinus* which occurs from Venezuela and Colombia to southernmost Texas (Galindo and Trapido, in preparation). It is present on both the Atlantic and Pacific slopes. Other forest-inhabiting *Haemagogus* of Middle America are two of the three subspecies of *Haemagogus mesodentatus*, the typical race *mesodentatus* occurring on the Atlantic slope from western Panama to southeastern San Luis Potosi, Mexico, and the race *gorgasi* on the Pacific side from El Salvador to southern Sinaloa, Mexico (Galindo and Trapido, 1956 c).

In explanation of certain epidemiological aspects of the disease in Panama we have been much interested in determining the possible role of the sabethine mosquito, *Sabethes chloropterus*. Early in the work it was realized (Galindo, Trapido and Carpenter, 1950) that if this mosquito were shown to be a vector there would be a possible explanation for survival of the virus over the dry season. This matter of the mechanism of the survival of virus over the dry season has been elaborated in recent papers (Galindo, Trapido, Carpenter and Blanton, 1956 and Trapido and Galindo, 1957).

Of the species tested in this study *Haemagogus spegazzinii falco* and *H. equinus* previously had been the subjects of experiment. Both the typical subspecies of *spegazzinii* and the race *falco* had repeatedly been shown to transmit in the laboratory, and had been implicated in transmission in nature. The work with this species has recently been reviewed by Whitman (1951). In the case of *H. equinus*, material of Colombian origin was used by Waddell and Taylor (1945 and 1947) in various experiments in which successful transmissions were obtained. In the field studies made in South America virus was never recovered with certainty from naturally infected individuals. In Colombia Boshell-Manrique and Osorno-Mesa (1944) demonstrated the presence of virus in a group of three species of *Haemagogus*, one of which was *H. equinus*, but it is uncertain which of

[1] Present address: The Rockefeller Foundation, 49 West 49th Street, New York, N. Y.

these species may have been infected. The role of the latter species in nature has therefore been in doubt.

Interest in the sabethine mosquitoes stems from the recovery of yellow fever virus from a pool of four genera of this tribe by Shannon, Whitman and Franca (1938). One mosquito of the tribe, *Trichoprosopon frontosus*, is mentioned as having experimentally transmitted by bite (Waddell, 1949 and Whitman, 1951) but the details of the experiments have not been published. The difficulty in keeping these mosquitoes alive in the laboratory and inducing them to bite has limited the work with them.

## MATERIALS

*Virus strains.* It seemed desirable to obtain a recently isolated strain of yellow fever virus which had undergone as few passages in experimental animals as possible. As the Trinidad Regional Virus Laboratory had made a number of isolations during the recent outbreak there, such a strain was solicited from the director, Dr. Wilbur Downs. He kindly forwarded to us the two strains used in the experiments to be described.

The first strain was received in dry ice on December 4, 1954 as mouse brain suspensions representing the first and second mouse passages of specimen # 4754, the serum of a nonfatal case of yellow fever. This strain failed to produce fever or other symptoms in any of three rhesus monkeys injected by the subcutaneous route and circulating virus titers were low, although the monkeys later showed a high level of immunity. This strain was used only in the first experiment.

The second strain of yellow fever virus was received on March 14, 1955 as a suspension of the spleen of the original *Alouatta* (howling monkey), specimen # 4204, found dead in the forest on July 30, 1954. This strain has proven uniformly fatal to rhesus monkeys and gives high circulating titers. It was used in the second and third experiments.

*Experimental animals.* White Swiss mice, usually 21 to 30 days old, were used for virus titrations and for isolation of virus from mosquitoes by injection. The intracerebral route of inoculation was employed.

Monkeys were used as sources of infective blood for the initial engorgement of the mosquitoes and as hosts for experimental transmission by bite. In the majority of the experiments juvenile rhesus monkeys were used. In a few experiments locally captured red or black spider monkeys (*Ateles geoffroyi robustus* and *panamensis*) were employed. All monkeys were previously tested for neutralizing antibodies by the intracerebral mouse protection technique.

*Mosquitoes.* As *Aedes aegypti* has presumably been eradicated from Panama, a colony of this species is not maintained at the Gorgas Memorial Laboratory. In view of the known efficacy of *Haemagogus spegazzinii* and *equinus* in transmitting yellow fever virus, these species were considered as controls.

The *Haemagogus spegazzinii falco* used in the experiments were the first generation progeny of females collected at Cerro La Victoria, near Panama City. This species was used only in the first experiment since it could not be colonized and since the numbers of females which could be collected was not large.

In the first experiment, the *Haemagogus equinus* used were both the progeny

of females collected at Cerro La Victoria, and stock from a colony which had been established with material from the same place. For the subsequent experiments the mosquitoes used were reared from eggs obtained at two localities in Guatemala, El Salto in the Department of Esquintla and El Remate in the Department of Peten. Large numbers of *equinus* eggs had been obtained at these localities in the course of collecting the material of *mesodentatus mesodentatus* and *mesodentatus gorgasi*, forms in which we were primarily interested.

The *Haemagogus mesodentatus mesodentatus* and *mesodentatus gorgasi* were reared from eggs obtained respectively at El Remate and El Salto, Guatemala.

The material of *Haemagogus lucifer* was obtained at Cerro La Victoria, Panama. As this species was not colonized and the number of eggs gotten from wild caught females was not large, only limited numbers were available and this species was used only in the first and third experiments.

A colony of *Sabethes chloropterus* had been established by the time the second and third experiments were performed. The wild stock had been obtained at Cerro La Victoria, Panama. In the second experiment both colony-reared individuals and wild caught material, also from La Victoria, was used, while in the third experiment all the *chloropterus* were colony stock. Insofar as we know this is the first time a sabethine mosquito has been colonized. While the species with which we worked lives well and mates readily in captivity, great difficulty was experienced in getting them to feed in the laboratory, although they attack freely in the field. It was particularly difficult to obtain refeeding.

## METHODS

For feeding the mosquitoes several methods were used. Usually the *Haemagogus* were first put in Barraud cages, eight inches square, and the shaved foot of the monkey inserted. When adequate feeding was not obtained by this method the mosquitoes were transferred to one-pint cylindrical cardboard cartons which had been provided with netting at either end. The cartons were then applied to the shaved belly of the monkey. Mosquitoes which had not fed by either of these methods were transferred individually to shell vials and these were then put on the shaved belly of the monkey. The *Sabethes chloropterus*, which in the field bite primarily about the head and similarly fed in the laboratory, could not be induced to bite by the methods used for the *Haemagogus*. They were released in a cage three feet long, two feet wide and 18 inches high, and the entire body of the trussed monkey was put in the cage. Under these conditions a portion of the specimens would feed on the face of the monkey when he did not move too much, and there was also some feeding on the hands.

On the day before feeding experiments were scheduled, food (sugar-soaked cotton balls) was removed from the stock cages. After feeding, all mosquitoes were placed in one-pint cardboard cartons provided with netting at both ends, and these were then held on a shallow pan covered with moistened cotton and filter paper. Balls of sugar-soaked cotton were kept on the tops of the cartons at all times until the day before refeeding was attempted. As there was an abundant growth of mould on the feces excreted in the days after the first feeding, the mosquitoes were usually transferred to fresh cartons after about a week.

In the first experiment the mosquitoes were held in an outdoor insectary where the daily range in temperature was from 72°F. to 90°F. and the bi-hourly mean was 78°F. For the second and third experiments an enclosed insectary was available in which the temperatures ranged from 76°F. to 85°F. and the bi-hourly mean was 79°F.

Mosquitoes were allowed to engorge on monkeys on the 2nd to 5th day after subcutaneous injection with yellow fever virus. Immediately prior to exposure to the bites of the mosquitoes, blood was drawn from the femoral veins of the monkeys and the serum diluted serially in 10 per cent inactivated, filtered, negative rhesus serum-saline for titration in mice. Six mice were injected intracerebrally with each decimal dilution. At the end of the 30-day observation period surviving mice were challenged for immunity by the intracerebral injection of between 5,000 and 10,000 LD/50 of the French neurotropic yellow fever virus. As the Trinidad strain of virus isolated from the spleen of a howling monkey showed relatively low pathogenicity for mice, the minimal infective titers (ID/50) obtained may not represent accurately actual virus titers.

The mosquitoes were maintained for an incubation period of 22 to 44 days. Any found dead or dying during the first five days after engorgement were discarded. Those found recently dead or dying subsequent to the first five days were triturated in 10 per cent rhesus serum-saline for injection into mice, either singly or in small pools of 2 to 4 mosquitoes. Mice were kept under observation for 30 days. Those surviving this period were challenged for immunity by the intracerebral injection of 5,000 to 10,000 LD/50 of French neurotropic yellow fever virus.

At the end of the incubation period the surviving mosquitoes of each species were allowed to bite a normal rhesus or spider monkey. Temperatures of the monkeys were taken twice daily, morning and afternoon. The animals were bled every other day and the serum injected intracerebrally into mice to detect circulating virus. Pathological study of the livers of monkeys dying of the infection was made by Dr. Carl M. Johnson who in each instance confirmed the diagnosis of yellow fever. Surviving monkeys were bled at three, four and six-week intervals and their serum tested for mouse protecting antibodies. Mosquitoes which probed or engorged on the test monkeys were emulsified with serum-saline and injected into mice.

*Identification of virus.* Mice developing suggestive symptoms during the 30-day observation period were sacrificed. Impression smears stained by the method of Giemsa and cultures in blood agar plates and in thioglycollate broth were made from their brain tissue.

Virus isolated in mice from the blood of monkeys bitten by infected mosquitoes was identified by cross-immunity and protection tests. For the former a group of normal mice and a group of mice previously immunized against the French neurotropic yellow fever virus were injected intracerebrally with first mouse passage virus. Results were considered positive if the immunes were alive and well after the non-immunes had sickened or died. In the protection tests the intraperitoneal technique was employed combining mouse passage virus with known positive and negative monkey serums.

Due to the very large number of isolations of virus from mosquitoes feeding on infected monkeys, protection tests were not done. In view of the known history of these mosquitoes, identification of yellow fever virus was considered established when bacterially negative smears and cultures were obtained and cross-immunity experiments gave results as previously described.

The experiments were performed in three groups. The general technique previously described was followed in each instance.

### EXPERIMENT 1

In the first experiment (see Table 1) the virus strain isolated in mice from specimen # 4754, serum of a nonfatal human case of yellow fever, was used. This virus had been passaged three times in mice and once in a rhesus monkey prior to subcutaneous injection of the rhesus monkey used for infecting the mosquitoes. Mosquitoes used were *Haemagogus mesodentatus gorgasi, spegazzinii falco, lucifer* and two strains of *equinus*, one from Panama and one from Guatemala. The mosquitoes were fed on the rhesus monkey on the second, third and/or fourth days after inoculation. Virus titers were low, the highest being $10^{-2.5}$ on the second day after inoculation.

Only *H. mesodentatus gorgasi* and *spegazzinii falco* survived long enough to be used for bite transmission attempts, each species being allowed to engorge

TABLE 1

*First transmission experiment with a Trinidad strain of yellow fever virus isolated from human serum (specimen # 4754)*

| Species of Mosquito | Day of Feeding | Titer of Circulating Virus ID/50 | Results of Mouse Injections | | | Results of Refeeding on Monkeys | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total no. mosquitoes injected | No. virus isolations | Per cent positive (Min. & Max. possible) | No. of mosquitoes biting | Incubation period (days) | Outcome |
| *Haemagogus mesodentatus gorgasi* | 2nd | $10^{-2.5}$ | 16 | 2 | 12.5–37.5 | | | |
| | 3rd | $10^{-1.8}$ | 2 | 0 | | | | |
| | 2nd & 3rd (pool) | $10^{-2.5}$ & $10^{-1.8}$ | 18 | 1 | 5.6 | 12* | 29–33 | Negative |
| *Haemagogus spegazzinii falco* | 2nd | $10^{-2.5}$ | 3 | 0 | | 1 | 31 | Negative |
| | 3rd | $10^{-1.8}$ | 10 | 2 | 16.6 | 6† | 28–32 | |
| *Haemagogus lucifer* | 2nd | $10^{-2.5}$ | 3 | 0 | | | | |
| | 3rd | $10^{-1.8}$ | 3 | 0 | | | | |
| | 4th | $<10^{-1}$ | 2 | 0 | | | | |
| *Haemagogus equinus* (Panama) | 2nd | $10^{-2.5}$ | 10 | 0 | | | | |
| *Haemagogus equinus* (Guatemala) | 2nd | $10^{-2.5}$ | 17 | 1 | 4.2–12.5 | | | |
| | 3rd | $10^{-1.8}$ | 7 | | | | | |

* Subsequent injection of mice with suspensions of individual mosquitoes gave one positive.

† Subsequent injection of mice with suspensions of individual mosquitoes gave two positives.

on a normal rhesus monkey. *Haemagogus mesodentatus gorgasi* which had fed on the second and third days were put together for refeeding in the transmission attempt. Results are tabulated in Table 1. No transmission by bite was obtained, which was to be expected in view of the low virus titers in the infecting monkey. Neither monkey showed fever or other symptoms, circulating virus or subsequent immunity. However, this experiment was not without interest because virus was recovered from a small proportion of the mosquitoes by injection of mice, in spite of the low titers in the circulating blood. Of the *H. spegazzinii falco* which had engorged on the third day, when the virus titer was $10^{-1.8}$, 16.6 per cent were positive. The exact proportion of positive *H. mesodentatus gorgasi* could not be determined since pools of mosquitoes were used in several instances. However, those engorging on the second day, when the circulating virus titer was $10^{-2.5}$, showed a proportion of positives somewhere between 12.5 and 37.5 per cent depending on whether only one or all of the mosquitoes in the positive pools contained virus.

EXPERIMENT 2

For the second transmission experiment (see Table 2) the strain of yellow fever virus isolated from the spleen of a howling monkey, specimen № 4204, was used. Three monkeys, two rhesus and one spider monkey, were injected subcutaneously with this strain in its first experimental monkey passage; i.e., with serum of a rhesus injected with the original *Alouatta* spleen suspension. The following species of mosquitoes were employed: *Haemagogus mesodentatus gorgasi, mesodentatus mesodentatus, Sabethes chloropterus* and two strains of *H. equinus*, from El Remate and El Salto, Guatemala. (The *Haemagogus equinus* from El Remate died in less than 26 days and therefore were not used for bite transmission.) After an incubation period of 26 to 34 days each species was allowed to bite a normal rhesus monkey. Transmission by bite was obtained in each instance. The rhesus monkeys bitten by the three *Haemagogus* forms all developed fever in three to five days after the first day of refeeding and died in six to seven days. It is of interest that the rhesus bitten by only one *H. mesodentatus mesodentatus* suffered a rapidly fatal infection. The monkey (*Macaca mulatta*) on which *Sabethes chloropterus* was refed was exposed each of five days. It developed fever 15 days after the first exposure and ten days after the last, and died two days later. This prolonged incubation period may indicate infection with a minimal amount of virus. Virus was isolated, by injection of mice, from a high percentage of the *Haemagogus* mosquitoes (see Table 2). Virus was not obtained from any of the 49 *Sabethes chloropterus* injected into mice after periods of incubation varying from 12 to 44 days. The discrepancy between successful transmission by bite and unsuccessful isolation by injection must be resolved in future experimentation.

EXPERIMENT 3

The strain of virus obtained from *Alouatta* spleen was again used in the third experiment. It had been passaged twice in rhesus monkeys. The following mosquitoes were used in this experiment: *Haemagogus mesodentatus gorgasi, lucifer,*

TABLE 2

*Second transmission experiment with a Trinidad strain isolated from* Alouatta *spleen (specimen # 4202)*

| Species of Mosquito | Day of Feeding | Titer of Circulating Virus ID/50 | Results of Mouse Injections | | | Results of Refeeding on Monkeys | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total no. mosquitoes injected | No. virus isolations | Per cent positive (Min. & Max. possible) | No. of mosquitoes biting | Incubation period (days) | Outcome |
| *Haemagogus mesodentatus gorgasi* | 3rd (Rh #1) | $10^{-6.2}$ | 2 | 2 | 100 | 4 | 26–28 | Positive |
| | 4th (Rh #1) | $10^{-5.3}$ | 0 | | | 2 | | |
| | 3rd & 4th (pool) | $10^{-6.2}$ & $10^{-5.3}$ | 8 | 5 | 62.5 | | | |
| *Haemagogus mesodentatus mesodendatus* | 3rd (Rh #1) | $10^{-6.2}$ | 0 | | | 1 | 27 | Positive |
| | 4th (Rh #1) | $10^{-5.3}$ | 3 | 2 | 66.7 | | | |
| | 3rd (Rh #2) | $10^{-5.4}$ | 16 | 2 | 12.5–50 | | | Positive |
| *Sabethes chloropterus* | 3rd (Rh #1) | $10^{-6.2}$ | 4 | 0 | | 7 | 27–34 | Positive |
| | 4th (Rh #1) | $10^{-5.3}$ | 4 | 0 | | 2(4)* | | |
| | 3rd (Rh #2) | $10^{-5.4}$ | 10 | 0 | | 0(1) | | |
| | 3rd (RS #1) | $10^{-6.8}$ | 1 | 0 | | 2 | | |
| | pool of the above | | 30 | 0 | | | | |
| *Haemagogus equinus* (Peten, Guatemala) | 3rd (Rh #1) | $10^{-6.2}$ | 1 | 1 | 100 | 0 | | |
| | 3rd (Rh #2) | $10^{-5.4}$ | 20 | 7 | 35–80 | 0 | | |
| *Haemagogus equinus* (El Salto, Guatemala) | 3rd (Rh #1) | $10^{-6.2}$ | 68 | 30 | 44–70 | 10 | 26–28 | Positive |
| | 4th (Rh #1) | $10^{-5.3}$ | 4 | 0 | | 16 | | |

* Parentheses indicate specimens which probed but did not feed. Rh = Rhesus (*Macaca mulatta*). RS = Red spider monkey (*Ateles geoffroyi*).

*Sabethes chloropterus* and *H. equinus*, the last from El Salto, Guatemala. All species were fed on a single rhesus monkey on the third, fourth and fifth days after injection when the circulating virus titers were $10^{-5.8}$, $10^{-4.2}$, and $10^{-3.8}$ respectively.

After an incubation period varying from 22 to 29 days, surviving mosquitoes were allowed to feed on normal monkeys. *Haemagogus equinus*, *lucifer* and *Sabethes chloropterus* were each fed on rhesus monkeys. *H. mesodentatus gorgasi* was fed on a black spider monkey. An infection fatal in seven days was produced in the rhesus bitten by *H. equinus*. The spider monkey on which *H. mesodentatus gorgasi* was fed did not die but showed circulating virus and was subsequently immune. Only one *H. lucifer* attempted to feed on the rhesus monkey. It is uncertain whether it simply probed or actually fed. In either case results were negative.

In this experiment only the *H. lucifer* and *S. chloropterus* were injected into mice as it was thought that sufficient data had already been accumulated on the other two species. The exact percentage of *H. lucifer* positive by mouse injection could not be determined due to the use of some pooled specimens. However,

TABLE 3

*Third transmission experiment with a Trinidad strain isolated from* Alouatta *spleen (specimen #4204)*

| Species of Mosquito | Day of Feeding | Titer of Circulating Virus ID/50 | Results of Mouse Injections | | | Results of Refeeding on Monkeys | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total no. mosquitoes injected | No. virus isolations | Per cent positive (Min. & Max. possible) | No. of mosquitoes biting | Incubation period (days) | Outcome |
| *Haemagogus mesodentatus gorgasi* | 3rd | $10^{-5.8}$ | 0 | | | 5(11)* | 27–29 | Positive |
| | 4th | $10^{-4.2}$ | 0 | | | | | |
| | 5th | $10^{-3.8}$ | 0 | | | | | |
| *Haemagogus lucifer* | 3rd | $10^{-5.8}$ | 32 | 11 | 34–59 | 1† | | Negative |
| | 4th | $10^{-4.2}$ | 4 | 0 | | | | |
| | 5th | $10^{-3.8}$ | 10 | 0 | | | | |
| | pool of the above | | 4 | 1 | 25–50 | | | Negative |
| *Sabethes chloropterus* | 3rd | $10^{-5.8}$ | 9 | 0 | | 3 | 27–29 | Negative |
| | 4th | $10^{-4.2}$ | 0 | | | | | |
| | 5th | $10^{-3.8}$ | 0 | | | | | |
| *Haemagogus equinus* (El Salto, Guatemala) | 3rd | $10^{-5.8}$ | 0 | | | 3–5 | 22–24 | Positive |
| | 4th | $10^{-4.2}$ | | | | | | |
| | 5th | $10^{-3.8}$ | | | | | | |

* Parentheses indicate specimens which probed but did not feed.

† Uncertain whether probed or fed.

of those originally fed on the monkey on the third day when the circulating titer was $10^{-5.8}$, between 34 and 59 per cent were positive by mouse injection.

No virus isolations in mice were obtained in this experiment with any of nine *S. chloropterus*, all fed on the third day, nor was transmission by bite obtained.

## DISCUSSION

In view of the many uncontrollable variables in an experiment of this kind, it is difficult to make an accurate interpretation of all results obtained. The principal objectives, however, were achieved. Fair to high proportions of all *Haemagogus* species tested harbored virus. The exact proportion varied with the titer of virus in the infective meal. Successful transmission by bite was obtained twice in three trials with *H. mesodentatus gorgasi*, twice in two trials with *H. equinus* and once in the trial made with *H. mesodentatus mesodentatus*. The failure of *H. mesodentatus gorgasi* to transmit by bite in the first experiment may be attributed to use of a mouse-passaged strain of yellow fever virus which developed only very low titers in the blood of the source monkey. The direct correlation between virus titer in the infective meal and subsequent capacity to transmit by bite has been amply demonstrated by Waddell and Taylor, 1947, and Bates and Roca-Garcia, 1946, among others. It is significant that under equal circumstances, the control species, *H. spegazzinii falco*, also failed to transmit by bite, although two of the seven engorging mosquitoes were positive by mouse injection.

The experiment involving bite transmission by *H. lucifer* must be considered unsatisfactory as only one mosquito bit or probed.

No *Sabethes chloropterus* were found to harbor virus, at least in sufficient amounts to infect mice. No mouse injected with suspensions of this species showed symptoms or developed a subsequent immunity. One experimental transmission by bite was obtained in two attempts. In the successful transmission experiment, 11 mosquitoes fed and five probed. In the unsuccessful experiment, only three fed. The interpretation of this result is difficult and further experimentation is required.

## SUMMARY

Successful transmission from monkey to monkey of a strain of yellow fever virus isolated in Trinidad was obtained with the following Middle American *Haemagogus: H. m. mesodentatus, H. m. gorgasi* and *H. equinus*. All the above mentioned species as well as *H. lucifer* and *H. spegazzinii falco* were found by intracerebral mouse inoculation to harbor yellow fever virus from one to four weeks after the infective meal. No virus was isolated from mice injected with pools of *S. chloropterus* even though the species transmitted the virus by bite from monkey to monkey on one occasion.

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