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## ETIOLOGY OF YELLOW FEVER.

### IX. MOSQUITOES IN RELATION TO YELLOW FEVER.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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That a certain species of mosquito serves as a carrier of yellow fever has long been suspected. Finlay<sup>1</sup> supported this theory as early as 1881 and went so far as to advise conferring immunity upon non-immune persons by causing them to be bitten by mosquitoes (*Stegomyia calopus*) which had previously fed on a yellow fever patient. Thenceforth *Stegomyia calopus* became the center of attention in yellow fever investigations, the theory being experimentally confirmed finally by Reed, Carroll, Agramonte, and Lazear,<sup>2</sup> who successfully transmitted yellow fever to non-immune human subjects by allowing the infected stegomyias to bite them. The successful eradication of yellow fever by Gorgas<sup>3</sup> from various endemic centers in the western hemisphere through anti-stegomyia campaigns amply attests the correctness of this important discovery. Reed and his coworkers established the fact that the virus exists in the peripheral blood during the first 3 days of the illness and that the mosquitoes, which have fed on a yellow fever patient during this period, become capable, after about 10 days, of transmitting the infection by their bite to a normal person. Hence they assumed that the virus required a period of at least 10 to 12 days of extrinsic incubation during which it must pass through a life cycle, as was known to be the case with the parasite of malaria. The necessity for an extrinsic incubation of about 12 days was pointed out by Carter<sup>4</sup> in his epidemiological studies of yellow fever. This theory, coupled with the improbability of a bacterial origin of the disease, brought about the general impression that the virus was a protozoan, although nothing definitely objective was adduced in support of this view.

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<sup>1</sup> Finlay, C., El mosquito hipotéticamente considerado como agente de transmisión de la fiebre amarilla, *An. r. Acad. cien. méd. Habana*, 1881-82, xviii, 147.

<sup>2</sup> Reed, W., Carroll, J., Agramonte, A., and Lazear, J. W., The etiology of yellow fever, *Am. Pub. Health Assn., 28th Meeting, 1900*; also *Senate Doc. No. 822, 61st Congr., 3rd Sess., 1911*, 56.

<sup>3</sup> Gorgas, W. C., *Sanitation in Panama, New York and London, 1915*.

<sup>4</sup> Carter, H. R., Note on the spread of yellow fever in houses. Extrinsic incubation, *Med. Rec.*, 1901, lix, 933.

Reference has already been made<sup>5,6</sup> to the fact that the virus of yellow fever passed through the pores of a Berkefeld or a Chamberland filter. This point is important in excluding as the inciting agent of the disease any organism incapable of passing through these filters which might be found incidentally in the blood or tissues of yellow fever patients. Some persons believe that the virus is ultramicroscopic, at least with the magnification possible at the present time.

The characteristics of the organism isolated from the yellow fever cases in the present investigation conformed with all the other known characteristics of the yellow fever virus, and it became necessary to determine whether or not it behaved like the latter in relation to mosquito transmission. That it does, under certain conditions, is shown in the experiments to be described.

#### *Material and Mode of Experiments.*

Larvæ of *Stegomyia calopus* collected from houses in Guayaquil were brought to the laboratory.<sup>7</sup> They varied in stage from the very young to the pupal form.

For the purpose of maintaining the imagos which emerged from these larvæ special cages were constructed consisting of a square wooden frame with wire net on all but two opposing lateral walls, which were solid pieces of wood with a round opening in the center for the insertion of the arm or a glass of water from either side of the cage. The opening was covered by a well fitting sliding door which could be drawn to one side and slipped back in place. A long glass cylinder about 8 inches long and 2 inches in diameter with one end closed was used for handling individual mosquitoes. When the specimens were to be killed for examination a piece of cotton saturated with chloroform was put at the bottom of the cylinder. For histological study the mosquitoes were placed in fixing fluid such as sub-

<sup>5</sup> Reed, W., and Carroll, J., The etiology of yellow fever; a supplemental note, *Senate Doc. No. 822, 61st Congr., 3rd Sess.*, 1911, 149.

<sup>6</sup> Marchoux, Salimbeni, and Simond, La fièvre jaune, *Ann. Inst. Pasteur*, 1903, xvii, 665.

<sup>7</sup> These were obtained through the cooperation of the officials of the Municipal Board of Health of Guayaquil.

limate-alcohol, Zenker's fluid, or 10 per cent formaldehyde, after the wings and legs were trimmed off. Mosquitoes engorged with blood usually sink to the bottom in a short time, but those which are unfed or have already digested the blood do not sink for many hours or even days, and it was necessary to keep them within the fixing fluid by forcing them down with a piece of absorbent cotton. In some instances a minute needle puncture into the abdomen or thorax, or both, facilitated the prompt penetration of the fixative agents. The specimens fixed in sublimate-alcohol or Zenker's fluid were washed in water for 48 hours and then transferred to 50 per cent alcohol, in which they were kept until worked out later for serial sections.

The larvæ showed a surprising resistance to phenol (3 per cent), formaldehyde (10 per cent), sublimate-alcohol (two parts of saturated aqueous solution of mercuric chloride and one part of absolute alcohol), and Zenker's fluid, being capable of surviving for at least 15 minutes. Some lived as long as 30 minutes in 10 per cent formaldehyde.

Monkeys and infected as well as normal guinea pigs, when not allowed to defend themselves, are eagerly bitten by the stegomyias. Rats, however, are seldom bitten by these mosquitoes, and bats have never been seen to be attacked when placed in a cage with hungry stegomyia females.

*Transmission Experiments with Stegomyia calopus from Man to Animals.*

Attempts were made to reproduce the appearances of yellow fever in the guinea pig by permitting the stegomyias to bite the animals after having fed upon the blood of yellow fever patients during the early stage of the disease. The procedure consisted in putting one arm of the patient into a cage containing 100 to 300 mosquitoes which had been hatched from the larvæ in captivity, and allowing the mosquitoes to feed until most of the females were engorged with blood. The cage containing the insects was then carefully kept in the shadow; sometimes the females were transferred to a separate cage. A glass of water containing a pea and a green twig was put into the cage, and each morning a slice of banana or papaya was placed upon the wire wall.

Since an enormous amount of labor would be required to test the infectivity of each female mosquito, summary experiments were made; that is, a dozen or more mosquitoes were allowed to bite one and sometimes two animals at the same time. The animals were then placed in separate cages and kept under daily observation for a period of at least 1 month, in case no infection took place within a shorter time. The tests for infectivity were usually made 2 weeks after the feeding on the patients, but sometimes earlier.

As the protocols show, positive leptospiral transmission was obtained in one of the following experiments.

*Experiment 1 (Negative).*—Aug. 5, 1918. 40 recently hatched females of *Stegomyia calopus* were allowed to become engorged by feeding on the right arm of Case 9 (G. C.), a severe case of yellow fever, admitted on the 3rd day of disease to the hospital. The patient recovered. Aug. 10. Sixteen females surviving. Eggs were found on the wet leaves in the glass. The sixteen mosquitoes surviving were allowed to feed on a very young normal guinea pig on that day; that is, 5 days after feeding on the patient's blood. All became engorged within the period from 12 m. to 2 p.m. The guinea pig was removed for observation into another cage. It remained well with normal temperature (38–39.5°C.) for 11 days, when it died of an intercurrent disease. There was no jaundice or change in the liver or kidney.

*Experiment 2 (Positive Transmission).*—Aug. 15. Twelve of the sixteen mosquitoes used in the above experiments still surviving. Larvæ in the glass. Aug. 28. Eight still surviving (23 days after feeding on the patient). A normal guinea pig was placed in the cage with these eight mosquitoes, which immediately attacked the animal, all becoming engorged. The guinea pig was removed to another cage and kept under observation. Temperature 38°C. Aug. 29, temperature 38°C.; Aug. 30, 40°; Aug. 31, 38.6°; Sept. 1, 39.3°; Sept. 2, 38°; Sept. 3, 37.5°; Sept. 4, 36°. Death occurred in 6 days.

*Autopsy.*—Moderately marked general jaundice. The lungs showed several small ecchymoses; the liver was yellowish brown and mottled; the kidneys were congested and showed ecchymoses; the stomach contained blood-stained food; the intestines showed numerous serous and mucous ecchymoses, and the stool was mixed with blood; the bladder was half full of yellowish brown urine which contained an abundance of albumin and casts; the adrenals were congested; the spleen, pancreas, and testes were apparently unchanged; the heart showed a few minute ecchymoses on the surface.

*Microscopic Examination.*—Examinations of emulsions of the liver, kidney, and blood for the leptospira were negative. The infectivity of the liver and kidney was tested on another guinea pig by injecting organ emulsions into the subcutaneous tissue. This animal died under the same symptoms in 7 days.

The leptospira was demonstrated in the kidney in small numbers, but not in the liver or blood.

Experiment 2 shows that the mosquitoes which sucked the blood of a yellow fever patient on the 3rd day of the disease were infective to the guinea pig after a period of 23 days. That they were unable to produce a typical infection when tested 5 days after the feeding is shown in Experiment 1.

*Experiment 3 (Negative).*—Aug. 5, 1918. Twenty-eight females were allowed to become engorged on the right arm of Case 22 (A. M.), a fatal yellow fever case, admitted to the hospital on the 4th or 5th day of the disease. The patient died on the 10th day with typical symptoms. Aug. 10. Twelve surviving. Aug. 12 (7 days after feeding). The mosquitoes were placed on a normal guinea pig. Several became engorged, and several were killed by the animal. This guinea pig showed no symptoms within the period of observation and was discarded.

Aug. 28. The surviving females were allowed to bite a normal guinea pig. The temperature rose to 39.9°C. on the 5th day but soon returned to normal and the animal showed no symptoms afterwards.

*Experiment 4 (Doubtful).*—Aug. 14, 1918, 4.15 p.m. A large number of stegomyias were placed on the left arm of Case 10 (M. N.), admitted on the 2nd day of the disease. 60 females which had become fully engorged were put into another cage. The patient died of typical yellow fever on the 7th day. Aug. 15, 9.30 a.m. Another feeding by the same mosquitoes on the same patient. Aug. 20. 34 surviving. Aug. 27 (13 days after feeding). 29 surviving. These were allowed to bite a normal guinea pig, and all became engorged. The guinea pig showed the following symptoms: Aug. 28, temperature 37.6°C.; Aug. 29, 38°; Aug. 30, 38.2°; Aug. 31, 39.5°; Sept. 1, 39.8°; Sept. 2, 40°; Sept. 3, 38.4°; Sept. 4, 38.1°; Sept. 5, 37.8°; Sept. 6, 38°; Sept. 7, 38.2°; Sept. 8, 38.1°. There was a trace of icterus in the scleras on the 8th and 9th days, which faded during the following day.

As the animal returned quickly to normal without further symptoms, a positive diagnosis was not possible, but it is probable that there was a mild infection.

*Experiment 5 (Negative).*—Aug. 14, 1918, 5 p.m. The right arm of Case 8 (R. V.), admitted on the 2nd day of disease, was put into a cage containing about 50 mosquitoes. Only five females sucked the blood within about 30 minutes. The case was severe but recovered. Aug. 15, 12 m. The feeding was repeated, 32 females becoming engorged immediately. Aug. 25. Many minute larvæ were found in the vessel containing water. Aug. 28, 10 a.m. The surviving females, thirteen in number, were allowed to feed on a normal guinea pig. All eagerly

engorged. The guinea pig was placed in a separate cage for observation. As no symptoms developed within a month it was discarded.

*Experiment 6 (Negative).*—Aug. 28, 1918, 12 m. 80 female stegomyias were allowed to engorge on Case 52, a girl of 10 years, whose brother had died in the hospital 4 days previously with yellow fever, but who was showing only a suspicion of icterus in the scleras which disappeared on the following day. The case was so mild that the patient never was confined to bed and left the hospital in 7 days. The temperature did not exceed 39°C. and remained at that point for the 1st day only. If the case was one of yellow fever it was extremely mild. Sept. 11. 23 females surviving. These were allowed to feed on a normal guinea pig; all became fully engorged immediately. The guinea pig was put into a separate cage for observation for 10 days. No symptoms developed and the animal was discarded after 3 weeks.

*Transmission Experiments with Stegomyia calopus from Animal to Animal.*

In earlier experiments<sup>8</sup> on *Leptospira icterohæmorrhagiæ* transmission of the disease from one guinea pig to another by means of *Culex pipiens* was unsuccessful. The present study with *Leptospira icteroides*, however, brought out the fact that *Stegomyia calopus* is capable of transmitting the experimental disease resembling yellow fever from an infected to a normal guinea pig. The positive results are few, notwithstanding the numerous attempts made, but are sufficiently conclusive to establish the main point, that this mosquito may serve as an intermediary host of *Leptospira icteroides*. The term intermediary host, however, is not used here in the sense understood in the case of certain protozoan organisms, which require an extrinsic host in which to pass their life cycle, but denotes that a certain length of time is necessary for multiplication of the organisms to such numbers that the mosquito may transmit enough to produce infection. From the biological and cultural properties of the organism this hypothesis seems reasonable, though the possibility of a stage of development in the mosquito has not been excluded. The following experiments indicate the positive transmission of *Leptospira icteroides* from animal to animal by the bite of the stegomyia mosquito.

<sup>8</sup> Noguchi, H., The survival of *Leptospira (Spirochæta) icterohæmorrhagiæ* in nature; observations concerning microchemical reactions and intermediary hosts, *J. Exp. Med.*, 1918, xxvii, 609.

*Experiment 7 (Negative).*—Aug. 4, 1918. 50 female stegomyias were engorged with the blood of a guinea pig which had been infected with the Case 1 strain of *Leptospira icteroides* 6 days previously. The animal was showing the organisms in the blood and had all the characteristic symptoms. Aug. 8. The mosquitoes fed on another guinea pig with beginning fever and albuminuria. The blood contained occasional leptospiras. Aug. 10. The mosquitoes which were used later in the experiments recorded below were allowed to bite a normal guinea pig on the 6th day after the first feeding. Only five mosquitoes attacked the animal. No symptoms followed the biting, and the guinea pig was discarded as negative on Sept. 13.

*Experiment 8 (Positive).*—Aug. 16 (12 days after the first and 8 days after the second feeding). Two normal guinea pigs were placed in the cage with the mosquitoes. Guinea Pig 544 was bitten by nineteen mosquitoes. The highest temperature was 39.6°C. on the 5th day, and the animal became mildly icteric on the 8th day. On the 12th day the temperature dropped to 37° and death ensued.

*Autopsy.*—Fatty degeneration of the liver, recent hemorrhagic spots in the lungs, semidigested bloody contents in the stomach and intestines, acute parenchymatous nephritis, and some ecchymoses in the lymph glands. The icterus was very slight. The leptospira was demonstrated under the dark-field microscope.

Guinea Pig 545 was bitten by nine other mosquitoes on the same day. The highest temperature was reached on the 6th day, but the animal gradually returned to normal without any icterus. It remained well for 24 days and was then subjected to a second infection with the same strain of the organism on Sept. 7. It proved refractory to the infection.

*Experiment 9 (Negative).*—The mosquitoes used in Experiment 8 were kept for another experiment, but within 5 days only eight of the twenty-eight still survived. These were allowed to feed on another normal guinea pig (No. 571) on Aug. 21. The animal showed no symptoms after the biting and was discarded as negative on Sept. 7.

*Experiment 10 (Positive).*—Aug. 13, 1918. In this series four guinea pigs (Case 1 strain) infected at different stages of the disease, 6th, 7th, 8th, and 9th days after the inoculation, all showing the symptoms, some intensely icteric, and some showing the leptospiras in the blood, were used to infect the mosquitoes in four separate cages. Three of the guinea pigs died subsequently, and one recovered. 142 engorged females in all were collected and put together in one cage for further experiments.

Aug. 21 (8 days after feeding on the infected guinea pigs). There were 83 mosquitoes surviving, and these were allowed to bite a normal guinea pig. All the mosquitoes sucked the blood eagerly. The protocol of this animal follows.

*Guinea Pig 570.*—Aug. 21. Bitten by 83 stegomyias. Temperature Aug. 22, 38.2°C.; Aug. 23, 38°; Aug. 24, 38.1°; Aug. 25, 39.8°; Aug. 26, 38°; Aug. 27, 37.2°; Aug. 28, 36.8°; Aug. 29, 37.6°; Aug. 30, 37°; Aug. 31, 36.2°; Sept. 2, 37.2°; Sept. 3, 36.5°. Death occurred during the night 13 days after the animal had been bitten.

*Autopsy.*—Jaundice general but mild; hemorrhages in the lungs, stomach, and intestines; liver yellowish brown; kidney intensely congested, containing some minute ecchymoses, yellowish; bladder filled with yellowish brown urine with albumin and casts. Ecchymosis in the serosa and mucous membranes in general. Very few of the leptospiras in the kidney, none in the liver or blood.

*Experiment 11.*—To supplement the foregoing experiments, on Aug. 23, 1918, that is 2 days later, the same mosquitoes, twenty-five in all, were crushed in a mortar and emulsified in Ringer's solution. The emulsion was examined for the leptospira under the dark-field microscope and also smeared over the scarified surface of the skin of a normal guinea pig. Occasional specimens of leptospira were found in the emulsion after long search.

The guinea pig, No. 544 A, came down with the typical symptoms on Aug. 31; that is, 8 days after the inoculation. In the liver and kidney the leptospira was demonstrated in small numbers under the dark-field microscope.

This experiment conclusively proves that the leptospira was present in the body of the stegomyia mosquitoes which had been fed on the infected guinea pig and were capable of transmitting infection by their bite to another animal. The course of the infection produced by smearing the mosquito emulsion over the skin was more rapid than that caused by the bite.

*Experiment 12 (Negative).*—Some of the mosquitoes used in Experiment 10 which had caused a positive transmission of *Leptospira icteroides* after 8 days from the time of feeding on infected guinea pigs were kept another week. As already stated, twenty-five of these 83 mosquitoes were crushed on Aug. 23 for a supplementary confirmation (Experiment 11) of Experiment 10. Subsequently most of the remaining females laid eggs, and some perished. On Aug. 28, 7 days after Experiment 10, only thirteen were left. On that date a normal guinea pig (No. 633) was placed in the cage, and all the mosquitoes became engorged. The guinea pig was kept under daily observation for 13 days, but there was no suspicion of infection, and it was discarded on Sept. 10.

*Experiment 13.*—Aug. 11, 1918, 11 a.m. An infected guinea pig (Case 1 strain) showing the typical symptoms was placed in a mosquito cage. Twenty-four engorged females were collected and put into another cage. Aug. 14. Sixteen additional engorged females were also put into the cage, making the total 40. Aug. 21 (10 days later). A normal guinea pig was placed in the mosquito cage. The mosquitoes fed eagerly, all becoming engorged within 10 minutes. This animal (Guinea Pig 572) remained apparently well for 11 days. It was found dead on Sept. 2. At no time did the morning temperature exceed 38.4°C., which was about normal for this animal.

*Autopsy.*—A trace of jaundice throughout the body; liver highly degenerated and pale yellow; a few ecchymoses in the lungs; kidneys much congested. There was too scanty a quantity of urine in the bladder to examine for albumin. Diagnosis: probably a mild infection with *Leptospira icteroides*.

## SUMMARY.

The foregoing experiments show that symptoms and lesions closely resembling those of yellow fever in man may be induced in guinea pigs by the bite of female stegomyias that have previously sucked the blood of a yellow fever patient or of an animal experimentally infected with *Leptospira icteroides*. With mosquitoes infected directly from a yellow fever patient the infectivity seems to become manifest after a longer period of incubation than with those infected with the animal blood. In the former, at least 12 days are said to be necessary before they become infectious, and this hypothesis seems to be borne out by the present experiment. On the other hand, the mosquitoes which were engorged with the infected blood of the guinea pig were found to be capable of transmitting the disease within 8 days after the feeding. This discrepancy may be explained by the fact that the number of leptospira existing in experimentally infected guinea pigs is far greater than that in human blood.

The frequency with which positive transmission by the stegomyia was obtained in both instances was very small indeed, in view of the number of mosquitoes employed. It appears that even under natural circumstances the percentage of mosquitoes that eventually become infected with the yellow fever microbe by sucking the blood may be very small. It has already been shown by previous investigators<sup>2,5</sup> that to transmit yellow fever from a patient to a non-immune person requires from 0.1 to 2 cc. of blood at the height of disease. According to my estimate a female stegomyia may take up 0.01 cc. or even less. Apparently a mosquito occasionally becomes infectious by taking up the one or two organisms which happen to be circulating in the peripheral blood of man, and it is these occasionally infected few which carry the disease. It is not difficult to realize the extent of ever increasing danger from a constant supply of the microbic virus which an endemic center or an epidemic of yellow fever can provide. One infected mosquito may mean many patients, and the life of such a mosquito is usually longer than that of the persons whom it fatally infects.

Finally, it is of interest to note that the development and maintenance of *Leptospira icteroides* are indispensably associated with the blood constituent, the serum, and this is amply supplied by the blood-

sucking insect. The organism is one of the most fragile of all the pathogenic parasites and cannot survive the concurrence of other less fastidious organisms such as bacteria. The comparatively aseptic body cavity of the stegomyia<sup>9</sup> furnishes a secure shelter for the parasite, which undoubtedly penetrates the zone of safety as soon as it is taken into the stomach of the insect. Unlike many other parasites this organism is capable of penetrating the intact skin or a bacteria-proof filter, and hence it is probably an easy matter for it to pierce the tissue of the visceral organs of the mosquito. Whether or not *Leptospira icteroides* can survive and multiply only in the body of *Stegomyia calopus* and not in other varieties or genera is yet to be determined.

Another interesting fact with regard to the extrinsic life of this organism is that it can multiply steadily at a temperature from 18–37°C. The optimum temperature, at which it remains viable for many months, is 26°. The climate in most of the tropical countries offers optimum conditions both for *Leptospira icteroides* and for the mosquito which carries and nourishes it.

<sup>9</sup> This refers to the presence of bacteria and not certain higher plant parasites (yeast, moulds, etc.) or protozoa which have been occasionally found in stegomyia mosquitoes. These non-bacterial organisms may exert no adverse influence upon *Leptospira icteroides*.