The benzidine test detects blood in traces corresponding to a dilution of 1 in 300,000 (McNally, 1939). It is best performed thus: to a small cutting or scraping of the stain placed on a white dish are added a drop of a saturated solution of benzidine in glacial acetic acid and a drop of 3 per cent hydrogen peroxide; a brilliant blue coloration develops immediately (Lloyd, 1935). The basis of the reaction is the oxidation of the benzidine by oxygen liberated from the peroxide by a peroxidase present in hæmoglobin or its derivatives.

That fruits and vegetable juices, particularly the potato juice, also provide the oxidase for the oxidation of the benzidine is known. That the plant juice contains auto-oxidizable substances of a phenolic nature which are capable of combining directly with atmospheric oxygen to form organic peroxides from which oxygen is readily transferred to an oxidizable substance by a peroxidase present in the same juice, is also known (Parsons, 1933).

The clay bentonite has also been found to react with benzidine. It reacts like the plant juice containing the auto-oxidizable substances and also like the potato juice. The scrapings from its exposed surface turn the benzidine greenish blue, even in the absence of hydrogen peroxide. The same scrapings after the removal of (loosely bound ?) oxygen, by submersion in an excess of physiological saline overnight, cease to affect the benzidine in the absence of the peroxide: the coloration now develops only on the addition of the latter reagent. The scrapings after they have been dried and exposed to air react without hydrogen peroxide again.

The clay does not give the reaction of thallium with benzidine.

The initial chemical reaction of the clay, although slower than that of blood, creates a presumption in favour of presence of blood. The presumption is not disposed of until a negative spectroscopic test is obtained. This simulation of the reaction of blood by an inorganic substance, even at only one step of the routine, is recorded in the interest of forensic chemistry.

The writers are indebted to the Geological Survey of India, Calcutta, for the identification and the following brief description of the clay :—

'Bentonites are clays that have the clay mineral "montmorillonite" with the chemical composition of $(MgCa)O.Al_2O_3.5\ SiO.nH_2O$, for their chief constituents. These clays are of very small grain size and absorb large quantities of water, swelling enormously in the process, and they have the property of remaining in suspension in their water dispersions.

The swelling property is reversible; the clay can be dried and re-swelled an infinite number of times; this activity remaining unaffected by temperatures below 450°F. It can be readily distinguished from other clays by its reaction with benzidine.

*Uses.*—Bentonites are used in metal foundries as a bonding material for moulding sands, in drilling muds, as a detergent in the laundry, as a plasticizing agent in ceramic materials, as a standard suspending, spreading and adhesive agent in horticultural sprays and

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## STUDIES ON *LEPTOSPIRA ICTERO-HÆMORRHAGIÆ* IN RATS IN BOMBAY CITY

By M. N. LAHIRI

(*From Haffkine Institute, Bombay*)

ACCORDING to Ido, Hoki, Ito and Wani (1916 and 1917) it was Miyajima who first drew attention to the finding of spirochætes resembling *Leptospira icterohæmorrhagiæ* in the kidneys of the field mouse. The discoverer subsequently reproduced the disease in the guinea-pig by inoculation of the infected kidney of the field mouse. These workers, acting on this suggestion, examined a number of wild rats. The presence of similar spirochætes were detected in the kidney or urine of the rats and were also demonstrated by means of guinea-pig inoculations. Since then, investigations have been carried out in different parts of the world by different workers. High prevalence of infection among rats has been observed both in places where human cases have been known to have occurred and in places where the disease in man is unknown. Langworthy and Moore (1927), for instance, detected a high incidence of the infection among rats in the New York State, 40 per cent by direct examination and 60 per cent by serological tests, and yet as far as is known no human cases have been reported from that area.

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insecticides, in clarifying turbid waters and purifying sewage, in gelatinizing wet-mash poultry foods, for clarifying wines, in cosmetics, and pharmaceuticals.'

The original specimen of the clay which was found to react with benzidine was an exhibit designated 'stone' in a murder case. Other specimens resembling it in appearance, consistency and reaction were obtained from the aforesaid department. It was a compact lump of a bluish-white substance which could be easily scraped and ground.

The use of the clay in cosmetics is worthy of note. A brand of talcum powder was found to react with benzidine. It is also noted that the reaction between benzidine and bentonite is known to geologists.

*An acknowledgment*

The writers are indebted to V. P. Sondhi, Esq., M.B.E., M.Sc., F.G.S., of the Geological Survey of India, Calcutta, for the aid received.

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Anderson and Wagle (1933) first pointed out the existence of infection in rats in the city of Bombay. The present investigation was undertaken to carry out a systematic examination of rats not only to demonstrate the infection but also to isolate and identify the strains with particular reference to their serological classification. Because, if the murine strains and human strains when obtainable are serologically identical, it might throw some light on the possibility of the rat playing a part in the transmission of the disease. For this purpose, rats from a particularly congested area of this city were trapped and brought alive to the laboratory. In all a series of 125 rats was examined.

## *Methods*

1. *Direct examination.*—It has been shown by several workers that the leptospiræ in rats are confined to the convoluted tubules of the renal cortex where they are discretely localized. Therefore, major portions of the cortex of both the kidneys from each animal were emulsified in sterile normal saline and care was taken to break up the tissues thoroughly. The emulsion thus prepared was examined by dark-ground illumination. Three preparations were examined before returning a sample as negative.

2. *Animal inoculation.*—As a measure of economy it became necessary to pool the kidney emulsions of three or four rats in which leptospiræ could not be detected by dark-ground illumination. Three c.cm. from each of the mixtures thus made were inoculated intraperitoneally into a young guinea-pig, whereas in those emulsions showing leptospiræ a separate guinea-pig was used in each case for the infective material. The peritoneal fluids of the guinea-pigs were aspirated and examined on the 4th, 6th and 7th day after inoculation for the presence of leptospiræ. As a rule, the animals were kept under observation until six weeks after inoculation.

3. *Culture of the kidney cortex.*—Small pieces of the tissue were inoculated into Vervoort's medium and incubated at room temperature (21° to 29°C.). The cultures were examined regularly at intervals from the 8th day onwards to the end of three weeks after inoculation.

4. *Serological testing of the rat's serum.*—As soon as the rat was killed by means of chloroform the chest wall was opened aseptically and blood was collected from the heart with a fine capillary pipette. The rat's blood in general clots very quickly after death. The sera of rats were tested for the presence of agglutinins and lysins against a classical strain of *L. icterohæmorrhagiæ* kindly supplied by Dr. B. M. Das Gupta of the School of Tropical Medicine, Calcutta. Varying dilutions of each serum ranging from 1 in 10 to 1 in 80 were first made. An equal volume of rich young live culture in Vervoort's medium was added to each of the dilutions of the serum. The final dilutions of the serum, therefore, were 1 in 20 to 1 in 160. As a control, rabbit or normal human sera in dilution of 1 in 10 was used. The tubes were incubated at 37°C. for 2½ hours and at the end of this period samples from each tube beginning from the highest dilution of serum were examined by dark-ground illumination. Further dilutions were not necessary except in only one instance when dilutions up to 1 in 640 had to be made.

## *Observations*

1. The leptospiræ were demonstrated in only eleven specimens of the kidney emulsions examined by dark-ground illumination. Incidental to the search for leptospiræ in these animals it will be of interest to record that *Trypanosoma lewisi* were seen in the blood of five rats.

2. Forty guinea-pigs were inoculated with the kidney emulsions. These animals may be arranged in the following groups :—

(*a*) Ten guinea-pigs were injected with infective material positive by the above method. One specimen, although it showed leptospiræ, could not be inoculated as guinea-pigs were not available. Of these ten, four failed to show any evidence of leptospiral infection and remained alive and well during the observation period. Four animals succumbed to the infection with typical signs, from the culture of the heart's blood of three animals, strains of leptospira were obtained. With regard to one the animal died late in the night hence its liver emulsion showing numerous leptospiræ was inoculated into a second animal for the isolation of the strain which, however, did not show any evidence of infection. A month later, far beyond the usual fatal period, this animal received a massive inoculation with heavily infected guinea-pig liver, but it remained insusceptible to infection. In the remaining two animals, although they died within the usual fatal period, there was no jaundice, and characteristic lung hæmorrhages as well as hæmorrhages in the groin were the visible signs of infection. No leptospiræ were seen either in the body fluids or in any organs. Buchanan (1927) also recorded similar findings in his studies.

(*b*) Of the remaining thirty animals which received the pooled emulsion only two died of infection and at autopsy exhibited the complete picture of the disease. The heart's blood collected almost immediately after death was cultured and thus two strains of leptospira were obtained. This brings out an important fact that, if only very few leptospiræ are present in the kidneys, an infected rat may escape detection by the direct method but infection can be demonstrated by animal inoculation.

3. Kidney substance of seventy-eight rats was cultured. In all twenty-seven were free from bacteria of which only two gave a pure growth of leptospira. The positive cultures obtained were, however, from rats which showed leptospiræ in their kidneys. The rest showed bacterial growth and in several instances a motile organism was isolated which on culture was identified as *Bact. pseudo-asiaticum.* Evidently, the rats had this bacterial infection. Das Gupta (1940) also reported similar heavy infections with salmonella-group organisms among rats in Calcutta.

4. Sixty-eight samples of sera were examined of which only six gave positive reaction. Four samples reacted in a dilution of 1 in 20 and one in a dilution of 1 in 80. The sixth sample agglutinated up to a titre of 1 in 640. Of these six positive specimens four were collected from the animals in whose kidneys leptospiræ were seen. It is difficult to draw any conclusion from such a low titre. It may be coagglutinins or that the serological properties in rats may persist only for short periods. With titre of 1 in 80 it

is possible that the rat was infected with the classical strain whilst the titre of 1 in 640 is definitely suggestive of infection.

The five strains of rat leptospira recorded above, all reacted up to the full titre with anti-leptospiral serum (classical).

*Comment*

From the above studies it is evident that the dark-ground method of examination yields the best results for the demonstration of infection. But for the purpose of isolation of the organism either the kidney cortex must be cultured or an emulsion of the ground-up kidney tissues inoculated into a guinea-pig. As regards culture, although a large number of positive results have been recorded by many observers, in my experience the results were far from satisfactory. This was in most instances due to the simultaneous infection by *Bact. pseudo-asiaticum* in rats, already referred to.

With regard to the animal inoculation, it may be mentioned that the method has obvious advantages. Even infected rats which may escape detection by the direct method when scanty leptospiræ are present often show infection by this method. But the drawback is that in certain guinea-pigs the disease could not be reproduced. It may be either due to variations in virulence of the organism or due to individual resistance towards susceptibility to infection. Observation recorded under animal inoculation lends strong support to the later contention. Several workers by means of serological tests have demonstrated infection in rats and some investigators reported the titre to be very high. It is shown, however, that in the series most of the infected rats reacted in a very low titre, whereas positive reactions were obtained in two rats not carrying leptospiræ. These observations, therefore, confirm the findings of Zimmermann (1930). Thus, the importance of the combination of various methods is stressed.

*Summary*

The kidney cortex of one hundred and twenty-five rats trapped in one congested area of the city were examined by dark-ground illumination and by animal inoculations. Seventy-eight specimens of kidney tissues were cultured in Vervoort's medium. Sixty-eight samples of sera were tested for the presence of agglutinins and lysins.

Leptospiræ were detected in the kidneys of eleven rats by direct method and of only two by animal inoculations. Two other animals showed evidence of infection serologically. The rats found to be infected by the above methods were all of the species *Rattus norvegicus* except one which was of the species *Rattus rattus* and whose serum was found to contain anti-leptospira agglutinins with a titre of 1 in 640. The five strains of rat leptospira isolated all belong to the classical group.

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# OBSERVATIONS ON THE RELATIONSHIP BETWEEN MALARIA AND PISTIA IN TWO VILLAGES IN MURSHIDABAD DISTRICT

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and

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ONE of the writers (P. C. R.) during the course of his duties as health officer of Murshidabad district has made extensive observations since 1928 on the epidemiology of malaria in relation to the presence of pistia and finally applied the results to practical tests in several isolated highly endemic localities in the district. From the success obtained by systematically removing pistia from the breeding places, he concluded that malaria and pistia were interrelated at least in the district of Murshidabad.

He suggested that mosquitoes bred out from larvæ which have opportunities to feed on pistia become susceptible to plasmodial infection. According to him, excepting *Anopheles subpictus* and *A. vagus*, any and every anopheline mosquito breeding in association with pistia is dangerous to the community. It should, however, be recalled that it is not known whether larvæ really feed on pistia.

Laboratory experiments, on the other hand, conducted by one of the writers (D. N. R.) with *A. stephensi*, showed no difference whatever in the comparative infectivity in two samples, one bred out with pistia and the other without it. In these experiments larvæ were collected in nature and were not reared from eggs.

It is known that mosquito larvæ are dependent for their breeding chiefly on the ecological conditions of the water which are set up by

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