THE SELECTION OF A STRAIN OF *BACILLUS PESTIS* FOR THE PREPARATION OF VACCINE, WITH SPECIAL REFERENCE TO THE EFFECT OF ANIMAL PASSAGE ON VIRULENCE.

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In 1926 a number of experiments were carried out at the Medical Research Institute, Accra, in connection with the preparation of plague vaccine. In this paper are described only those which illustrate certain factors governing the selection of a strain of *B. pestis*.

I. METHODS.

Experimental Animal. The animal used was *Cricetomys gambianus*, the pouched rat, which is now the commonest domestic rat of Accra. It is very susceptible to inoculation with plague, as the following figures show. During the period April–August, 209 unvaccinated specimens were infected by hypodermic inoculation of pure culture. Of these, 67 received one of the so-called avirulent cultures, to be described presently, and 10 (15 per cent.) recovered. The remaining 142 were infected with unmodified cultures, some of which had not been subjected to animal passage for as much as nine months. Of this number, one died $3\frac{1}{2}$ hours after infection without any sign of plague, two were killed after 44 hours, their spleen films showing heavy plague infection, and the remaining 139 all died spontaneously in an average time of 53 hours, spleen films showing numerous plague bacilli in every case. Unmodified cultures therefore gave a mortality of 100 per cent.

Methods of observation and recording. The majority of vaccinated animals also died after infective inoculation, consequently a mere statement of mortality would furnish but little result. The time elapsing between infection and death (survival period of an animal or killing time of a culture) is therefore considered in estimating the virulence of a culture, susceptibility of an animal or protective value of a vaccine.

Virulence is assumed to be inversely proportional to killing time, a period of 408 hours (17 days) being allotted to animals which recover.

In estimating the protective value of a vaccine, the difference between the survival period of each animal and the average survival period of the controls is first obtained, allotting a difference of 360 hours to animals which recover.

The average difference is then worked out and the ratio of this to 360 gives the efficiency of the vaccine. An example is given later.

The figure 408 was chosen on the assumption that an animal which lived 408 hours after infection had definitely recovered. It was obtained by adding 48 hours, as a margin, to the longest survival period (360 hours) obtained out of all the animals infected (351), excluding those which recovered. The maximum "difference" allotted to vaccinated animals was $408 - 48 = 360$, because 48 hours was the average survival period of controls.

Observation was interrupted between 9.30 p.m. and 6 a.m., and to adopt a uniform rule animals which appeared well at 9.30 p.m. and were found dead at 6 a.m. were regarded as having died at 4 a.m.

Two strains of *B. pestis* were used. They were both from human cases. One was obtained by Dr Young at Sekondi in March 1924, and the other by Dr Connal at Lagos in December 1925. In this paper, cultures are distinguished by the letter *L* or *S* (Lagos or Sekondi) and a number. A new number (not usually consecutive) was given to a culture after each animal passage.

Douglas' culture medium, prepared without salt, was used in most of the experiments.

II. CERTAIN FACTORS AFFECTING VIRULENCE.

In the selection of a strain of organism for the preparation of vaccine, virulence is regarded as of prime importance, and the experiments to be described support this view. Certain factors affecting virulence will first be considered.

THE EFFECT OF LONG CULTIVATION.

Two experiments with old agar cultures, which had been kept at room temperature and sub-cultured at infrequent intervals, did not indicate any loss of virulence.

(1) Strain *S* was used, and the last passage was through a guinea-pig nine months previously.

June 29th. 8 *C. gambianus* were infected by hypodermic inoculation of 0·2 c.c. of a 4-day broth culture.

All died in the following times: 40, 40, 42, 45, 46, 53, 56, 64 hours.

Average survival period, *i.e.* killing time of culture = 48 hours.

(2) Strain *L* was used, and the last passage was through a guinea-pig, which died in 96 hours, seven months prior to the experiment.

July 10th. 8 *C. gambianus* were infected by hypodermic inoculation of 0·2 c.c. of a 4-day broth culture.

All died in the following times: 40, 46, 47, 49, 49, 56, 64, 64 hours.

Killing time of culture = 52 hours.

The virulence was not tested at the beginning of the period of culture, but it will be seen that the final virulence was not less than the average virulence of all the so-called virulent strains (average killing time = 53 hours), many of which were 3 days or less ex body, and moreover the killing times

(48 and 52 hours) are so short, that the virulence cannot be far short of the maximum.

The Effect of Animal Passage.

Loss of virulence on passage through an immune animal. A marked fall in virulence occurred when a strain was subjected to passage through a vaccinated animal, which died of plague a long time after infection, *i.e.* an animal possessing well-marked but incomplete immunity.

The following experiments illustrate this:

(1) April 19th. 7 vaccinated *C. gambianus* and 5 unvaccinated ones, serving as controls, were infected by hypodermic inoculation of broth culture 31 *L*, the dose being 20 million bacilli.

All the controls died in the following times: 44, 57, 57, 57, 70 hours.

Killing time of 31 *L* = 57 hours.

The organism was recovered in culture (38 *L*) from a vaccinated animal which lived 186 hours, and after two days' growth was inoculated (dose 0·4 c.c. broth culture) into 8 unvaccinated *C. gambianus* with the following results:

2 recovered (re-inoculated with virulent culture after 32 days, again recovered).

6 died in the following times: 75, 87, 105, 108, 149, 213 hours.

Killing time of 38 *L* = 194 hours.

Thus the killing time of the culture was increased from 57 to 194 hours by passage through an immune animal.

(2) May 6th. 8 vaccinated *C. gambianus* and 6 controls were inoculated with 0·4 c.c. of a 2-day broth culture, 44 *L*.

All controls died in the following times: 36, 36, 36, 44, 44, 50 hours.

Killing time of 44 *L* = 41 hours.

The organism was recovered in culture (57 *L*) from a vaccinated animal which lived 300 hours (*C. g.* 27). The virulence of this culture was tested on three occasions with the following results:

(*a*) May 22nd. 7 *C. gambianus* received 0·4 c.c. broth culture 57 *L*, 3 days ex *C. g.* 27.

1 recovered (killed after 20 days).

6 died in the following times: 108, 132, 170, 258, 276, 276 hours.

Killing time of 57 *L* = 233 hours.

(*b*) June 29th. 6 *C. gambianus* received 0·2 c.c. of a 2-day broth culture 57 *L*, 41 days ex *C. g.* 27.

All died in the following times: 33, 162, 162, 165, 178, 316 hours.

Killing time of 57 *L* = 169 hours.

Films from the animal which lived only 33 hours showed *B. pestis* in the lumbar glands, but none in liver or spleen. It is probable therefore that plague was not the sole cause of death. If this animal is excluded the killing time becomes 197 hours.

(*c*) July 14th. 10 *C. gambianus* received 0·2 c.c. of a 12-day agar growth emulsion of 57 *L*, 56 days ex *C. g.* 27.

2 recovered (re-inoculated with virulent culture after 21 days, died 140 and 252 hours later, but not of plague).

8 died in the following times: 71 (*C. g.* 215), 94, 102, 113, 143, 144, 161, 185 hours.

Killing time of 57 *L* = 183 hours.

Thus, by passage through an animal with well-marked acquired immunity, the killing time of the culture was increased from 41 to about 200 hours. It then remained approximately constant during 56 days cultivation.

Failure to restore virulence. An attempt was made to restore the virulence of the culture by infecting a series of *C. gambianus*, recovering the organism from the one which died first, *i.e.* the most susceptible, infecting another series and so on. Three series were used but the attempt failed, as the following details show:

(**1**) July 22nd. 8 *C. gambianus* were injected with 0·2 c.c. of a 2-day broth culture, 92 *L*, 5 days ex *C. g.* 215, which was infected with 57 *L* and lived 71 hours.

2 recovered (re-inoculated with virulent culture after 21 days, 1 recovered and 1 died 168 hours later, but without signs of plague).

6 died in the following times: 64 (*C. g.* 232), 109, 148, 156, 183, 288 hours.

Killing time of 92 *L* = 220 hours.

(**2**) July 29th. 8 *C. gambianus* were infected with 0·2 c.c. of broth culture, 98 *L*, 4 days ex *C. g.* 232, which lived 64 hours.

All died in the following times: 89 (*C. g.* 274), 89, 89, 95, 108, 113, 144, 185 hours.

Killing time of 98 *L* = 114 hours.

(**3**) August 4th. 8 *C. gambianus* were infected with 0·1 c.c. of broth culture, 106 *L*, 2 days ex *C. g.* 274, which lived 89 hours.

One died in 58 hours and is excluded from the calculation, because there was no definite evidence of plague *post mortem*. Spleen and lumbar glands were quite normal macroscopically, films from spleen, liver and lumbar glands showed a few putrefactive bacilli, and of 4 cultures 2 were contaminated and 2 remained sterile. The autopsy was made 12 hours after death.

Details of the remaining 7 are as follows: 1 recovered (killed after 22 days).

6 died of plague in the following times: 114, 120, 128, 160, 175, 236.

Killing time of 106 *L* = 192 hours.

The successive killing times were therefore 183, 220, 114 and 192 hours. Thus the last culture appeared to be no more virulent than the first, but inferences drawn from this are weakened by the wide fluctuation of the intermediate figures.

Virulence for guinea-pigs. To ascertain whether loss of virulence, produced by passage through an immune *C. gambianus*, applies to other animals, only one experiment has so far been made. Guinea-pigs were used, and, to determine their susceptibility to virulent culture, 5 were inoculated (July 16th) with 0·2 c.c. of broth culture 75 *L*, 33 days ex *C. g.* 153:

All died in the following times: 64, 123, 125, 126, 196 hours.

Killing time of 75 *L* for guinea-pigs = 127 hours.

At the same time 5 guinea-pigs were inoculated with 0·2 c.c. of a 2-day broth culture, 57 *L*, 58 days ex *C. g.* 27:

All died in the following times: 114, 138, 138, 258, 294 hours.

Killing time of 57 *L* for guinea-pigs = 188 hours.

C. g. 153, from which culture 75 *L* was taken, lived only 37 hours after infection with 72 *L* (mentioned later), and it was therefore supposed that 75 *L* would be highly virulent. But, judging from the length of their survival periods, it was not very virulent for guinea-pigs, and a subsequent test showed that it was not very virulent for *C. gambianus* either, for three *C. gambianus*, infected with it, had an average survival period of 96 hours. The experiment is rather spoiled by this unexplained loss of virulence, but as far as it goes it indicates that loss of virulence, produced by passage through an immune *C. gambianus*, applies not only to this species, but to guinea-pigs as well, though not perhaps to the same degree.

References in the literature to the effect of animal passage on virulence. The loss of virulence caused by passage through an immune animal may explain certain conflicting experimental results obtained by the earlier investigators.

Hankin (1898), working in India, found that the plague bacillus when transmitted directly from rat to rat lost virulence rapidly and failed to kill the third or fourth rat. From his account it appears that the rats were wild ones and that only one was used at each passage. On the other hand, passage through white mice increased the virulence of the organism.

Otto (1902), working in Berlin, obtained quite different results and succeeded in making 64 successive passages through rats without lessening the virulence of the organism. As a rule he used two rats at each passage.

To put the matter to further test, the Indian Plague Commission (*Ind. Pl. Com. J. of Hygiene*, 1906, pp. 496, 502) made two series of experiments. In each of these 26 passages were made from rat to rat, with no indication of loss of virulence. Wild Bombay rats were used. In the first series inoculation was subcutaneous and usually 3, and sometimes 6, rats were infected at each passage, the animal showing the greatest number of *B. pestis* in films being used to infect all the animals of the next passage. In the second series the animals were infected by scarification and a larger number (6 to 45) was infected at each passage. The report states that 59 per cent. of animals were immune to cutaneous infection.

As suspected by Hankin, the reason for his failure to pass *B. pestis* through

more than 3–4 rats in succession appears to be the frequent immunity of Indian rats. As he used only one rat at each passage, he fell foul of an immune one in the course of three or four successive inoculations, whereas the Indian Plague Commission, by using several at each passage and selecting one with well-marked septicaemia for further passage, avoided the immune ones. Again, Hankin's white mice were presumably laboratory bred and Otto's rats were obtained in Germany, and consequently neither had been exposed to plague infection and there were no immune animals among them.

Cultural and morphological characters. Dr H. Schütze, of the Lister Institute, has kindly examined some of the cultures mentioned in this paper. He informs me that, judging from salt-stability tests and behaviour in broth culture, the avirulent strain 57 *L* appears to be a rough variant.

Previous to this it was noticed that avirulent cultures in Douglas salt-free broth differed from virulent ones in their greater tendency to become clear. At the end of 24 hours at 30° C., the avirulent growth is usually confined to the surface and bottom of the liquid, the bulk of the medium being perfectly clear, whereas virulent broth cultures are usually somewhat turbid for the first 2 or 3 days.

Morphological differences are more definite, avirulent forms being characterised by greater length and breadth and greater irregularity in shape. In broth cultures of virulent strains the bacilli, as seen in the dark field, occur mostly in chains about 10μ long. Individual bacilli are 1·5μ to 4μ (usually 2·5μ) in length and less than 1μ broad. Their ends are rounded, the shorter bacilli being oval. Avirulent bacilli on the other hand are of much greater and more variable length. Individuals of 20μ are not uncommon and chains 40μ long, consisting of only 3 or 4 bacilli, occur frequently. The breadth is usually 1μ to 1·5μ, and may vary along the length of a single organism giving it an irregular bulging outline.

Appearance on salt agar. Avirulent bacilli in fact are similar in appearance to the involution forms which develop on salt media. The effects of salt on a virulent and an avirulent strain were compared, by growing each on Douglas agar, to which 2 per cent. salt had been added, for 4 days at 30° C. Before the experiment the morphological differences between the two were well marked, but, while the salt medium scarcely affected the avirulent bacilli, it made the virulent ones longer, broader and more irregular in shape, so that at the end of the period of cultivation there was scarcely any difference between the two.

III. VACCINATION EXPERIMENTS.

In addition to ordinary vaccine tests, in which virulent antigen and virulent infecting cultures were used, the protective values of (1) virulent antigen against avirulent infection, and (2) avirulent antigen against virulent infection were investigated. By virulent antigen is meant vaccine prepared from

virulent culture, and by avirulent antigen, vaccine prepared from culture rendered relatively avirulent by passage through an immune animal.

Vaccines were made from cultures grown on Douglas salt-free agar, sterilised with 3 per cent. phenol and standardised to contain 3000 million bacilli per c.c., a dark field counting method being used. Doses of 0·5 c.c. and 1 c.c. were injected with an interval of one week.

To test the protection afforded by virulent antigen against avirulent infection, a vaccine (No. 26) made from a culture with a killing time of 41 hours was used.

As a preliminary the following experiment is given to show the protective value of vaccine No. 26 against virulent infection.

Virulent antigen v. *virulent infection.* 7 *C. gambianus* were vaccinated. None died during the immunisation period. These 7 animals together with 8 controls were infected (June 11th), 14 days after the second vaccination, with 0·2 c.c. of a 3-day broth culture, 72 *L*, injected hypodermically.

All the controls died in the following times: 37, 46, 50, 50, 51, 52, 61, 61 hours.

Average survival period of controls = 51 hours.

Of the 7 vaccinated animals:

1 recovered (killed after 24 days).

6 died in the following times: 43, 47, 51, 66, 81, 152 hours.

The difference between each of these survival periods and the average survival period of the controls is: − 8, − 4, 0, 15, 30, 101 hours, and allotting a "difference" of 360 hours (as described under "Methods") to the animal which recovered the average difference is 70·5 hours, and the ratio of this to the greatest possible difference (360 hours) which would exist if all the animals recovered is $\frac{1}{5}$ or 20 per cent.; 20 per cent. then represents the efficiency of the vaccine.

It may be remarked that this is one of the poorest results obtained with vaccines similarly prepared. Efficiency factors were usually over 30 per cent., and in one experiment, in which 12 animals were infected and 5 recovered, it was 49 per cent..

Virulent antigen v. *avirulent infection.* Three weeks after the last experiment was begun 12 *C. gambianus* were inoculated with vaccine No. 26; 3 died in the immunisation period. Seven days after the second vaccination (June 29th) the remaining 9, together with 6 controls were inoculated with 0·2 c.c. of a 2-day broth culture of the avirulent culture, 57 *L*.

Details of the controls have already been given, but may be repeated to facilitate comparison.

All the controls died in the following times: 33, 162, 162, 165, 178, 316 hours.

Average survival period of controls = 169 hours.

Details of the 9 vaccinated animals are as follows:

All died in the following times: 64, 75, 80, 88, 100, 100, 127, 135, 350 hours.

Average survival period of vaccinated animals = 124 hours.

The average survival period of the vaccinated animals is actually shorter than that of the unvaccinated ones, and the efficiency of the vaccine worked out according to the method given above has a negative value, viz. − 12. It would appear therefore that, instead of increasing resistance to avirulent infection, vaccination with virulent antigen had diminished it. There are two factors, however, which may account for the shorter survival period of the vaccinated animals, and render unnecessary the assumption that vaccination had favoured infection. One is the longer period of captivity of the vaccinated animals, and the other is the delayed toxic action of the vaccine.

Owing to lack of accommodation the controls were not kept alongside the vaccinated animals, but were infected usually after two or three days' residence in the laboratory, whereas the vaccinated animals had to be kept at least two weeks before being infected. It is believed that the longer period of confinement produced merely a general impairment of health, but the possibility of an obscure epidemic was not forgotten. This possibility however seems remote, for no such epidemic was ever suspected and conditions were unfavourable to the spread of disease, as the animals were well separated in concrete wall cages or in wire cages kept on iron racks, were housed in a concrete building and were rid of fleas by means of a lysol bath immediately on admission.

As regards the toxic action of antigen, a certain proportion of animals died within an interval of 36 hours after vaccination, death being preceded by great muscular relaxation and coma. Less frequently deaths of this kind occurred at longer intervals after vaccination, right up to the time of infection; and some of the deaths occurring after infection may have been partly of this nature. One of Rowland's (1911) experiments shows this delayed mortality. The survival periods of 8 rats treated with his chloroform-nucleoprotein-toxin in doses ranging from 1·6 mg. to 0·2 mg. were 1 (survived), 8, 8, 5, 4, 27, 10 days respectively. MacConkey (1912) also mentions it as occurring after the administration of toxin-antitoxin mixture, and refers to the symptoms as "marasmus." Toxic action then may be regarded as tending to balance protective action, and if the latter is absent, the vaccinated animals will show the higher mortality.

However, from the comparative success of some of the ordinary vaccine tests, the influence of the two factors, just considered, appears to be slight, and scarcely sufficient to account for the much shorter life of the vaccinated animals in this case. It therefore seems safe to conclude that vaccination with virulent antigen gives no protection against avirulent infection.

Avirulent antigen v. *virulent infection.* To test the protective value of

avirulent antigen against virulent infection, a vaccine was made of culture 57 *L*, when 44 days ex *C. g.* 27.

11 *C. gambianus* were vaccinated and 2 died during the immunisation period.

The remaining 9, together with 8 controls, were infected with 0·1 c.c. of a 1-day broth culture (118 *S*); 5 were infected eight days and 4 eleven days after the second vaccination.

All the controls died in the following times: 42, 42, 49, 51, 51, 52, 58, 66 hours.

Average survival period of controls = 51 hours.

Of the vaccinated animals all died, their survival periods being: 47, 49, 49, 55, 55, 64, 64, 68, 84 hours.

Average survival period of vaccinated animals = 59 hours.

The vaccinated animals thus lived very little longer than the controls, and the efficiency factor of the vaccine is only 2·3. It is so low that, even if allowances are made as in the previous experiment, we may conclude that the protective value of avirulent antigen against virulent infection is negligible.

Virulent infection of vaccinated animals compared with avirulent infection of unvaccinated animals. It is of interest to compare the results obtained in these two cases.

Collecting all the experiments in which the vaccine was prepared from virulent culture and sterilised by phenol, and in which a large infecting dose (0·1 c.c. to 0·4 c.c. of broth culture) was used when testing, the following figures were obtained:

Number of *C. gambianus* tested	61
Number which recovered	14
Percentage recoveries	23 per cent.
Average survival period	158 hours

In these experiments 38 controls were used. All died, with an average survival period of 48 hours.

The results obtained on inoculating unvaccinated animals with the avirulent cultures, 38 *L* and 57 *L*, are as follows:

Number of *C. gambianus* tested	33
Number which recovered	6
Percentage recoveries	18 per cent.
Average survival period	196 hours

The results of these two groups of experiments are thus somewhat similar. It would seem that a virulent strain of *B. pestis* loses some element of virulence on encountering antibodies within the animal body, and this element is not regained either on keeping the strain in culture, or on passage through susceptible animals. When vaccinated animals are infected, the bacillus meets the antibody in the animal under test, whereas, in the case of unvaccinated animals infected with an avirulent strain, *i.e.* a strain obtained from a vaccinated animal, the bacillus has met the antibody prior to the test; and, as regards immunity, the results are similar in the two cases. These results

however apply only to killed cultures, for infection with living avirulent cultures does produce immunity, as shown in the next section.

Immunity conferred by infection with living avirulent cultures. As noted above, 67 animals were infected with avirulent cultures and 10 recovered. The cultures used were 38 *L*, 57 *L*, 92 *L*, 98 *L*, 106 *L* and 83 *L*; the last (not yet mentioned) was derived from 57 *L* by passage through a *C. gambianus* which lived 316 hours. Of the 10 animals which recovered, 8 were re-infected with virulent cultures. The fate of these and the length of interval between the two infections is given in the following list:

3	re-infected	after	29	days,	killed	28	days later
1	,,	,,	21	,,	,,	22	,,
1	,,	,,	20	,,	,,	28	,,
1	,,	,,	21	,,	died	252	hours later
1	,,	,,	21	,,	,,	140	,,
1	,,	,,	20	,,	,,	168	,,

Films from spleen and lumbar glands and cultures were made from the animals which died spontaneously, and spleen films from those which were killed, but neither these nor the macroscopic *post mortem* appearances gave rise to any suspicion of plague infection.

SUMMARY.

(1) Strains of *B. pestis*, kept in culture for 7 and 9 months, showed no loss of virulence.

(2) Virulent strains, when subjected to passage through an immunised *Cricetomys gambianus* (African pouched rat), suffer distinct loss of virulence for this species, and virulence is not regained in culture or on repeated passage through susceptible *C. gambianus*. The so-called avirulent plague bacillus, obtained in this way, appears to be a rough variant. It differs in microscopical appearance from the ordinary virulent forms, tending to the appearance of involution forms, which develop in salt media.

(3) As regards immunising properties:

(*a*) Vaccine prepared from virulent strain gives considerable protection against infection with virulent strain.

(*b*) Vaccine prepared from virulent strain gives no protection against infection with avirulent strain.

(*c*) Vaccine prepared from avirulent strain gives little or no protection against infection with virulent strain.

(4) Animals, which recover from infection with an avirulent strain, are immune to subsequent infection with virulent strain.

CONCLUSIONS.

If the results obtained with pouched rats can be applied to man, the loss of virulence caused by passage through immune individuals would appear to be an important factor in determining the subsidence of an epidemic of plague. For, as immune individuals increase in number, the plague organism

has a greater chance of becoming attenuated, without losing its immunising power, and thus is established a cycle antagonistic to the virulence of the organism.

But the *killed* avirulent organism has no immunising power, and therefore it is of importance to avoid using it in the preparation of vaccine. It would appear that, for this purpose, one should not be too ready to discard a stock culture in favour of a fresh body strain, without a thorough test of the virulence of the latter. This applies more particularly to fresh human strains. Plague patients are not always readily available and have often been vaccinated, and if a culture from a vaccinated case is used, the resulting vaccine *is likely to be inefficient.*

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