LABORATORY NOTES ON PLAGUE IN KENYA.

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This paper presents a summary of the work on Plague carried out in the Bacteriological Section of the Kenya Medical Research Laboratory, during the period August, 1926, to December, 1928, in connection with the preparation of prophylactic plague vaccine. It is divisible under the following sections:

A. Preparation of a vaccine of Haffkine type at Nairobi.

B. Tests on rats of the immunising potency of the vaccine.

C. Notes on the application of plague vaccine for human prophylaxis:
   I. Experience in Kenya.
   II. Discussion of the relative values of agar-grown and Haffkine's plague vaccines.

D. Notes on bacteriology and serology of *Bacillus pestis*:
   I. Growth in fluid cultures under various conditions, etc.
   II. Agglutinating sera and absorption tests.

A. PREPARATION OF A VACCINE OF HAFFKINE TYPE AT NAIROBI.

Towards the end of 1926 it was decided, by sanction of the Deputy Director of Laboratory Services, to alter the type of anti-plague prophylactic made for issue to the Colony, and to commence manufacture as closely as possible in imitation of the process used at the Haffkine Institute in Bombay. Plague being both endemic and epidemic in Kenya, and of considerable industrial and political significance there, it was a matter of special anxiety to select a prophylactic which should be the most effective for its purpose, and also amenable to manufacture in sufficient quantities with the unsatisfactory laboratory accommodation up to now in existence. It is not a matter for regret, up to the time of writing, that our choice was the vaccine of Haffkine, the value of which has been so amply proved by the past 30 years of experiment and practice in India.

General considerations.

In its rate of growth we find *Bacillus pestis* to be comparable with the more freely growing streptococci, luxuriant growth appearing on the surface of agar in about 30 hours at 30° C. In broth, cultivation is uncertain unless special precautions are adopted, by reason of the marked aerophilic character of the bacilli: after forming a preliminary diffuse turbidity, they speedily

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sediment entirely and then soon autolyse. This may be overcome by aeration of the cultures by frequent agitation or otherwise; or by the classical method of "stalactite" culture—inoculation being made into shallow broth with a large surface area bearing a film of oil from which, if complete stillness of the fluid is maintained, the growth comes to depend in streamers of great fragility. With such culture in a narrow tube, the upper part of the growth is found to consist of healthy bacilli actively multiplying in chains; while at the bottom of the tube, where conditions soon become practically anaerobic, the organisms pass through remarkable involution changes before autolysis.

Since Haffkine's vaccine consists of the accumulated products of many weeks' growth of plague bacilli in broth, it is essential to adopt some means of maintaining the vitality of cultures at high level over a prolonged period.

Conditions for preparing Haffkine vaccine in Nairobi compared with Bombay.

It is unnecessary here to describe the well-known process of manufacture used in the Haffkine Institute; but advantage is taken there of the very uniformly warm climate of Bombay for raising cultures for vaccine at room temperature; temperatures of 25° to 30° C. being most suitable. In Nairobi, the wide variations of temperature which characterise the district—which is about 6000 ft. above sea level, and about $1\frac{1}{2}°$ south of the equator—are shown by the following extracts from the Health Office records: maximum 89° in February to 63° in June; minimum 59° in March to 45° in July. Night and day variations are commonly as much as 20° to 30° F. Climatic conditions in Nairobi are therefore unfavourable, as compared with Bombay, to cultivation of plague bacilli outside the incubator; and this, coupled with the absence of a room which could be devoted entirely to plague vaccine culture, considerably modified the process of manufacture.

Culture medium, apparatus, cultivation, sterilisation and storage.

The broth used is that called "P_3" by G. S. Graham-Smith, who originated it and kindly gave me the formula in 1918 for experiment with the spore-bearing anaerobes, when working on this group for the Food Investigation Board (1920, 1923). This is an auto-digest of bullock's pancreas in water, and extremely rich in the lower protein-cleavage products, especially tryptophane. It is now used in various dilutions as the basis of all culture media in the Nairobi Laboratory, being easily, quickly, and very inexpensively made in large quantities. For plague vaccine, the 20 per cent. strength of the broth is used, with 1 per cent. of peptone and 0·5 per cent. of NaCl added, at reaction of pH = 7·0. Experiments are described under Section D of this paper, which decided on the strength and quality of broth to be used, and the method of cultivation under Nairobi conditions.

For culture, ordinary conical flasks of 2 litre capacity are used, each containing 1·1 litre of broth, to which is added not more than 0·5 c.c. of olive

oil. Each brew of vaccine comprises ten such units, the extra litre above ten being intended to compensate for loss by evaporation and final bottling of vaccine. In view of the adopted method of standardisation, described below, perfect clarity of the medium is secured before inoculation.

For inoculation, preliminary cultures are made with five recently isolated strains of *B. pestis* in each of ten tubes containing 5 c.c. of broth; after 48 hours incubation, at 30° C., each tube is emptied into one of the large flasks, which are then well shaken and transferred to an incubator kept at 28° to 30° C.

The strains of plague bacillus used are obtained from dead rats sent to the Laboratory for autopsy from the district, and from human subjects alive or post-mortem. The income of fresh cultures being sufficiently regular, it has seldom been necessary to employ a strain as much as 20 weeks old; and new strains are put into vaccine as soon as possible after isolation and testing. But an immunity test with rats, detailed under Section B, performed with vaccine made with cultures 4 to 5 months old, tends to negative the idea that extreme recency of cultures is essential for Haffkine vaccine. Before using it for vaccine, each strain on isolation is verified by agglutination tests against serum made with a standard strain of *B. pestis*.

Incubation is continued for as long as possible, with a minimum of 3 weeks, until by reason of limited incubator space the cultures are crowded out on to shelves in the Laboratory; here they are exposed to the vicissitudes of temperature described above, until the elapse of a period of 12 weeks from their inoculation. The Laboratory being in a constant state of vibration during working hours, stalactite growth in these cultures is impossible; the expedient of shaking the cultures every 3 or 4 days was therefore adopted with success, on the results of experiments on fluid culture described in Section D.

It is clear that this procedure of cultivating vaccine is empirical and haphazard. It could scarcely be otherwise with the variable of atmospheric temperature and limited accommodation for the cultures to contend with. The result has been that a large proportion of the output of vaccine failed to pass the standardising tests given below, and had to be discarded.

Sterilisation is effected by adding 10 c.c. of pure phenol to each 1·10 litre of culture. This is not too large an addition of preservative for vaccine in broth issued in rubber-capped bottles of 25 or 30 c.c. which are very liable to contamination by careless use. Live cocci have been found in abundance in broth vaccine containing 0·5 per cent. of phenol, after storage for a considerable period in the cold chamber.

For *storage*, glass aspirating bottles of 10 litre capacity are employed. These are previously sterilised by standing filled with 5 per cent. phenol for several days, and are sealed with paraffin after charging with vaccine. After a minimum period of 5 weeks storage in the ice chamber in darkness, to weaken toxicity, bottling is done directly from the aspirating vessels.

The usual control cultures for sterility are made both from the storage vessels and from 5 per cent. of the small bottles of each brew of vaccine.

As will be seen from the above, the whole process is simplified to suit the decidedly primitive conditions at present obtaining for preparation of vaccine on any large scale.

STANDARDISATION OF NAIROBI PLAGUE VACCINE AND CONSTANCY OF QUALITY.

The process of standardisation by estimating the number of organisms per unit volume is not, of course, applicable to Haffkine's vaccine by reason of the fact that the solids in suspension consist almost entirely of amorphous granular material representing the remains of disintegrated bacilli; while, as shown by Naidu, Morison and Avari (1926), the immunising antigen, as also the toxic principle, resides practically entirely in the liquid portion of the vaccine.

The decisive standardising test of a prophylactic is, of course, that of its immunising power upon the animal organism which it is desired to protect. In the case of plague, the quite undoubtable parity between the results of vaccinating humans and rats has established the invaluable advantage of deputising these rodents for reliable estimations of immunising potency of vaccine for application to man.

In the Haffkine Institute, the rat immunity test is wisely relied upon for controlling the quality of plague vaccine issued; and the long and admirable series of experiments on induced immunity achieved there are classical. In our Laboratory, circumstances have debarred the application of such tests to more than a very few representative samples of plague vaccine; a simple chemical and physical standard of quality has therefore been relied upon, itself verified by those few immunity tests which have been done.

The method consists simply in estimation, by the opacity scale, of milligrammes of suspended solids per c.c. of the whole vaccine, and also determination approximately of the content of protein in solution in the vaccine, after centrifugalising clear and filtering through paper. The values so obtained are proportionate to the amount of growth which has occurred during cultivation of the vaccine, and therefore may be assumed to be proportionate also to the amount of antigen present. The standard to be aimed at was decided first by submitting to such tests mixed samples of Haffkine vaccine received from Bombay. For estimation of protein, Esbach's method is quite satisfactory for the purpose, in a modified tube calibrated for readings in tens of milligrammes.

A good average sample of vaccine contains, by these estimations, from 0·45 to 0·55 mg. of bacillary sediment per c.c., and from 40 to 50 mg. of dissolved protein per cent. Vaccine containing less than the minimum of 0·75 mg. of total solids—sediment and protein—per c.c. is regarded as being sufficiently below par to be rejected. An immunity test is detailed in the next section, performed with such vaccine which failed to pass the physical standard.

The matter of dissolved protein and of sediment will be touched on again in Sections C and D of this paper

B. TESTS ON RATS OF THE IMMUNISING POTENCY OF THE VACCINE.

Only four experiments have been carried out up to date, using altogether 84 rats. These have been Albino cage rats, failure having met all attempts to induce into captivity the wild house rats of the district, which are *Rattus rattus*. Cage rats have the advantage in a district where plague is endemic of being reliably virginal in respect of previous infection. The number of experiments has been limited therefore by the number of rats which could be reared in the department under conditions obtaining, and reserved for plague work. The animals selected for each experiment were of uniform size, about three-quarters fully grown, and uniformly in good condition.

Vaccine dosage. In Bombay, the standard dose for rats is 0·5 c.c., which, as I am informed by Dr B. P. B. Naidu of the Haffkine Institute, is found convenient for testing both immunising power and toxicity. A high but varying percentage of the test animals succumb to the effects of this dose. The standard human dose of Bombay Haffkine is 3 to 4 c.c. In Nairobi, the standard human dose being 2·0 c.c., the rat dose is set proportionately lower, viz. 0·30 c.c. subcutaneous.

Test dose of virus. The Indian test dose is 0·003 mg. of plague-infected spleen, administered a week after vaccination. In Nairobi the test dose is approximately 0·02 to 0·03 mg. of young agar culture in saline or dilute broth suspension, of a recently isolated strain of *B. pestis*; it is given subcutaneously on the 14th day after vaccination. It should be mentioned that none of the animals died, nor showed visible signs of sickness, during the period of immunisation.

Immunity tests. The experiments were observed at 12 and 24 hourly intervals, in order to record the power of the vaccine to impede and arrest epidemics of plague. The times of the various deaths given below are those when the animals were found dead.

Exp. 1.

Twelve rats *A*: given 0·3 c.c. of vaccine brew B. 26, on 1. ix. 27.

Twelve rats *B*: not vaccinated. On 15. ix. 27, rats *A* and *B* given 0·02 mg. of *B. pestis* obtained 3 weeks previously from the spleen of a native who had died of plague. Rats *A* and *B* were then placed in separate chambers under identical conditions. Results as below:

Rats *A*	Rats *B*
Three dead, evening of 19. ix. 27 Case mortality: 24·7 %	Ten died during 18–19. ix. 27 Case mortality: 83·3 %
The 9 survivors seem well, 20. ix. 27	No. 11, died 20. ix. 27 Case mortality: 91·6 %
No. 4, died morning of 21. ix. 27 Mortality: 33·3 %	No. 12, died morning of 21. ix. 27 Mortality: 100·0 %

No further deaths occurred among rats *A*, whose immunity rate was therefore 66·7 per cent. These, and the animals in the ensuing experiments, were

kept under observation daily for a period of 3 weeks from the date of test dose; the experiments were then regarded as concluded.

Exp. 2.

This was designed to test a brew of vaccine made with strains of *B. pestis* which had been maintained on agar for 4 to 5 months since isolation. Unfortunately rats could not be spared for the non-vaccinated controls, three fully grown hutch rabbits and one spring hare (*Pedetes surdaster larvalis*) being used instead. A minimal dose of vaccine was here experimented with.

Twelve rats each injected with $1\frac{1}{2}$ minim (0·09 c.c. approx.) of vaccine brew B. 39. The test dose 14 days later was 0·028 mg. of bacilli obtained 19 days previously from the spleen and bubo of a plague-dead native; the spring hare and 3 rabbits were inoculated with this at the same time.

The spring hare died 2 days later; post-mortem signs were indefinite, and microscopical examination failed to reveal *B. pestis* in the organs. As should be well known, such a result may occur in the case of an animal inoculated with plague; and death from the intrinsic toxicity of the inoculum cannot always account for it. In human autopsy, also, it has been our experience to fail to demonstrate *B. pestis* in spleen and liver in cases where evidence was strong for septicaemic plague.

Rabbits seem to be less susceptible than rats to plague, and are probably to be judged as less so than man, all things being equal; but individually they vary widely in their resistance to plague infection, as they do to typhoid. In this experiment, one of the rabbits died on the 8th day; autopsy showed a much enlarged spleen, and a much enlarged inguinal gland; plague bacilli were abundant in the spleen, but could not be identified in the gland. The first rabbit had died on the 7th day, with plague bacilli scanty in the spleen. While the third rabbit never became obviously sick, it died 41 days later with *B. pestis* extremely abundant in the spleen; this seems noteworthy in view of the possibility of plague carriers among humans and other animals.

The behaviour of the rats was as follows:

Death no. 1, evening of 3rd day. Spleen much enlarged, bacilli moderately plentiful.

Deaths nos. 2, 3 and 4, morning of 4th day. All spleens much enlarged, *B. pestis* scanty in nos. 2 and 3, extremely abundant in no. 4.

Death no. 5, morning of 7th day. Spleen much enlarged, bacilli scanty.

Eighth, ninth and tenth days: no further deaths, and all seven survivors seem well.

Eleventh day: death no. 6, though all seemed well the previous evening. Spleen definitely enlarged and engorged, but microscopical examination reveals only some gram-positive cocci.

No further deaths or sickness occurred during the remaining ten days of quarantine or subsequently. A 50 per cent. survival rate from the minimal dose of vaccine may be based on the assumption that the 6th rat died of

plague *per se*, and that if 12 control rats had been used they would all have died from the heavy test dose of virus.

Exp. 3.

Twelve rats *A*: given 0·3 c.c. of plague vaccine brew B. 38, on 26. i. 28.

Twelve rats *B*: not vaccinated. These, with rats *A*, inoculated with 0·028 mg. of *B. pestis* isolated 13 days previously from a human bubo, and kept on agar since without subculture. Rats *A* and *B* then placed in separate chambers under the same conditions: 10. ii. 28.

Rats *A*	Date	Rats *B*
All well in evening	12. ii. 28	Death no. 1, evening
Death no. 1, morning Death no. 2, evening Another rat sick	13. ii. 28	Death no. 2, morning Deaths nos. 3 and 4, evening Several others sick
No further death, morning Death no. 3, 12.30 p.m. Case mortality: 24·9 %	14. ii. 28	Deaths nos. 5, 6, 7, 8 and 9, morning Death no. 10, 12.30 p.m. Case mortality: 83·3 %
All survivors seem well throughout day	15. ii. 28	Death no. 11, 12.30 p.m. Surviving rat sick Case mortality: 91·6 %
Death no. 4, midday The 8 survivors well and active, evening	16. ii. 28	The one survivor seems subdued throughout day
The 8 survivors well Case mortality: 33·3 %	17. ii. 28 to 2. iii. 28	The one survivor well Case mortality: 91·6 %

No further deaths nor sickness occurred. Corrected immunity rate of the vaccinated animals here is 63·6 per cent.

Since Exps. 1 and 3 gave closely similar results, and were strictly comparable in the manner of their conducting, they may be combined in one table, as below, for comparison with Exp. 4 given in Table II. The figures denote case mortalities on successive days after the test dose of virus, and the survival on the 20th day: rats *A* being vaccinated, rats *B* the non-vaccinated controls.

Table I.

Rats	2nd day %	3rd day %	4th day %	5th day %	6th day %	Survival 20th day %
A 24	—	16·6	24·9	24·9	33·3	66·7
B 24	8·3	33·3	83·3	91·6	95·8	4·2

The vaccine used for the above tests conformed to the standard for solids in suspension and dissolved protein, *i.e.* about 40 mg. of protein per cent., and 0·55 mg. of sediment per c.c. Table II gives case mortalities of Exp. 4 performed in exactly the same way as Exps. 1 and 3, but with vaccine of only 25 mg. of protein per cent., and 0·28 mg. of sediment per c.c. Rats *A* were vaccinated, *B* the controls.

Table II.

Rats	2nd morning %	2nd evening %	3rd day %	4th day %	5th day %
A 12	16·6	58·3	91·6	91·6	100·0
B 12	33·3	58·3	100·0	—	—

All that this sample of vaccine achieved was to impede the progress of the "epidemic" a little. But the strain of *B. pestis* used here for the test dose seems to have been an exceptionally virulent one.

C. APPLICATION OF PLAGUE VACCINE FOR HUMAN PROPHYLAXIS.

Parity is close between vaccine immunity rates for humans and for rats, as may be seen from the Indian literature (B. P. B. Naidu and others, 1925–7) from which we gather that immunity rates for rats treated with the 0·5 c.c. dose of Haffkine may touch 76·1 per cent., and are commonly between 40 and 60 per cent. While for example of human induced immunity rates, we find (1909) figures which may be tabulated as below, where *A* represents people who contracted plague later than a week from date of vaccination, and *B* non-vaccinated individuals. The survival rate of the former is corrected.

Table III.

Humans	Vaccine dose c.c.	Plague deaths %	Survival rate %
A 3,164	3–4	38·2	61·8
B 56,240	Nil	85·5	14·5

I. EXPERIENCE IN KENYA.

In Kenya, because of the floating character of native populations, and for other reasons, reliable inoculation statistics are difficult to collect; and so far time for this has been short. Table IV below refers to hospital cases, and is constructed on material for which I am indebted to Dr Martin, M.O.H. of Nakuru.

Table IV.

Humans	Vaccine dose c.c.	Plague deaths %	Survival rate %
A 24	2·0	33·3	65·1
B 43	Nil	95·3	4·7

The resemblance of the survival figures here to those of the rats in Table I is remarkable. But it should be remembered that whereas for experimental rats a uniformly lethal dose of plague virus is used, human infections naturally received from the bites of fleas are necessarily variable in intensity, and on the average must be only moderate. Immunity rates induced by vaccine should therefore tend to be higher with humans than in experiment with rats. Accordingly, Table IV is modified by further details from Dr Martin, to the effect that one of the patients *A* escaped from hospital and walked 10 miles, then died; while several others were probably in the grip of plague when inoculated; his opinion therefore is that the corrected survival rate should be 73·9 per cent. On the other hand, in Kenya usually only the severe cases of plague come to hospital, milder cases being apt to be passed over.

Protection against attacks of plague. This naturally interests the public more than protection against death from plague after vaccination. In Kenya, suggestive details have been furnished by the Nairobi Health Office, pointing

to the arresting of various more or less serious outbreaks by inoculation with Nairobi vaccine; these have been published elsewhere (1928).

In the middle of 1928, an extensive rat epizootic occurred in the Indian quarter of the town; in view of an imminent epidemic, extensive inoculations were performed; the number of human cases recorded was negligible. But it is commonly impossible to collect more than suggestive information on the point of protection by inoculation.

An important source of error in estimating results of anti-plague inoculation among natives is that commonly only the severe cases come under medical observation, the milder cases—such as those modified by inoculation—often not being reported or escaping diagnosis.

II. THE RELATIVE VALUES OF AGAR-GROWN AND HAFFKINE'S PLAGUE VACCINES.

Discussion of this from the human standpoint necessitates reference to experimental tests with rats, since I am unaware of the existence of any published statistics relating to human inoculations with agar-grown vaccines.

Important work has been done at the Haffkine Institute (1924–5) and by H. Schutze at the Lister Institute (1925) in estimating the immunising potency and toxicity for rats of agar-grown plague vaccines, and comparing these with Haffkine's. There are also Pirie's experiments in immunising gerbils with South African agar-grown plague vaccine, which gave negative results (1927).

The agar-grown vaccines are popular because of the mildness of the symptoms induced by injection. Haffkine's prophylactic is unpopular for the converse reason. But it is doubtful whether the agar-grown vaccines are of any prophylactic value for humans when injected in the doses prescribed, viz. 0·1 to 1·5 mg.; it is doubtful too whether the really effective human prophylactic dose would not be even more disturbing to the system than 3 or 4 c.c. of Haffkine's vaccine carefully administered.

Below is reproduced a table compiled (1927) by averaging all the published tests performed on rats with agar-grown vaccines at the Haffkine Institute (1924–5).

Table V.

Rats	Av. vaccine dose mg.	Vaccine deaths %	Plague deaths %	Survival rate %
225	5·9	25·3	61·4	38·6

It is seen that, as regards case mortality and survival rate, this very nearly reverses the results obtained in Exps. 1 and 3 (Table I); while the vaccine dose is an enormous one for rats, and the mortality effected by it is correspondingly high.

The next table presents experiments performed only with agar-grown vaccines sterilised by heat at 60° C.

Table VI.

Rats	Av. vaccine dose mg.	Vaccine deaths %	Plague deaths %	Survival rate %
145	9·2	54·5	33·6	66·4

For the enormous dose of vaccine, this is an excellent result as regards immunity rate, comparable with the results of experiment with Nairobi vaccine. But it may also be regarded as a very good result for the agar-grown vaccine when regarded as an active poison.

The question of the ratio between the effective vaccine dose for rats, and that for humans, arises now. The Indian rat dose of 0·5 c.c. of Haffkine is unnecessarily high for testing induced immunity; it is designed to test toxicity as well, and a considerable proportion of the wild brown rats used in Bombay die from the effects of it. The Nairobi dose of 0·3 c.c. is evidently the correct immunising dose for rats, since it achieves about the highest possible immunity rate, and no rat has been known to die from it. The Nairobi human dose of 2·0 c.c. is on the low side; the Indian dose of 3·0 c.c. prescribed for freshly issued Haffkine may be regarded as the correct human dose, since though quite severe effects may follow upon it, it is not likely to cause death of a healthy subject.

On this basis, the effective immunising dose of plague vaccine for humans may be fairly said to be at least ten times the dose for the rat. Therefore, if it were wished to achieve with agar-brown vaccine a survival rate with humans of about 60 per cent., as in Tables I, III, IV and VI, a dose of about 90 mg. of this vaccine would be needed; and from the immediate toxic effects of this some 50 per cent. of the subjects injected might be expected to die.

It could be rejoined that some much lower dose of agar-grown vaccine might effect a high immunity rate, without in the process committing so much murder. But though in the extensive series of Bombay tests the toxic effects lessened logically with lower doses, the induced immunity rates lessened also in proportion to nearly nil. For example, the dose of 1·42 mg. brought about a survival rate of less than 9·0 per cent. of rats; this is about one-third the usual number of human survivors from attack by plague in Kenya, without the assistance of vaccine; and the 1·42 mg. dose is only 0·08 mg. less than the usually prescribed dose of agar-grown vaccine for human prophylaxis.

The experiments on rats by Schutze with agar-grown vaccine made by the Lister Institute have been similarly averaged below in that part of Table VII above the dotted line. The figures in the lower half of the table illustrate his comparative tests with Haffkine vaccine obtained from Bombay; the dose of Haffkine he used is not stated by him, but it must have been 0·5 c.c. or less, judging by the toxic deaths from it. The dose of agar-grown vaccine employed is stated by him to be "half that given to humans."

Table VII.

Rats	Vaccine dose mg.	Vaccine deaths %	Plague deaths %	Survival rate %
87	0·75	1·2	44·2	55·8
42	??	4·7	44·5	55·5

It is not stated by Schutze whether he used cage rats, or wild rats which are prone to die in captivity and thus cause uncertainty in estimating deaths

from vaccine toxaemia. We therefore judge that the 0·75 mg. above forms a satisfactory dose of Lister Institute plague vaccine for rats. Assuming on our Exps. 1 and 3, it may be said that 0·3 c.c. of Nairobi Haffkine vaccine would probably have effected a higher survival rate than in the above table, without any vaccine death; and that with one-tenth the effective dose for humans. Why, therefore, should only 1·5 c.c. of this agar-grown vaccine be regarded as a proper human dose? We conclude that the effective human prophylactic dose of Lister Institute vaccine for plague should be at least 7·5 mg.; and that for some people would be a lethal dose.

In general, it is logical that the antigenic factor in immunity should be more concentrated in Haffkine's than in vaccine made simply by washing off growth on agar. For taking endotoxin alone, one cubic centimetre of the broth vaccine contains that derived from the disintegration of many generations of many millions of bacilli; and in young agar culture it should be that the immunogenetic factor which is endotoxin bears a much smaller ratio to the toxic nucleoprotein (Sydney Rowland, 1911) than in Haffkine's broth vaccine wherein the protein may be largely hydrolysed by enzyme during cultivation and storage.

It is a mistaken idea that the broth itself is toxic. Young mice injected with the comparatively huge dose of 1·0 c.c. of the broth used for Nairobi plague vaccine suffered no manifest ill effects, while similar mice injected with culture in the same broth of a coliform organism to the strength of 0·4 mg. per c.c. all died with violent symptoms of toxaemia within an hour or two.

The toxic principle, and possibility of removing it.

Sydney Rowland (1911) extracted a highly toxic nucleoprotein from plague bacilli, and found the immunising antigen to be closely combined with this. The nucleoprotein-antigen complex possessed powerful immunising properties; he found that this after hydrolysis by a ferment of plague bacillary cells had lost toxicity while gaining in immunising power.

The protein in solution in Haffkine vaccine being the result of autolysis of the bacilli, it occurred to the writer that this might be the toxic principle: that the fact of reduction of toxicity of the vaccine by storage might be explained by action of proteolytic ferment upon the dissolved protein: and that this protein might be destroyed by means of pepsin or trypsin added to the vaccine, thus perhaps greatly lessening toxicity without affecting the immune antigen.

Experiments were accordingly made with trypsin, since the end reaction of the vaccine culture is about *p*H 7·5, and trypsin acts in an alkaline medium. It was found that 0·1 per cent. of trypsin acted well upon boiled egg-albumen in the broth at *p*H 7·4, containing 1 per cent. of phenol, at 37° C. At room temperature—average about 20° C.—action was of course slower. 0·1 per cent. of trypsin powder was used, and clear centrifugalised top fluid of vaccine, containing 40 mg. protein per cent., was then experimented with. Tests on rats

with 1 and 2 c.c., and personal injection, of the trypsinated top fluid certainly showed it to be less toxic than the same vaccine untreated. But immunity tests with it could not be performed, and results of these experiments were on the whole undecided or anomalous. For instance, it was once doubtful if trypsination proceeded any further than the stage of metaprotein; and a sample of vaccine which had been stored in the cold for a few months appeared to contain only alkali-metaprotein in its top fluid.

Experiments on this point having to be discontinued, the above is given in case it should be of suggestive value.

Dosage and duration of protection.

The toxicity of Haffkine type vaccine being considerable, dosage in Kenya has been set at 2·0 c.c., as against the Indian 3 to 4 c.c. This dose subcutaneously causes sufficient local and general reaction to incapacitate a healthy subject for a day or two. On the basis of the one-tenth ratio of rat to human dosage, and of a high degree of immunity having been induced in rats (Exp. 2) with one-twentieth of the standard 2 c.c. dose, it is considered that a human dose of 1·0 c.c. should be sufficient for the usual purpose in Kenya of providing protection in emergency; it being usually difficult to induce natives to be inconvenienced by inoculation except when alarmed by an outbreak of plague. The absence of a negative phase has been demonstrated by the Indian workers with Haffkine's vaccine.

D. NOTES ON BACTERIOLOGY AND SEROLOGY OF *BACILLUS PESTIS*.

I. NOTES ON GROWTH IN FLUID CULTURE, ETC.

The medium used throughout was a digest of pancreas employed originally by G. S. Graham-Smith in investigations of the rate of growth of bacteria in fluid cultures (1920), and subsequently designated by him "P_3." Details of its preparation are as follows:

Bullock's pancreas stripped of fat and fascia, minced, weighed, and mixed with 2·5 c.c of tap water for each gramme of weight, in a wool-plugged flask. Two per cent. of chloroform is added, to inhibit putrefaction, and the whole incubated at 37° C. for 24 to 48 hours. The flask is then steamed at 100° C. for one hour (using for this the autoclave at low pressure at high altitudes such as that of Nairobi), the contents filtered clear through cloth followed by paper, and the filtrate steamed or autoclaved to sterilise.

The 48 hours digest is used in Nairobi in various dilutions for all culture media that need a nitrogenous basis. The initial acidity is about *p*H 6·4, like ordinary meat extract; but it gives a formol titration reading which is practically constant at 200·0 c.c. of *N*/10 NaOH per cent. The 20 per cent. dilution in water, with 1 per cent. of peptone and 0·5 per cent. of salt, is used for agar and broth for ordinary and vaccine culture purposes, and makes an excellent medium. The 10 per cent. strength, of pure P_3, a pale or nearly

colourless fluid, is valuable as the basis for "sugar" media, and to replace peptone water also for testing for indol production; it is valuable also for plague and other cultures for agglutination tests, having growing powers nearly equal to peptone meat extract. The 50 per cent. strength with agar is used for ordinary and vaccine control cultures for the liquifying spore-bearing anaerobes, including *B. tetani* which I have found to grow exceptionally well in this medium (1923).

The convenience of a concentrated medium, of wide application by dilution, and simple and inexpensive of preparation, is the reason for exclusive use of P_3 rather than any particular superiority of it over other culture media. The addition to it of something of the nature of the "protective colloid" of McLeod, Wheatley, and Phelon (1927) seems to be essential.

Growth of B. pestis *in various dilutions of* P_3.

Cultures were made in small flasks of the same shape, all containing the same volume of P_3 in strengths ranging from 5 per cent. in water to 100 per cent., at the same *p*H value, the flasks about half full of broth and each inoculated with the same number of drops of a shaken young broth culture of a recently isolated strain of *B. pestis*. The cultures were in duplicate series.

In these, growth appeared earliest in the weaker strengths; lag phase was marked in the 40 to 100 per cent. strengths in proportion to the concentration, and during this growth came to the maximum in the 20, 25, 30 and 35 per cent. strengths between the 3rd and 8th day of incubation at 30° C. Later on, growths in the higher concentrations appeared to equal those in the intermediate ones, cultures in the 5 and 10 per cent. broths remaining weaker. Estimations of growths were uncertain, but it was sufficiently evident in repeated experiments that concentrations of 20 to 30 per cent. of P_3 were the most suitable for growth of plague bacilli.

Estimation of growth in fluid culture.

By reason of its strongly aerophilic character, *B. pestis* does not lend itself well to the method of plating out units of fluid culture. Also the extraordinary liability of plates to gross contamination in the present Nairobi laboratory was too great an obstacle. Estimation of growths by the opacity method was therefore relied upon. In the above experiments, the difficulty introduced by variations in tint was overcome to some extent by centrifugalising, pipetting off roughly proportionate volumes of supernatant fluid, and replacing these with equal volumes of saline in which the deposits were shaken up before estimation.

The best that can be said of the estimations in these and the following experiments is that they corresponded to differences in growth which were distinct to the eye.

Enrichment of P_3 with added substances.

P_3 broth of 30 per cent. strength to which 1·5 per cent. of gelatin had been added yielded within a given time of incubation a growth of 0·60 mg. of plague bacilli, as compared with 0·20 mg. in the plain 30 per cent. broth at the same reaction. Similar results were obtained by addition of 15 per cent. of neutralised alkali-metaprotein (Lieberkuhn's Jelly). The action of these substances was doubtless that of "protective colloid," as described by McLeod and Co. (1927).

Peptone had a similar effect. Comparison was made between 30 per cent. P_3 plus 1 per cent. of peptone, and 25 per cent. P_3 which gave the same formol titration reading; growths in the former were 0·34 mg. per c.c. as compared with 0·14 in the latter.

Influence of temperature, shape of flask, aeration and surface oil.

Exp. 1.

Cultures in flasks of 200 c.c. capacity, each containing 100 c.c. of the same brew of P_3 broth of 30 per cent. strength, at *p*H 7·3. Uniformly inoculated with 6 drops of a young broth culture of recently isolated *B. pestis*. Growths estimated after 8 days, in mg. per c.c.

A. Globular flask, shaken daily to aerate, at 37° C.	0·27 mg.
B. Globular flask, shaken daily, at 30° C.	0·27 ,,
C. Globular flask, not shaken, at 30° C.	0·24 ,,
D. Conical flask, not shaken, at 30° C.	0·20 ,,
E. Conical flask, shaken daily, at 30° C.	0·35 ,,
F. Conical flask, 6 drops of olive oil added, shaken every 4th day, at 30° C.	0·62 ,,
G. Globular flask, shaken daily, at 25° C.	0·24 ,,
H. Conical flask, shaken daily, at 25° C.	0·20 ,,

Dissolved protein in centrifugalised top fluid of culture F was estimated to be 10·0 mg. per cent.; that in all the other cultures mixed together was 3·0 mg.

Exp. 2.

Performed similarly to the above. Growths estimated after 10 days incubation at 30° C.

A. Globular flask, 6 drops of oil, shaken daily	0·55 mg.
B. Globular flask, 6 drops of oil, not shaken	0·40 ,,
C. Conical flask, 6 drops of oil, shaken daily	0·60 ,,
D. Conical flask, 6 drops of oil, not shaken	0·48 ,,
E. Globular flask, without oil, shaken daily	0·28 ,,

The main conclusions that could be drawn from such experiments were that (*a*) richer growths might be secured by regular strong agitation of cultures under a film of soil, and (*b*) that for some reason such cultures might be more dense in conical flasks than in globular. In these, growth occurs among the film of oil upon the surface, much as on the surface of agar; after standing undisturbed for a day or two, the culture is divisible into three distinct zones: surface growth, bottom sediment, and clear intermediate broth containing fragments of growth detached from above. Could freedom from vibration have been secured, stalactite growth would doubtless have occurred.

Fermentation reactions of B. pestis.

Fifty strains were tested in this respect, four of these being some years old and derived from the Lister Institute, four received from Bombay, the remainder having been collected recently in Nairobi district. All these gave closely similar results, which the table below typifies. P_3 broth of 10 per cent. strength was employed as the basis of the sugar media. The acid to alkaline change in the substances not permanently fermented was a characteristic and striking feature, which has not been found elsewhere recorded.

Test substance	24–48 hours	48–96 hours at 30° C.
Glucose	A. A.	A.
Maltose	a. A.	A.
Dextrin	a. A.	A.
Galactose	A. A.	A.
Mannite	a. A.	A.
Lactose	a. a. or neut.	Alk.
Saccharose	a. a. or neut.	Alk.
Dulcite	a. a. or neut.	Alk.
Sorbite	a. a. or neut.	Alk.
Isodulcite	a. a. or neut.	Alk.
Indol		Nil

The "a." signifies slightly acid; the "A." strongly acid. Gas is not formed. The above key group of fermentable compounds is sufficient for confirming identity of *B. pestis* in ordinary practice. It was found that the fermentation cultures in tubes, not being shaken to aerate, were rather liable to die out before typical reactions were complete; anomalous results, such as permanent acidity of lactose, were thus sometimes observed with strains which had been verified by agglutination tests.

Method for obtaining cultures from buboes in living subjects.

This has been found useful in Nairobi, where plague patients have usually to be reached by car from the Laboratory over extremely rough roads.

The wool plug of a narrow sterile test tube is scorched and pushed in about half way to the bottom of the tube, which is at once closed with another sterile plug. A Dreyer's agglutination tube is sterilised by flaming, and placed inside the large tube to rest upon the lower plug, with its mouth close against the upper plug. With a sterile, wool-plugged Wright's pipette and teat, broth is introduced into the little inner tube up to within a few millimetres of its mouth; in such a narrow tube the fluid forms an immobile column which cannot easily be spilled out. The whole may be carried in the breast pocket, together with a small syringe sterilised with olive oil at 140° C., the needle protected with a sterile sealed-off piece of quill glass tubing, enclosed in a sterile container or else simply wrapped up in cotton wool. At the patient's side, the skin over the bubo is painted with iodine, the gland aspirated, the plug removed from the culture tube container which may be held in the horizontal position, and a little of the broth is drawn up into the syringe to wash out the infected material from the needle into the culture tube. A trace of oil is washed out as well, enough for the requirements of stalactite growth, which

may be well exhibited if the culture tube container be immobilised in the incubator. Subculture is then made on to agar.

An advantage of this method is that it permits of culture being easily made from the traces of gland material which are often all that can be aspirated from plague buboes; and the easy introduction of viscid material from the aspirating needle into culture medium.

A type of streptococcus associated with B. pestis.

A rather small, short-chained streptococcus appears very commonly in the sediment of Nairobi plague vaccine, and what appears to be the same organism has been seen in the centrifugalised deposit of Haffkine vaccine received from Bombay. Similar streptococci associated with plague bacilli have been seen in and cultivated from the spleens of Nairobi plague rats; and in one human case both the cervical bubo and the spleen contained such streptococci together with *B. pestis*. It tends to lie latent in agar cultures of *B. pestis*; but in one case what appeared at first to be a pure primary culture of *B. pestis* became in the course of some weeks a growth of plague bacilli and the streptococcus in about equal proportions. The constancy of its appearance in association with plague has been sufficient to warrant its provisional designation as *Streptococcus pestis*; it can be fairly readily separated from its mixed growth, and exhibits very small rather slowly growing colonies on the surface of nutrient agar. It does not appear to be haemolytic; but it has not yet been at all fully investigated (1928).

Stab culture of B. pestis.

A striking demonstration of the aerophilic nature of *B. pestis* can be given by stab culture in a tube half filled with nutrient agar. Inoculating heavily with a fine needle say 4 inches in length, the growth is confined to within the uppermost inch of the needle track, and comes to assume by spreading laterally the form of a short, truncated sword with curiously furbelowed edges; while on the surface of the column of agar the growth spreads to form a disc like the large head of a nail. An interesting contrast is made by a similar culture of a strict anaerobe, say *B. tetani*, whose growth extends to the bottom of the needle track and leaves the top $\frac{3}{4}$ inch or so of the column of agar clear.

II. Agglutinating sera and absorption tests.

Agglutinating sera of more or less high titre have been made by the writer (1928) by subcutaneous inoculation of rabbits with increasing doses of attenuated living culture. The strain of *B. pestis* used, designated "S.P.D.," is several years old, and derived originally from the Lister Institute. Initial doses of about 0·30 to 0·50 mg. of young agar culture were given, increasing by about the same amount at fortnightly or monthly intervals up to 3, 5, 7, or as much as 10 mg. of live culture. Sera of titre 1/1000 to 1/5000 for standard

formalinised S.P.D. culture were easily obtained; but individual rabbits varied in their response. Of two similar rabbits which received a similar scale of dosage, one failed to produce a serum of titre higher than about 1/5000; while the other yielded a serum of titre 1/17,000 for standard culture, and 1/25,000 for culture made from a smooth colony of S.P.D. The strongest serum so far obtained has been of titre 1/25,000 for standard mass culture.

In this connection, rough and smooth colonies of S.P.D. tested against this serum showed titres ranging from about 1/2500 to 1/12,000 for the rough, and 1/12,000 to 1/25,000 for the smooth colonies.

The fact is well known that injections of killed plague culture fail to engender any but an inconsiderable amount of agglutinin, or none at all. In this work, strong doses of phenolised Haffkine type vaccine, or of heat killed agar culture, in rabbits, failed to produce agglutinin in serum dilution of 1/25, this appearing only on commencing inoculations with live plague bacilli. This phenomenon appears to rule out the question of heat-stable and heat-labile agglutinins which is now so prominent in typhoid-paratyphoid immunology.

Absorption tests. A series of 71 strains of bacillus with the cultural characters of *Bacillus pestis* has been subjected to absorption tests, with the 1/17,000 S.P.D. serum, in view of the possible existence of a specific serological variant. Of these strains, 53 were collected in Nairobi district from plague rats and human plague cases, 6 were old stock cultures originally obtained from the Lister Institute, 4 came from Bombay, and 8 from the Bacteriological Laboratory of Uganda. A further series of about 30 recently collected Kenya strains have been put to agglutination tests only.

Agglutination tests of all these, in mass culture as isolated, gave titres varying widely, from 1/500 only, up to 1/15,000 or so. In one human case, cultures from the spleen and from the cervical bubo both showed standard agglutination at 1/500 of the serum.

To economise serum, an original method was devised for absorption tests, which has been described elsewhere (1928). They are done in ordinary Dreyer's agglutination tubes, 3 drops only of pure serum being needed for each test, and the method permits of control on the ratio of density of absorbing suspension to agglutinin content of serum. The possibilities of the method have not yet, however, been fully worked out.

The result of these absorption tests was failure to discover among the 71 cultures examined any specific variant from the type strain of plague bacillus S.P.D.

Serological affinities of the Pasteurella *group.*

It was of interest to discover whether the other members of this, the "haemorrhagic septicaemic" group, gave cross agglutination with the type *B. pestis.* Only one tentative experiment has been done. Cultures of *P. aviseptica, suiseptica, boviseptica* and *muriseptica* were obtained from the National Type Collection and put up against S.P.D. serum of titre 1/17,000, in dilutions

commencing at 1/25. Agglutination did not occur of any of these types. It can only be said, therefore, that either the serum used happened to be naturally a purely specific one, or at least very poor in group elements; or that all four cultures were remarkable in consisting mainly of specific elements; or that these members of the group have no serological affinities with *B. pestis*.

Therapeutic possibilities of high-titre plague serum.

In January, 1928, it was planned to test the curative power of high-titre rabbit plague serum on artificially infected rats. But the project had to be abandoned for lack of laboratory animals. The underlying idea was that the antitoxic factor in high-titre serum might be proportionate to its agglutinin content. And agglutination tests with plague culture against two well-known brands of anti-plague serum had given positive results in dilution of only 1/30 with one of these, and negative results with the other; though it may be that this latter was made by injections of bacillary extract which might possibly not engender agglutinin.

According to the report of the Haffkine Institute for 1928, however, and information kindly furnished by Dr B. P. B. Naidu, experiments there with high-titre plague sera in this direction have given encouraging results.

In conclusion, my thanks are due to the Editors of the *Kenya and East African Medical Journal* for their courtesy in permitting me to use some of the material which has appeared in a non-technical style in that Journal.

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