

METHODS OF LABORATORY DIAGNOSIS

EXAMINATION OF RODENTS AND FLEAS

Before discussing the laboratory methods available for the detection of plague in individual rodents, it should be pointed out that a properly functioning intelligence service is of outstanding value for this work, since the laboratory staff is thus kept informed of the occurrence and extent of unusual rodent mortality. In the case of the commensal rodents in particular, the finding of an appreciable number of animals which have succumbed spontaneously may ordinarily be taken as *prima facie* evidence for the existence of a plague epizootic, while marked scarcity of live rodents in a locality which previously showed heavy infestation is indicative of a past major epizootic. In view of what has been discussed in chapter 4, every possible effort must be made to examine the rodents found dead because they offer the strongest chance of obtaining frankly positive evidence of infection. This point is well illustrated by the recently published observations made during the period 1940-50 by Gross & Bonnet⁴⁹ in the Hamakua district of Hawaii. These workers detected plague 358 times in 4,808 rodents found dead as against 23 times in 20,623 killed animals and 20 times in 304,807 trapped rodents.

Evaluation of Methods

The principal methods used to detect plague infection in rodents are as follows :

- (1) autopsy;
- (2) bacteriological tests (smears and cultures);
- (3) serological tests;
- (4) animal experiments with material from individual rodents or with cultures isolated from them;

- (5) animal experiments with pooled organs;
- (6) animal experiments with pooled fleas.

Autopsy

Bearing in mind that the pioneer work of the Plague Research Commission⁸⁸ was done at the time when acute plague was rampant among the rats of India, it is not surprising to find that the Commissioners, comparing in 1907 the various methods available for the diagnosis of rodent plague, emphasized the outstanding value of the macroscopic findings. In the opinion of the Plague Commission, it was justifiable to diagnose the disease in rats on the basis of the presence of even one of the pathognomonic gross signs, regardless of whether or not subsequent smear examinations, culture tests, or animal experiments yielded positive results.

It is no longer possible to evaluate the results of macroscopic examinations as highly as the Plague Research Commission did—not only because animals may be found which, for the reasons set forth in the preceding chapter, show no gross signs of the infection, but also because observations made outside India have proved that morbid appearances macroscopically indistinguishable from plague may be produced by infective agents other than *Pasteurella pestis*. It is noteworthy in this connexion that, in the experience of Macchiavello⁷⁰ at Antofagasta, Chile, rats which afterwards proved to be plague-infected were less apt to show suggestive gross signs than a large number of macroscopically suspect but actually plague-negative animals.

Nevertheless, the presence of signs such as typical buboes, a characteristic mottled or waxlike appearance of the liver, and clear pleural fluid has been found to be strongly suggestive in most plague areas; as has been noted before, frequency of rats showing such pathognomonic signs proves the presence of an acute epizootic, while their rarity is suggestive of a quiescent stage of the infection. In order to make reliable macroscopic observations, it is necessary, however, to perform dissection before putrefaction has set in.

Bacteriological tests

While subsequent workers, following the lead of the Plague Research Commission, continued to lay stress upon the direct results of rat dissection, they usually considered it necessary to confirm the diagnosis with the aid of laboratory methods, especially smear examination and culture tests.

Although, as will be discussed later (page 237), great care has to be exerted in interpreting the results of smear examination, the abundance of characteristic bipolar-stained bacilli or involution forms in smears prepared from the organs of the dissected rodents goes a long way towards supporting a positive diagnosis in the course of outbreaks previously confirmed by fully reliable methods.

Culture methods are fully satisfactory whenever it is possible to examine fresh material, but the presence of putrefaction renders it difficult or even

impossible to obtain reliable results from the buboes or internal organs of plague-suspect animals by such tests. However, the possibility of proving the existence of plague in putrified rodents was greatly increased by the recommendation—first made in 1926 by Pons (quoted by Murdock⁸¹), then by Fusco (quoted by Devignat²³) and Micheletti,⁷⁸ and again independently by Uriarte et al.¹¹¹—that in such cases the bone-marrow of the tibia or femur, rather than the internal organs, should be used for bacteriological examinations.^a

While the great value of this method was confirmed by most field workers and also through experiments made with rat and guinea-pig carcasses by Russo,⁹³ a few investigators came to less satisfactory conclusions. Macchia-vello & Paracampos⁷³ in particular, working in the hot climate of north-east Brazil, had many failures with this procedure and expressed an urgent warning against the exclusion of the possibility of plague on the grounds of negative results thus obtained. They admitted, however, that the impossibility of cultivating *P. pestis* from many of their rodents appeared to be largely due to the fact that the plague strains prevailing in north-east Brazil were often too attenuated to cause septicaemia. Bone-marrow cultures made from animals suffering from acute or subacute plague were invariably positive and less contaminated than those from internal organs. Moreover, since the viscera of dead rodents were apt to be destroyed by insect larvae, bone-marrow examination was often the only method practicable for laboratory investigation.

Serological tests

Since serological tests yield results far more rapidly than do culture tests, they are of outstanding value for the confirmation of the *prima facie* evidence obtained from dissection and smear examination. The methods suitable for such tests have been referred to in Chapter 3 and are dealt with in greater detail on page 243.

Animal experiments

Individual tests. Animal experiments performed with the organs of individual rodents or with the growths isolated from them yield fully reliable results if conducted in an adequate manner. They are, therefore, of the greatest value in confirming the diagnosis both in incipient plague epizootics and in sporadic instances of rodent infection. However, it is hardly possible to perform experiments with material from each individual animal during the routine examination of large numbers of rodents.

^a Petraghani,⁸⁶ while endorsing the value of bone-marrow examination, considered it still more advantageous to cultivate the brain of putrefied rats in a medium consisting of eight parts of ordinary and two parts of liver bouillon.

Pooling-tests.

(a) *Organs* : A most important innovation, permitting of the large-scale use of animal experimentation for the diagnosis of rodent plague, was introduced by Chapin¹⁹ in 1909. Finding that many of the rats dissected by him at Seattle, USA, were too putrified to show gross lesions, and wishing not to overlook instances of "inapparent" infection, he resorted to cutaneous inoculation of guinea-pigs with the organs of not one but a whole group of rats. Chapin himself, perhaps because he used "the organs of every rat of an entire catch . . . sometimes 30 or even more in number" to infect single guinea-pigs, failed to obtain positive results in the 400 rats tested in this manner. However, the method soon afterwards proved successful in the hands of other workers, such as Lloyd¹¹,⁶⁸ (also at Seattle) in 1914 and Creel¹¹ at New Orleans in 1915, and has been much used since.

Swellengrebel & Hoesen¹⁰⁴ seem to have been the first to replace Chapin's method by subcutaneous infection of guinea-pigs with suspensions obtained by collecting small pieces of the organs of the rats to be examined in a sterile mortar and grinding up this material with a little sterile saline. Handling in this manner 7,625 apparently normal rats, the two workers were able to detect plague in 54 pools.

The value of this method, which has remained one of the standard procedures for ascertaining the presence of plague in commensal rats as well as in wild rodents, has been well demonstrated by the following results reported at the 1934 Calcutta Conference of Medical Research Workers (quoted by Pollitzer⁸⁹) :

<i>Species</i>	<i>Number of animals used or group tests</i>	<i>Number of animals found smear-positive</i>	<i>Number of groups tested</i>	<i>Number of positive group tests</i>
<i>Rattus rattus</i>	2,107	2	141	13
<i>Mus musculus</i>	1,393	—	100	6
<i>Bandicota malabarica</i>	306	1	95	15

These results are all the more noteworthy because rodents showing suggestive signs of infection were not included in the group tests. Cultures made from the suspensions prepared for guinea-pig infection were often unsatisfactory.

An interesting method, taking advantage of bone-marrow examination as well as of pooling, was used by Devignat²⁵ in the course of monthly rodent surveys in the Belgian Congo. The procedure consisted in cutting off the legs of freshly killed rodents (mainly *Rattus natalensis*) just above the knee, aspirating the bone-marrow with the aid of a syringe provided with a suitably thin and short-bevelled needle, and suspending the material in a bottle containing 3 ml of sterile normal saline. Repeating this operation with each animal of the daily catch, it was thus possible to

obtain a pool containing material from up to 500 rodents. The suspension was immediately used for subcutaneous inoculation of a guinea-pig, which was then dispatched to the laboratory. In the case of animals the bones of which were too small to yield bone-marrow, the spleens were successively used for cutaneous inoculation of a guinea-pig.

Practising these methods, Devignat repeatedly succeeded in demonstrating the existence of plague in rodents which otherwise appeared to be free from the infection.

Van Riel & Mol,⁹² working in the Kivu region of the Belgian Congo, also obtained good results with bone-marrow pools. They used the subcutaneous method for infecting guinea-pigs in the field, but preferred the cutaneous route when making tests in the laboratory.

It deserves attention, however, that the experiences made by Heisch⁵³ in the Rongai area of Kenya, where inapparent plague seemed to be prevalent among the wild rodents, did not confirm the value of the above-described method. Heisch referred in particular to one instance, where the pooled spleens, bone-marrow, and fleas collected from a batch of *Arvicanthis* had been used separately for the inoculation of different guinea-pigs, and positive results had been obtained only with the spleens. He urged, therefore, that pools from the internal organs rather than those from the bone-marrow should be used to demonstrate the presence of inapparent rodent plague and recommended in particular the technique adopted by Meyer et al.,⁷⁷ who inoculated their test guinea-pigs with material collected from not more than five plague-suspect ground-squirrels.

(b) *Fleas*: Several observers like Kitasato, the Plague Research Commission, Petrie & Todd and Jettmar (quoted by Pollitzer⁸⁹) had shown that while, particularly at the end of an intense epizootic, it was often difficult or even impossible to find plague-affected rodents, it was usually comparatively easy to collect infected fleas for laboratory tests. However, since smear examination and culture methods are, as a rule, rather unsuitable for demonstrating the existence of the infection in these insects, it was often necessary to resort to animal experiments in order to get reliable results.

An early attempt to use pooling methods when examining fleas was made by Swellengrebel & Hoesen¹⁰⁴ who, working with 15,279 fleas, obtained positive results with 18 pools. However, the method attracted no attention until it was once more applied in 1936 by Eskey³² to detect foci of wild-rodent plague in California. Since then, ample use has been made of this procedure in order to ascertain the presence of the infection in commensal rats as well as in wild rodents.

No doubt can exist that the large-scale use of one or preferably both of the above-mentioned pooling methods is alone fully reliable for deciding whether or not plague is present in a given rodent population.

Comparison. Assessing the value of pooling-tests in comparison with other methods of rodent and flea examination, it must be realized that, paradoxical though it may seem at first glance, the extreme sensitivity of the pooling-tests limits their usefulness. Being apt to prove positive in the case of pools containing merely a few, or possibly even single, virulent plague bacilli, they furnish a reliable answer to the question of whether plague is present in the rodents or fleas examined, but do not show to what extent the infection is present. Although this second point may be rather immaterial under certain circumstances—for instance when making large-scale wild-rodent surveys—it is of paramount importance when dealing with plague manifestations in individual localities, where the proper conduct of anti-epidemic work depends on full awareness of the trend of infection. Under these circumstances, to neglect the methods available for the examination of individual rodents in favour of pooling-tests would be erroneous.

As has been noted above, the method of using flea pools has the great advantage of being applicable even when the number of rodents has become much reduced by an extensive epizootic. Moreover, since fleas are not merely vectors, but also to a considerable extent reservoirs of plague, positive results with flea pools may be obtained during the off-seasons, when it may be difficult or even impossible to prove the continued existence of rodent infection. It must also be admitted that examination of flea pools is more expedient and less dangerous than that of tissue pools.

It is therefore not surprising to find that some workers place sole reliance on the former method, considering the routine examination of tissue pools to be generally superfluous. Humphreys et al.⁵⁷ recently supported this opinion because they found that parallel examination of 5,019 tissue pools and 4,641 flea pools yielded only three instances in which the former proved positive and the latter negative. However, pointing out that in two of these three instances only one flea per animal had been obtained, Humphreys and his co-workers admitted the value of tissue-pool tests in cases where the rodents concerned were free, or nearly free, from ectoparasites. It must be realized as well that positive results with flea pools may prove misleading in so far as the rodents which appear to be concerned may have picked up fleas from plague-susceptible species without suffering from the disease themselves. It would appear, therefore, that examination of tissue pools, since it serves to check the results obtained with flea pools, should be used parallel with flea-pool tests periodically at least, if not as a matter of routine.

Techniques

Dealing with the technique of rodent examination, it seems permissible to concentrate attention on the methods suitable for commensal rats,

which in most plague areas form the bulk of the material to be handled ; the more so because the procedures to be adopted in the case of other rodent species do not differ in principle from those required for rat investigation.

Collection and delivery of rats

If rats found dead, or killed by the people, are available for examination, they should preferably be put into individual paper bags or be individually wrapped in paper for transport to the laboratory.

An alternative adopted in China was to place large tin buckets or wooden boxes with lids, or large, covered earthenware jars, at strategic points of the plague-affected settlements. The people were urged to place the dead rodents in these collecting vessels, which were inspected twice daily ; the rodents found were taken to the laboratory in covered tin boxes. In plague-affected localities where the people were un-co-operative, it was possible to obtain dead rats by paying rewards to the scavengers for the delivery of carcasses found by them in refuse.

Rats caught in snap traps should be transported to the laboratory in individual paper or canvas bags. An adequate procedure recently recommended in this connexion by Gross & Bonnet⁴⁹ was as follows : taking care not to touch the animals, the trappers freed them by picking up the trap by the base and releasing the striker, allowing the rodent to fall into a suitable receptacle. If it was proposed to examine the fleas, the retrieved rodent was placed in a paper bag and one-quarter of a teaspoonful of calcium cyanide was inserted ; the bag was then shaken and tightly tied by the neck. Otherwise the trapped rodents were put into gallon-cans containing kerosene.

Cage traps in which rats have been caught alive should be enclosed in canvas bags for transport to the laboratory.

Killing rats trapped alive

Although, as discussed by Omar,⁸⁵ a considerable number of procedures has been recommended for killing rats delivered live to the laboratory, the following deserve prime consideration :

Submersion. While the method of submerging the rodents in water or other fluids is the least attractive of the available procedures, it is cheap and effective, the more so as it facilitates collection of the fleas harboured by the rodents. The use of antiseptic fluids in place of pure water offers no essential advantages and must be avoided when it is planned to use the fleas for culture examination or pooling-tests.

Volatile fluids. A simple and effective method for killing rats in cage traps enclosed in canvas bags is to put each bag with the trap inside into a suitably sized box or tin, partly to open the bag and to push in a cotton pad

soaked in a volatile fluid such as chloroform, ether, or gasolene. The box or tin is then kept closed for about 20 minutes, a time sufficient to kill the rat and at least to stun its fleas. Gasolene is the cheapest of the suitable volatile fluids and is also preferable in so far as, according to Eskey & Haas,³⁴ chloroform or ether is apt to exert an adverse influence on the infectivity of the fleas.

Lethal gases. Trapped rats as well as their fleas may be conveniently killed with the aid of calcium cyanide. For this purpose the cage traps in their canvas bags, which should first be opened up, are put into a tin or wooden box provided with a well-fitting cover and an opening suitable for pumping in the dust after the receptacle has been closed.

The rats and their fleas are killed rapidly and, if a tin of sufficient size is provided, several traps may be dealt with at the same time.

Calcium cyanide is also suitable for dealing with dead rats delivered to the laboratory, particularly those not enclosed in bags. For this purpose a layer of the chemical is put into a large tin bucket provided with a well-fitting cover. The rat carcasses are placed on the calcium cyanide and should preferably be kept in the closed tin overnight. Before they are taken out in the morning, the tin should be left open for at least 15 minutes. In the case of an acute epizootic, calcium cyanide may be applied once more before ventilation of the tin.

To avoid accidents, these methods should be applied in the open air by workers trained in their use.

Collection of fleas

Before carrying out dissections, it is necessary to free the rodents from fleas and other ectoparasites. This can be easily done by thoroughly brushing and combing all parts of the fur of the carcasses. These operations are best performed either in a large, deep, white enamel pan or while holding or suspending the rats over a large, white enamel basin filled with water. Before starting the work, the rats may be first plunged into the water; some workers prefer to keep them submerged while they are brushed and combed.

The fleas and other ectoparasites collected from each rat should be put into a separate stoppered ampoule to await classification and indexing.

Dissection and preparation of material

It would be redundant to deal in detail with the technique of rat dissection, which is performed according to the methods generally adopted for the autopsy of small laboratory animals—although, as is described on page 245, with special precautions. However, attention must be drawn to the necessity of examining with special care the lymph-nodes of plague-suspect carcasses. The recommendation of the Plague Research Commission that

the pectoral muscles should be cut so as to make the axillary lymph-nodes visible has been referred to in Chapter 4, and the advice of the Commission to inspect the cut surface even of lymph-nodes which appear to be unaffected externally has also been noted. In addition to the superficial lymph-nodes, the deep cervical, mesenteric, retroperitoneal, and pelvic lymph-nodes should receive attention.

Smears or impression films should be made not only from abnormal lymph-nodes but also from the heart-blood, spleen, liver, and lungs, all of which should be placed on one slide in order to save labour and material. Cultures and material for animal experiments or pooling-tests should be taken from any bubo present as well as from one or more of the above-mentioned internal organs, or, in the case of putrefaction, from the bone-marrow.

Recording of results

As recommended in the antiplague instructions published by the Office International d'Hygiène Publique in 1937,⁸² daily records should be kept showing (a) the number of rats and other rodents examined, classified by species; (b) the number of males, females, and pregnant females in each species; (c) the number of rats found infected in each species.

These figures and their totals are used to calculate plague indices, i.e., the percentages of rodents found infected in each species and in the totals. If, as is advisable, graphs based on these values are prepared, it is preferable to use for this purpose the five-day averages instead of the daily figures (Shih & Pollitzer⁹⁶).

Flea examination

While animal experiments with pooled fleas are of outstanding value in plague work, possibilities for taking advantage of bacteriological methods for the examination of plague-suspect fleas are rather limited. It has been stated by Webster¹¹³ that smears of individual crushed fleas may be stained and examined for the presence of plague-like bacilli, but only a proportion of the plague-infected fleas will be detected thus, and the morphology is not really conclusive. Studying the suitability of culture methods for detecting plague in fleas, Tiflov¹⁰⁶ worked with suspensions prepared by crushing 26-100 living wild-rodent fleas (*Nosopsyllus consimilis* or *Ctenophthalmus wagneri*), each batch containing but one infected flea. Results obtained on the one hand by injecting part of the suspensions intramuscularly into guinea-pigs, and on the other by cultivation were as follows :

<i>Method</i>	<i>Number of tests made</i>	<i>Number of positive results</i>
Guinea-pig infection	40	39
Cultivation on agar	39	33
Cultivation on blood-agar	39	37

However, results obtained by most other workers when using suspensions prepared by crushing fleas for cultivation were so disappointing that it was recommended that the proventriculus and stomach of plague-suspect fleas should be dissected out and the contents used for cultivation. Satisfactory results were obtained in this way both with individual fleas (Gore⁴⁷) and when pooling the stomach contents of 60-70 fleas (Bichkov & Borzenkov¹⁰), but this procedure is far too elaborate to be suitable for routine work. Animal experiments therefore remain the method of choice for routine examination of plague-suspect fleas.

EXAMINATION IN HUMAN PLAGUE

Patients

Bubonic plague

Puncture. Although ample use has been made of bubo puncture to collect material for the bacteriological diagnosis of bubonic plague, various objections have been raised against this method.

Some workers pointed out that in the acute stage of the disease it was difficult, with the aid of this procedure, to obtain sufficient material for examination. It must be noted, however, that this difficulty may be easily overcome if, as recommended by Girard,³⁹ the needle and syringe are well washed out after puncture with a little saline and the latter is then used for the tests. The alternative, sometimes suggested, of first injecting a few drops of sterile saline or broth into the substance of the bubo and then aspirating, is less recommendable—if for no other reason than that this procedure considerably adds to the discomfort of the patient.

Another objection was that even in the acute stage of the disease the method was apt to yield negative results if, instead of the affected lymph-nodes themselves, the often conspicuous oedema hiding them were punctured.^b It was also pointed out that since, in the course of recovery, the causative organisms in the affected lymph-nodes first became scanty and then altogether disappeared, punctures carried out late in the disease often failed to confirm the diagnosis of plague. This held true particularly of suppurated buboes.

The fear was moreover expressed that puncture of plague buboes might prove harmful by facilitating entry of the infection into the lymph- or bloodstream. It is difficult to agree with this opinion if it is considered that extirpation of plague buboes has been successfully practised to treat the disease; it must, however, be admitted that the operation is rather painful.

^b As aptly pointed out by de Smidt,¹⁰¹ the punctate of plague buboes should contain numerous cells as well as plague bacilli. Absence of the former indicates that the buboes have not been reached.

Blood culture. Dissatisfied with the method of bubo puncture, several observers advised the use, instead, of early blood culture for the diagnosis of bubonic plague. Some, such as Bonebakker,¹⁴ recommended bile media for this purpose; South American workers, such as da Silva⁹⁸ and Barreto & Castro,³ preferred direct intraperitoneal inoculation of experimental animals with the blood of the patients.

As has been pointed out in chapter 4, particularly rapid results may be obtained with the latter procedure if, according to the proposal of Gotschlich,⁴⁸ material for microscopic and culture examinations is taken in vivo from the peritoneal cavity of the infected animals.

In addition to the above-mentioned methods, serodiagnostic procedures may be used (see page 243).

Evaluation. When trying to determine the comparative value of these methods, it must be kept in mind that, in order to take full advantage of the potent remedies now available for plague treatment by starting their administration at the first possible moment, an early diagnosis of the disease is indispensable. Considering that in bubonic plague development of the gland affection precedes that of septicaemia, it would seem at first glance that examination of material obtained through bubo puncture should be of greater value for making a quick diagnosis than blood tests, and still more than serodiagnostic procedures, which yield a positive result only after the morbid process has developed. It must be realized, however, that this holds true only as far as smear examination of the bubo contents is concerned, because confirmation of the diagnosis through culture tests and animal experiments is no less time-consuming when such material is used than when the blood of the patient is examined.

The finding of numerous and characteristic bacilli in smears made from the buboes goes a long way towards supporting the diagnosis, but it is questionable how far such results are superior in value to the *prima facie* evidence procurable in typical cases through clinical examination alone. It is felt, therefore, that bubo puncture followed by smear examination and confirmatory tests should be used only in clinically doubtful cases, or in order to establish the diagnosis in hitherto plague-free localities. Ordinarily a decision whether or not to initiate treatment can be reached on clinical grounds, and blood examination or (particularly in mild cases or those which have received early treatment) serodiagnostic methods should be used to confirm the diagnosis.

Primary pneumonic plague

A clinical diagnosis of primary pneumonic plague is easy as soon as the characteristic sputum is expectorated, and this evidence is strongly supported by the examination of sputum smears which in typical cases show an abundance of characteristic bacilli. Culture tests and animal

experiments carried out with such sputum yield comparatively rapid results.

As will be discussed later, primary pneumonic plague is ushered in by a stage lasting about 20-24 hours, during which cough is absent or insignificant and no characteristic expectoration occurs. At this stage, plague bacilli are either altogether absent from the sputum or saliva, or are present in such small numbers among other bacteria as not to be recognizable with certainty. Clinically, such patients show signs of an acute general infection but no lung involvement. To arrive at a definite diagnosis at this stage of the disease is therefore difficult or impossible. If plague is present, further clinical observation as well as repetition of sputum examinations at frequent intervals will eventually lead to a diagnosis. It is clear, however, that during epidemics, and particularly when dealing with patients who have been in contact with pneumonic-plague victims, it is imperative not to wait for such confirmation but to start energetic treatment as soon as the presence of the disease is suspected.

Septicaemic plague

A *prima facie* diagnosis of plague without manifest buboes or manifest lung involvement is not difficult in the ultimate stage of the disease, because it is then possible to detect large, or at least appreciable, numbers of plague bacilli in blood smears. Earlier in the disease the organisms are few and far between; the clinical signs are only those of a serious general infection and are hence without pathognomonic significance. However, as pointed out by Bouffard & Girard,¹⁵ puncture of the liver may be used with advantage at this stage, and it is also noteworthy that Goldstein⁴⁶ was successful when puncturing the lungs in cases of pulmonary plague where no manifest signs of pneumonia could be detected. Conseil & Durand²² recommended lung puncture also in cases of pneumonic plague where no sputum was available, especially in children who, as a rule, did not expectorate.

The laboratory diagnosis of septicaemic plague can be easily established through culture tests or animal experiments with blood specimens taken even early in the disease, but by the time the results of such examinations become available the patients will be beyond hope, if not already dead. Their only chance lies in drastic treatment started as soon as the presence of the infection is suspected.

Dead bodies

Whenever a complete autopsy is possible, no difficulty will be encountered in establishing a *prima facie* diagnosis in the case of typical bubonic plague, even if the presence of the disease had not been previously suspected, because the characteristic features of a haemorrhagic type of adenitis

are hardly ever found in morbid processes due to other causes (van den Berg & Vos ⁴).

Cases where the bubonic lesions are not marked may be far more easily overlooked unless the possibility of plague is kept in mind and adequate laboratory examinations are undertaken.

The same may be said to hold true of instances of primary respiratory infection where pneumonic changes are slight or even absent. Cases of typical primary pneumonic plague usually show such distinctive changes in lungs and pleurae that they should lead, if not to a *prima facie* diagnosis, at least to the suspicion that some peculiar infection, necessitating careful bacteriological examination, is present. Still, the fact should not be overlooked that certain other infective processes, especially influenza, may produce gross lung lesions not dissimilar to pneumonic plague, and that some instances are on record where micro-organisms closely resembling *P. pestis* were found in individuals showing pneumonic features at autopsy (Pollitzer ⁸⁹).

Not only for lack of facilities but mainly on account of popular prejudices, it is often impossible to perform complete autopsies in instances where death from plague is suspected. Various methods have been recommended to obtain, under such circumstances, material for laboratory examination.

Excision

Some workers, such as Lefrou ⁶⁴ and, more recently, Macchiavello,⁷¹ advised excision of the buboes and/or pieces of suitable internal organs (e.g., the liver, the spleen, or—if pneumonic plague was suspected—the lungs) in place of complete autopsies, but it must be feared that even this simplified procedure would be much resented in countries where any mutilation of dead bodies is abhorred. Serious consideration should be given, however, to the possibility of using the viscerotome in place of dissection procedures, as has been successfully done by plague workers in Ecuador.¹²

Puncture

A procedure much used in place of dissection methods for obtaining material for laboratory examinations was puncture of various organs of the dead bodies. In the case of bubonic plague, the buboes were usually advocated for this purpose, but satisfactory results were also obtained both in this form of the disease and in septicaemic plague by puncturing the liver (Bouffard & Girard ¹⁵). Opinions as to what organs should be chosen in the case of pneumonic plague were somewhat divergent, but most workers advised puncturing several parts of both lungs as well as the liver

or heart (Girard; ^{38, 40} Petrie; ⁸⁷ Kamal ⁵⁹). Such elaborate procedures were recommended because sometimes lung punctures failed when material was taken from non-consolidated parts of the organ (Wu Lien-teh ¹¹⁴).

Generally speaking, it must be realized that negative results with materials obtained by puncturing the organs of dead bodies do not exclude the possibility that the individuals in question might have succumbed to plague, particularly if smear examinations alone are used to support the diagnosis. On the other hand, owing to the not infrequent presence in the dead bodies of coliform or other bacilli resembling *P. pestis*, the exclusive use of smear examination may yield false positive results. It should be noted, in this connexion, that Gram-negative bacilli of a suggestive appearance are conspicuous in smears made with material from the gastrointestinal tract, which may be punctured in place of the liver or spleen by inexperienced workers. However, cells are practically entirely absent in such preparations.

A useful procedure recommended by Vincke ¹¹² and Girard ^{40, 41} for facilitating animal experiments with the materials obtained by puncturing dead bodies was to wash out the syringe, after puncture, with sterile normal saline and to use the fluid for the inoculation of guinea-pigs. Vincke repeatedly punctured the buboes and/or various organs, washing out the needle and syringe after each puncture with the aid of 3 ml of sterile saline which had been placed in a 10-ml flask, and using the resulting suspensions to inoculate guinea-pigs cutaneously with the aid of cotton plugs. He found that material taken in this way within 24 hours after the death of the victims remained suitable for animal inoculation for 5 days, provided that the temperature did not exceed 35°C. A similar method was used by Girard who took material from any bubo present as well as from the liver and the upper, middle, and lower parts of both lungs.

Bone-marrow examination

Several workers obtained satisfactory results when using the bone-marrow of plague-suspect corpses for laboratory tests. Thus Ramos Diaz ⁹¹ was able to make a retrospective diagnosis of a pneumonic-plague outbreak in Peru by examination of the bone-marrow from a rib of one of the victims who had been buried for two months.

Some of the workers in South America recommended obtaining bone-marrow for laboratory tests from dead bodies by "digitomy", i.e., amputation of a finger (preferably the most easily amputated forefinger) and opening the second or third phalanx. Lobo & Silvetti ⁶⁹ found this material, because scant in plague bacilli, unsuitable for smear examination, but obtained good results by cultivation or animal experiments.

Alvarado,¹ dealing with the technique of "digitomy", recommended disinfecting the finger, before amputation, with alcohol. After the opera-

tion, which could be safely performed without rubber gloves, the operation wound was covered up with a cotton pad dipped in formalin and fixed with a bandage, or the hand was simply placed under the clothing of the dead body. For transport to the laboratory, the amputated finger was placed stump down in a bottle containing absorbent cotton. Extraction of the bone-marrow was somewhat difficult but could be facilitated by opening the bones lengthwise instead of crosswise. Alvarado suggested that in the plague regions compulsory "digitomy" should be performed in all cases where death after an acute illness of less than ten days' duration had occurred.

The method was also recommended by Barreto & Castro,³ who advised using the bone-marrow extracted immediately after amputation for the preparation of a roll culture in sulfite agar which was forwarded to the laboratory. The same procedure was used to dispatch material obtained through bubo puncture or section of the veins.

Useful as these methods of bone-marrow extraction are, they would, one fears, meet with much opposition in some of the plague areas. It might be possible, however, under such conditions to obtain bone-marrow for postmortem examination from the sternal bone, as is done in clinical work. It is of importance to note in this connexion that Modica⁷⁹ obtained positive cultural results in 10 out of 12 instances with the bone-marrow obtained through sternal puncture from plague patients.

Preservation of Material

Not only in the case of wild-rodent infection but also in that of rat-caused plague, which shows an increasing tendency to evolve in remote rural localities, it has become a matter of great importance to ensure that the materials to be forwarded for examination reach the often distant laboratories in a fit condition.

As has been noted, some workers, instead of relying on methods of preservation, preferred to use the material for examination immediately after its collection for the preparation of cultures (see Barreto & Castro³) or the inoculation of test animals (see Devignat²⁵ and van Riel & Mol⁹²), but for various reasons it is often impossible to take advantage of these procedures. In such cases it is necessary to preserve the vitality and virulence of the materials to be sent to the laboratories either by physical means or by keeping them in fluids or other substances counteracting putrefaction.

Into the first-mentioned category fall various procedures for keeping the materials for examination at a low temperature pending transport.

A method adopted for this purpose in Argentina was to use, for the transport of rats, large metal drums provided with double walls and to put ice into the outer compartments of these containers, which had a capacity

for 20-60 rats.⁸⁰ Henriques⁵⁵ recommended putting the tissues to be forwarded into test-tubes with paraffined stoppers, and enclosing the tubes for transmittal in thermos flasks filled with broken ice. A similar method is used in the USA (Link⁶⁷). Macchiavello & Paracamos,⁷² studying the viability and virulence of *P. pestis* in the tropics, noted that if guinea-pig or rat organs were kept in the icebox, survival of the plague bacilli was better in the spleen than in the liver, in which apparently a rapid autolysis, inimical to the micro-organisms, took place. For this reason some laboratories did not recommend forwarding liver specimens for examination.

It is obvious that preservation on ice of plague-suspect carcasses or organs pending transit will prove useful only as long as the distances to be covered are not too great or if rapid means of transport are available. This is not universally the case and, moreover, in many of the rural plague foci, ice is not available. The recent proposal of Amies² to use common salt instead of ice deserves, therefore, due attention. His method consisted of putting the suspect carcasses into a dry jar with a cap which was screwed down tightly after 2-3 ounces (60-80 g) of salt had been added. The salt was then made to cover the body of the animal by rotating the jar. It was best to examine the bone-marrow of animals preserved in this manner. Positive results were obtained in this way even from a plague-infected animal kept for 67 days.

Broquet advocated the preservation of plague-suspect organs in a fluid containing 20% glycerol and 2% calcium carbonate, stating that *P. pestis* in organs kept in this manner remained virulent for periods up to 13 days.¹⁶ The usefulness of this method was confirmed by several workers. Uriarte & Morales Villazon¹¹⁰ recorded in this connexion that, in the spleens of experimentally infected animals which had been preserved in Broquet's fluid either at room temperature or in the refrigerator, plague bacilli usually remained virulent for 12-19 days, sometimes even longer. Prado jr.⁹⁰ found Broquet's fluid useful for forwarding the lymph-nodes of plague-suspect rats to the laboratory. After arrival the tissues were placed on sterile filter-paper to remove the excess of glycerol and were then used for cutaneous or subcutaneous inoculation of test animals. Issaly & de Issaly⁵⁸ recommended the following formula for preparing Broquet's fluid: 20 ml of pure neutral glycerol (Baumé 30°), 2 g of calcium carbonate, and distilled water to 100 ml. The fluid was sterilized for 10 minutes at 100°C and then kept in bottles, the corks of which had been sterilized by dry heat and afterwards impregnated with sterilized melted paraffin to prevent development of fungi. These authors found that the spleens removed from experimentally infected guinea-pigs even two days after death continued to harbour virulent plague bacilli up to 15 days when kept in the fluid. They recommended its use for forwarding the organs of plague-suspect animals, preferably spleen or bone-marrow, to the laboratory. To prevent acidification of the fluid which, like the formation of fungi, biased its efficiency, they advised buffering with

bisodium phosphate and citric acid (1.1046 g and 0.0230 g, respectively, per 100 ml of fluid).

As established by Devignat²⁴ and Henriques,⁵⁵ infection of experimental animals could be produced with plague fleas kept in Broquet's fluid for periods up to 6 days.

Satisfactory results were also obtained by using saline solutions, in place of Broquet's fluid, for the preservation of plague-suspect organs or fleas. Webster,¹¹³ who seems to have been the first to suggest this method, recommended that in the case of fresh carcasses a few drops of heart-blood be taken with a sterile pipette through a seared portion of the heart surface and added to one ml of sterile citrated saline in a small test-tube. Such samples were apt to remain suitable for laboratory examination for some days.

As established by Girard,³⁹ suspensions in normal saline obtained from experimental animals 3-10 hours after death by puncture and washing-out of the syringe remained virulent for up to 6 days when kept at a temperature of 16°-26°C, but for 3 days only when kept at 37°C. Suspensions from the carcasses of experimental animals which had been kept after death for 48 hours at 21°C remained virulent for 24 hours only, but the organs of such animals were still infective after 3 days.

Eskey & Haas^{33, 34} recommended 2% solutions of sodium chloride for the transport of fleas to the laboratory. This concentration, while inhibiting the growth of contaminating bacteria, was not harmful to the plague bacilli. Meyer⁷⁵ even used 3% saline for forwarding fleas during summer, but took care to wash them in normal saline before grinding them up for animal tests.

The methods of preserving plague-suspect organs in paraffin with a melting-point of 42°-44°C (Henriques⁵⁵) or in mixtures of vaseline oil and vaseline (or lanolin or paraffin), as recommended by Berlin & Bacheva,⁵ have not been widely used. For various reasons Girard⁴² considered the latter method of little practical value.

Synopsis of Diagnostic and Differential-Diagnostic Methods

Laboratory methods for the diagnosis and differential diagnosis of plague have already been discussed in chapters 3 and 4. Nevertheless, it seems advisable to summarize them here, at the same time indicating their characteristic values.

Motility tests

As has been mentioned when dealing with the bacteriology of plague, the differential-diagnostic value of motility tests is not absolute since *P. pestis* is invariably immotile while *P. pseudotuberculosis*, though typically

motile, may occasionally fail to show movement. Generally speaking these tests are, however, of importance, the more so since with their aid it is possible to establish quickly the fact that otherwise suspect strains which show motility are not those of plague.

In place of hanging-drop examinations some plague workers recommended the use of Levinthal's⁵⁵ method. A useful modification of the latter was described by Himmelfarb :⁵⁶

Small drops of broth cultures of *P. pestis* are placed without spreading on thin agar; the plates are then incubated for 24-48 hours at 20°C. A small coverglass is placed next to a suitable colony and a second coverglass, with one edge resting on the first, is put in a slanting position over the colony. A little saline is placed over the colony with a capillary pipette. Motility is then studied with the aid of a high-power dry objective or the immersion lens.

To facilitate a distinction between active Brownian movement and true motility, Himmelfarb recommended replacing the saline by a 1‰ solution of mercuric chloride which, while not interfering with Brownian movement, rendered actively moving microorganisms immotile.

Smears

Fixation. Many workers, both because the usual method of fixation by passing the preparations through the flame does not ensure killing of the plague bacilli and because specimens treated in this way show less clear bipolar staining, prefer fixation of plague smears with alcohol. For this purpose the air-dried smears are treated for at least one minute with 95% ethanol, an ethanol-ether mixture, or preferably absolute methanol. Unpurified or denatured methanol such as that used as fuel, may be substituted. If many specimens have to be handled, it is expedient to immerse them in staining jars filled with alcohol. After fixation, the alcohol is poured off and the specimens are dried. Some workers advise the washing-off of the alcohol with water before drying the smears in the air or with the aid of blotting-paper. If smears have to be sent to a distant laboratory for examination, it is, according to Webster,¹¹³ best to fix them in alcohol for 10 minutes and, after drying, to dispatch them unstained.

Staining. Although different workers recommend various and sometimes rather elaborate methods of staining plague smears, it is as a rule quite satisfactory to use, in addition to Gram's method, the simple stains ordinarily available in laboratories, such as dilute carbolfuchsin, Læffler's methylene blue, or 1% carbolthionin blue. Should bipolar staining be indistinct, the alcohol-fixed specimens may be treated before staining, for half a minute with 0.5% acetic acid (Gaffky, quoted by Dieudonné & Otto²⁸). Petraghani⁸⁶ found it advantageous to use, instead of Læffler's methylene blue, a stain made up by adding to 10 ml of distilled water 2-3 drops of cold-saturated methylene blue and 12 drops of a 1‰ solution of lactic acid, staining at slight heat for half an hour.

Gram's method of staining is particularly indicated when dealing with sputum, pus from buboes, or material from putrified carcasses or dead bodies, so as to facilitate the differentiation of the Gram-negative plague bacillus from Gram-positive micro-organisms, such as the pneumococcus and certain spore-bearers which may resemble *P. pestis* in simply-stained preparations.

Webster¹¹³ advocated using Jensen's modification of Gram's method for plague smears, while de Smidt¹⁰¹ was in favour of the following method :

Alcohol-fixed smears were stained for 15 seconds in a solution consisting of 1 g crystal violet, 10 ml absolute ethanol, and 300 ml distilled water ; after washing, the smears were treated for a few seconds with Gram's iodine until brown or iron-grey, and acetone was then directly applied until no more colour was given off. After rapid washing the smears were counterstained with 1% neutral red solution.

Evaluation. Modern workers are unanimously of the opinion that, particularly when dealing with incipient outbreaks or with sporadic instances of the infection, smear examinations alone are insufficient to establish the diagnosis of plague.

As has been discussed before (page 222), instances of "inapparent" infection, where smears yield a negative result but where animal experiments prove positive, are frequently observed in rodents. Similarly, culture tests or animal experiments may sometimes establish the diagnosis of human plague in instances where smear examinations gave a negative result. The presence of morphologically atypical plague bacilli may also confound the issue when sole reliance is being placed on an inspection of smears.

On the other hand, examination of smears is often apt to suggest the presence of plague in instances where the infection is absent. Pasteurellae other than the plague bacillus, showing morphological features similar to, or even identical with, those of the latter organism, have often been found in rodents, and occasionally also in man. Moreover, in decomposed carcasses or dead bodies, bacilli belonging to other genera may show microscopic appearances similar to those of the pasteurellae and, as claimed by some observers, e.g., de Smidt¹⁰¹ and Hennessey,⁵⁴ the presence of such organisms may prove misleading when sputum smears of patients suspected to be suffering from pneumonic plague are examined.

The limitations of smear examination are well illustrated by the following results obtained by Girard⁴⁵ through the combined use of this method and of animal experiments in the case of materials which had been collected from human plague victims with the aid of punctures :

Smears	Total number of combined examinations	Animal experiments		Positive inoculations (%)
		Positive	Negative	
Positive	781	690	91	88.3
Suspicious	962	409	553	42.5
Negative	2,642	124	2,518	4.7

In spite of these shortcomings a judicious use of smear examinations is indispensable in order to watch the trend of fully confirmed epizootics. Smear examinations are also of some importance for the recognition of human plague, particularly in clinically obscure cases of bubonic plague.

Cultures

The methods of cultivation which appear to be of prime importance for the identification of *P. pestis*, and for its differentiation from other bacterial species, may be grouped according to whether they are intended to take account of :

- (1) morphological and growth characters ;
- (2) growth requirements ;
- (3) selective-growth requirements.

Characteristics of morphology and growth. (a) *Involution forms* : It is of importance to note that, as first shown by Hankin & Leuman,⁵² cultivation of the plague bacillus on agar containing 3% sodium chloride leads to the production of marked involution forms. True enough, claims that this phenomenon is specific for the plague bacillus to the exclusion of *P. pseudotuberculosis* have not been substantiated. However, the morphological features noticeable when the latter is grown on 3% salt-agar are apt to be different from those manifest in the case of the plague bacillus.

Thus Topping et al.,¹⁰⁷ growing a pseudotuberculosis strain of human origin on 3% salt-agar, noted the presence of longer rods and of coccoid and some swollen forms but stated that "it was not the characteristic pleomorphism of plague". Likewise Haas,⁵¹ cultivating a pseudotuberculosis strain of rat origin on 3% salt-agar, obtained involution forms which were "usually long, slender, slightly curved rods, or medium-sized rods lying in bundles in a manner suggestive of the diphtheria bacillus; less frequently there were enlarged hollow staining organisms resembling 'balloon' forms of involuted *P. pestis*".

It moreover appears that, as first noted by Zlatogoroff (quoted by Dieudonné & Otto²⁸) and confirmed by other observers,¹³ involution of plague bacilli on 3% salt-agar becomes manifest in 24 hours—that is, more rapidly than in the case of other bacterial species.

(b) "*Stalactite*" growth : The peculiar crumbly and "stalactite" growth of *P. pestis* in broth can no longer be considered of differential-diagnostic importance, because such features, while not invariably present if this micro-organism is cultivated, may be produced by the rough form of the pseudotuberculosis bacillus. Broth cultivation of an otherwise identified plague strain is, however, of value in so far as absence of turbidity renders it likely to be a pure culture of the organism.

Requirements for growth. (a) *Plain agar* : In order to distinguish between plague and pseudotuberculosis bacilli, advantage may be taken of

the fact that the former generally grows sparsely on plain agar while the latter develops abundantly after 24 hours' incubation (Bezsonova et al.⁸). To carry out such tests, care must be taken to use inocula sufficient in size to permit growth of the plague bacillus. On the other hand, it must be kept in mind that if material for cultivation is taken directly from the animal or human body, admixture of blood or tissue-fluids may lead to an unusually abundant growth of *P. pestis* on plain agar.

(b) *Small inocula* : Among the numerous methods which have been utilized to induce growth of *P. pestis* from small inocula, the following simple procedure recommended in the *Report of the Haffkine Institute for the year 1931*¹⁰⁵ is fully satisfactory for diagnostic and differential-diagnostic purposes :

With the aid of a 1-mm loop, dilutions of the micro-organisms to be tested are made in tubes each containing 10 ml of normal saline. Blood-agar slants are then inoculated with 1-mm loopfuls of the dilutions, and plain agar slants with 5-mm loopfuls. In the case of the plague bacillus, discrete colonies appear on the blood-agar slopes and none at all on plain agar, while pseudotuberculosis bacilli develop equally well on both media.

(c) "*Exhausted*" media : Basing his recommendation on earlier work by Fabiani,⁸⁵ Petragani⁸⁶ advocated, for the differentiation of plague bacilli from other bacterial species, the use of old culture media on which plague bacilli had been grown, and which had been sterilized after the requisite amount of water had been added. Plague bacilli failed to develop on such "exhausted" media, while other micro-organisms developed freely.

(d) "*Hungry*" media : Bezsonova⁶ (see also Bezsonova et al.⁷) found "hungry" agar, prepared without peptone, suitable for differential-diagnostic work; pseudotuberculosis bacilli developed fairly well on such media while plague bacilli grew poorly, if at all.

(e) *Bile media* : It should be added that bile media, although not suitable for differentiating the plague from the pseudotuberculosis bacillus, have been used with advantage for the enrichment of primary blood cultures. Kirschner⁶⁰ found pure bile, previously recommended by La Rosa,⁶² sterilized either by filtration or by heating for 20 minutes at 110°C, satisfactory for preliminary cultivation of blood or pus. By this method good growth could be obtained with small inocula (10-40 plague bacilli per ml); in fact, even the addition of one drop of blood to 5 ml of bile was sufficient to show up a slight bacteraemia. Ohoto^{83, 84} first advocated the use of Conradi and Kayser's bile medium for taking blood cultures in plague work, but afterwards used a medium consisting of 500 ml of fresh ox bile to which 5 g of peptone had been added. This was steam-sterilized for two hours, divided into 50 test-tubes, and again heated for one hour.

For routine work the simple procedure recommended by Sokhey & Wagle¹⁰³ is, however, fully satisfactory. This consisted of using 0.5 ml of

blood, obtained from a vein of the patient, to inoculate two agar slants (0.25 ml per slant) which were then incubated for two days at room temperature.

Requirements for selective growth. (a) *Low temperatures:* In order to isolate plague bacilli from contaminated materials, advantage has been taken of the ability of this micro-organism to grow at low temperatures. Petragani⁸⁶ recommended for this purpose incubation at 10°C but, as has been noted when dealing with the growth of *P. pestis* on gelatin, even freezing of media inoculated with contaminated material has been employed.

(b) *Media counteracting contaminants:* Besides the above-mentioned method, cultivation on media able to counteract the growth of contaminants was recommended for work with such materials. Drennan & Teague²⁹ advocated an agar medium prepared from beef-heart to which 0.025% sodium sulfite and 0.00143% of crystal-violet had been added. Similarly Meyer and Batchelder⁷⁶ recommended addition of 0.025% sodium sulfite and 0.0025% gentian violet to hormone beef-heart agar. Kister⁶¹ found Endo's medium useful for work with putrefied rats, since it restricted the growth of *Proteus vulgaris*. However, it was necessary to establish empirically the amounts of sodium sulfite and of fuchsin most favourable for this purpose. The use of the two media mentioned above is therefore more expedient.

Biochemical reactions

Carbohydrate substances. (a) *Techniques:* As discussed by Pollitzer,⁸⁹ solid media have been used to study the action of *P. pestis* on carbohydrate substances, and Francis³⁷ obtained good results with the semi-solid medium of Enlows³¹; liquid media have, however, been utilized for such tests by most recent workers, and seem to be preferable. The technique adopted by Chen²⁰ on the advice of K. F. Meyer and Pollitzer was as follows: 1% peptone water (prepared with a brand of peptone not containing carbohydrate-like fractions which might give false positive results) served as base, to which the fermentable substances were added in 1% proportions. Andrade's indicator was incorporated and the final pH of the medium was adjusted to 7.4. The cultures to be tested were first streaked out on blood-agar plates to check for contamination. If found pure, loopfuls of the growths were transferred into the appropriate media. The tubes were then incubated at 37°C. As recommended by de Smidt,¹⁰⁰ the growths were aerated by shaking the tubes daily. Readings were taken daily for a period of 21 days. In order to make sure that this procedure kept the bacilli alive, subcultures on blood-agar plates were made once weekly from each tube under test.

Another possible source of error to be guarded against, in conditions prevailing in the tropics, is the spontaneous hydrolysis of saccharose;

constant control with Fehling's solution is therefore necessary (Devignat & Boivin²⁷).

In their recent studies, Devignat & Boivin²⁷ took advantage of the "micro-glucide dish" devised by the former author.²⁶ To utilize this expedient and economical method, Devignat & Boivin first grew the plague strains to be tested for 48 hours at 30°C in 12 ml of peptone water containing Andrade's indicator. 0.5-ml quantities of these growths were then placed in the tubes of the dish, each of which contained 0.5 ml of one of the carbohydrates to be used, in a 4% solution made with twice-distilled water. An observation period of 8 days was found sufficient when applying this method.

In addition to the tests described above, a simple and expedient method devised by Uriarte & Morales Villazon¹⁰⁹ deserves mention. These workers used for their test 2% peptone water, slightly alkaline to litmus, with 8 g of glucose or laevulose and 4-6 ml of a 1% aqueous neutral red solution per litre. After sterilization at 110°C, this was poured into Durham tubes. Uriarte & Morales Villazon claimed that growth of plague bacilli in this medium produced a characteristic triad of signs: (a) slight acidity with a corresponding colour change to yellow; (b) absence of gas production; (c) formation of a flocculent precipitate which settled down so that the fluid remained clear. They claimed that none of the micro-organisms likely to be confused with the plague bacillus showed this combination of reactions.

Devignat & Boivin,²⁷ while confirming these results in general, found in three out of 36 instances slight turbidity instead of formation of a flocculent deposit.

Another simple method, recommended by Webster,¹¹³ involved the use of 1% peptone water containing glucose, mannite, lactose, and saccharose respectively besides an indicator. After incubation at 37°C for 48 hours, there should be production of acid but not of gas in the Durham tubes containing glucose and mannite, and no change in those containing lactose and saccharose.

(b) *Evaluation*: As has been discussed previously, the claim made by earlier observers that tests with glycerol-containing media were of value for a differentiation between plague and pseudotuberculosis bacilli can no longer be considered as valid. The importance of tests with rhamnose-containing media is still stressed but it must be noted that: (a) according to Russian observers glycerol-positive plague strains isolated in south-east Russia and the interior of Asia were apt to dissociate into rhamnose-acidifying and rhamnose-negative variants; (b) late rhamnose acidification produced by plague strains has been observed; and (c) Devignat & Boivin,²⁷ working with 40 strains isolated in the Belgian Congo, observed slight rhamnose acidification in one instance. It would be unwise, therefore, to deny the plague character of an otherwise suspect strain merely because it shows activity towards rhamnose media. At the same time, however,

exceptions of this kind do not invalidate the rule that, in contrast to pseudotuberculosis bacilli, plague bacilli (as well as the pasteurellae in the strict sense) produce no acidity in rhamnose media.

Hydrogen sulfide and indole. Tests for the presence of hydrogen sulfide and indole are of limited differential-diagnostic importance, because both plague and pseudotuberculosis bacilli fail to produce these substances in the course of cultivation. A simple test devised by Webster¹¹³ is as follows: A tube containing 1% peptone water should be inoculated with the bacilli to be tested and a few drops of 5% lead acetate solution put on the inner end of the plug, which should be made of white cotton-wool. If hydrogen sulfide is absent, the plug should show no blackening after incubation at 37°C for 24 hours. To the inner end of the plug of the same culture (covering the plug if necessary with a new layer of white cotton-wool) a drop of 1% potassium persulfate and then a drop of Ehrlich's rosindole reagent^c should be added. If indole is absent, there should be no pink colour on the plug after incubation for 24 hours at 37°C.

Milk. Tests with litmus milk (see also chapter 2) cannot be considered as fully reliable for a differentiation of the plague from the pseudotuberculosis bacillus, because some strains of the latter species are poor alkali-producers. Results obtained through cultivation of the plague bacillus in litmus milk are also somewhat inconsistent, Devignat & Boivin,²⁷ for instance, finding that the medium remained unchanged in 33 out of 38 strains tested, while slight acidity was produced 4 times and slight alkalinity once.

Nitrates. As discussed in chapter 3, procedures demonstrating respectively a reduction of nitrates to nitrites, and production of nitrous acid, are not universally valuable for a differentiation of plague and pseudotuberculosis bacilli; even in areas where positive reactions were usually given by *P. pestis*, occasional negative results were noted. Webster's¹¹³ simple reaction test is as follows: A 5-mm loopful of the same culture recommended for his hydrogen sulfide test is placed on a white opal glass plate and mixed with a 2-mm loopful of Ilosvay's reagent.^d If nitrites are present, a pink colour should appear within a minute.

Attention has been drawn to the potential value of Fauconnier's³⁶ proposal to differentiate between plague and pseudotuberculosis bacilli with the aid of tests showing the urease activity of these organisms. However, the favourable results reported by this author require verification.

^c Ehrlich's rosindole reagent is prepared by taking 1 g of *p*-dimethylamino-benzaldehyde, 95 ml of absolute ethanol, and 20 ml of concentrated hydrochloric acid, and mixing the whole with an equal quantity of rectified spirit.

^d Ilosvay's reagent is prepared by mixing equal parts of (a) 1 g of sulfanilic acid, 14.7 ml of glacial acetic acid, 285 ml of distilled water, and (b) 0.2 g of naphthylamine, 14.7 ml of glacial acetic acid, and 325 ml of distilled water.

Serodiagnostic tests

As has been stated previously when dealing with the problem of serodiagnosis, advantage should be taken of agglutination to test unknown strains with plague immune sera of established potency, and to examine, on the other hand, the sera of suspect patients with the aid of known cultures.

While satisfactory procedures, including slide-tests convenient for the rapid diagnosis of human plague, have been worked out, the problem of to what extent agglutination may be relied upon for the identification of unknown strains, and consequently for a differentiation of plague and pseudotuberculosis bacilli, is not yet solved. The methods proposed by Bhatnagar⁹ and by Seal⁹⁵ appear to be promising but their differential-diagnostic value should be confirmed by large-scale investigation.

In addition to the methods of agglutination, haemagglutination, and complement fixation dealt with in chapter 3, the following serological tests seem to possess practical value for the diagnosis, particularly the retrospective diagnosis, of human plague: (a) the flocculation test, introduced by Girard⁴³ (see chapter 3), using the serum of patients or convalescents and toxic filtrates or extracts of *P. pestis*; and (b) the allergic reaction recommended by da Silva, jr & de Albuquerque⁹⁹ (see also da Silva, jr.⁹⁷). These authors used an antigen prepared by removing the bubo of a subcutaneously plague-infected guinea-pig immediately after death, boiling this material for two hours in normal saline, grinding it in a sterile mortar at the proportion of 1 g per 20 ml of saline, filtering through sterile gauze, and finally adding 0.5% phenol. If found sterile, this material was used in quantities of 0.1 ml for intradermal injection. A control injection with an equal amount of milk was made 4 cm from the site of the antigen injection. Readings were taken after 24, 36, and 48 hours. In positive cases a papule surrounded by an inflammatory zone developed, the reaction becoming maximal after 36 hours.

Some workers, such as Cambosu,¹⁷ Menezes,⁷⁴ and Tumansky,¹⁰⁸ recommended the use of serological methods for the rapid diagnosis of rodent plague. A further precipitin test was recently recommended by Larson et al.⁶³ These workers established, by preliminary investigations, that boiling of the materials to be tested, as formerly employed in carrying out thermoprecipitin tests (Piras and others, quoted by Pollitzer⁸⁹), led to a decrease in the antigen content. Diethyl ether was therefore used to sterilize the culture or tissue suspensions to be tested with plague immune sera. Precipitin tests performed with antigens obtained in this way from tissues of animals which had died of plague showed the presence of soluble antigens in sufficient quantities to be of diagnostic value in instances where the material had been stored at 37°C for periods of at least 14 weeks.

In places where adequate laboratory facilities are available, complement-fixation tests, carried out according to the method of Chen et al.²¹ (see chapter 3, p. 169), may prove valuable for the diagnosis of rodent plague.

Bacteriophage tests

As discussed when dealing with the bacteriophage problem, Gunnison et al.⁵⁰ recently found that tests carried out with plague phage at 20°C offered a means of differentiating between plague and pseudotuberculosis bacilli.

To facilitate the application of such tests, Cavanaugh & Quan¹⁸ soaked sterilized strips of filter paper in broth cultures of *P. pestis* which had been seeded with a potent plague phage and then subjected the strips to lyophilization or desiccation in vacuo. If such strips were applied to freshly made plague subcultures on blood-agar plates and these were incubated at 20°C, a 1-mm wide zone of lysis usually became visible in 18-24 hours round the bacteriophage-coated strips while no such clearance was produced round noncoated strips of filter paper used as controls. Tests made under identical conditions with growths of *P. pseudotuberculosis* gave negative results. As far as established up to now, the bacteriophage-coated strips preserved by lyophilization remained capable of producing lysis for periods of three months, if sealed under nitrogen and stored at room temperature. Desiccated strips did not display such keeping qualities.

Should the value of this procedure be confirmed by extensive trials, it would form an easy means of differentiating plague and pseudotuberculosis bacilli, the more so as it might be possible to supply field workers or persons operating in areas without facilities for bacteriophage work with test-strips prepared in central institutions.

Animal experiments

In evaluating the methods available for the laboratory diagnosis of rodent plague, stress has been laid on the importance of animal experiments performed with pooled organs or pooled fleas. At the same time, however, it was pointed out that, invaluable though these methods are for giving a true overall picture of the situation, they fail to yield information on the extent and degree to which plague is present. Other methods for filling this gap must therefore be used side by side with pooling-experiments. Merely to carry out animal experiments with material from individual infected rodents would not be sufficient to establish to what degree they had been infected, and would, moreover, be quite out of question in the course of considerable epizootics. However, such experiments are called for when dealing with initial or sporadic manifestations of rodent plague.

The position in regard to human plague is similar. Here also it is essential to verify the diagnosis in initial or sporadic cases by all available methods, including animal experiments; to continue these with each patient during a considerable epidemic would, however, be impossible.

The choice of experimental animals to be used for plague diagnosis depends upon the nature of the work to be performed. Generally speaking,

guinea-pigs should be chosen for pooling-tests and these animals are also most suitable for confirming the diagnosis in initial or sporadic plague manifestations. As recommended by Lloyd,⁶⁸ three guinea-pigs, infected respectively through the unbroken skin, subcutaneously, and intraperitoneally, should be used when dealing with initial or sporadic manifestations of the infection. While the most rapid results may be expected from the intraperitoneally infected animal, the likelihood of obtaining a pure culture is greatest in the case of the percutaneously infected one. In tropical climates particularly, it is advisable to kill moribund animals (i.e., those which lie down on their sides) with the aid of chloroform, because this procedure, besides saving time, increases the chances of isolating pure growths.

Intraperitoneal infection of white mice is a method of unsurpassed value for establishing the existence of a bacteraemia and, at the same time, confirming the diagnosis in instances of human plague. Moreover, it will often be possible to use these animals, which can be easily bred and kept at small expense, on a larger scale than guinea-pigs. A drawback is that white mice are far more susceptible than guinea-pigs to pneumococcus infections (Girard ⁴⁴).

White rats are of outstanding value for differentiation between plague and pseudotuberculosis bacilli. It is true that a few strains of the latter species have been found pathogenic for white rats, but such aberrant results may also be obtained when using most of the other methods available. When performing differentiation tests, the cumulative evidence obtained in various ways, rather than the results of any individual method, should be taken into account (Schütze ⁹⁴).

Precautions Advisable

The dangers confronting plague laboratory workers are threefold :

- (1) the possibility of contracting bubonic infection through the bite of blood-sucking insects, especially rodent fleas;
- (2) the possibility of contracting direct bubonic infection when performing postmortems or otherwise handling contaminated material;
- (3) the possibility of contracting pneumonic infection through splashing or spraying material laden with plague bacilli.

Bubonic infection through fleas

When it is necessary to enter plague-infected houses, or when coming into contact with persons or dead bodies, or with rodents possibly harbouring infected fleas, it is safest to wear a special costume, consisting principally of a gown similar to that used by clowns which, made of one piece, covers

the whole body except the head and hands. This is donned through the opening in the neck and is then tied firmly round neck and wrists. The costume is completed by rubber or high leather boots, a linen cap, and, as far as necessary, by rubber, canvas, or leather gloves. A mask must be added if there is danger of pneumonic infection.

Since it is rather trying to wear the above-mentioned gown under tropical conditions, long linen stockings covering the feet and legs and tied up above the knees may be substituted, or high boots (made from rubber or leather), into which the trousers are tucked, alone, may be used.

It has been recently recommended that protection against fleas should be ensured by impregnation of the underwear with DDT, or impregnation of the clothes with other modern insecticides (Elishewitz;³⁰ Linduska et al.;⁶⁶ Smith & Burnett¹⁰²). A combination of such applications with the use of long linen stockings and/or high boots would seem particularly effective.

Safe methods for handling rodents delivered to the laboratory for examination and for freeing them from fleas have been described earlier (see pages 225, 226). Experimental animals kept in the laboratory should be housed in rat-proof rooms, or at least in rat-proof cages. To protect infected animals against stray fleas, the containers accommodating them may be covered with gauze, surrounded by tanglefoot paper, or suspended.

Direct bubonic or pneumonic infection

If one works with the cleanliness and carefulness indispensable for laboratory work in general, no special precautions need be observed when examining plague material by bacteriological methods. To minimize the danger of accidents, glassware of the best quality should be used exclusively for plague laboratory work. Slides should be immersed immediately after examination in a jar containing an antiseptic fluid (preferably industrial spirit) and should be boiled before cleaning.

When performing human autopsies, a proper costume should be worn, including a rubber apron and high rubber-boots. Solid rubber-gloves are indispensable. In his autopsy work, done before sulfonamides and antibiotics had become available for treatment, the present writer used two pairs of rubber gloves, the cuffs of the gown being tied down over an inner pair of the usual medium-weight pattern, and hands and forearms then being covered by a long pair of solid postmortem-gloves.

To avoid danger from occasional splashing, masks and preferably also goggles should be worn.

Less stringent precautions are necessary when dissecting plague-infected animals, the more so because plague workers should learn to perform autopsies with the aid of suitably long instruments, without touching the carcasses. However, even if this is done, rubber gloves should be used as a precaution against accidental touching of the animals or splashing of infectious material.

The carcasses of the dissected animals should be burned immediately after autopsy, together with the contents of the containers where the animals have been confined. Cremation is also preferable in the case of dissected human plague victims. If this is impossible, the dead bodies should be wrapped in shrouds soaked in strong antiseptic solutions before being put into leak-proof coffins or, preferably, quicklime should be used to cover them.

Experimental infection of test animals should be carried out with the precautions recommended above for dissection. When using injection methods, care must be taken to avoid the danger of spraying about infectious material; it is best to wear masks whenever infecting test animals with the aid of syringes.

All possible care must be taken when manufacturing vaccines or sera with virulent plague strains, and when carrying out experimental research work, because, as shown by several deplorable incidents, it is in the course of such activities that pneumonic infections are most likely to occur.

A strict supervision of the assistant and lower staff employed in plague laboratories is essential to make sure that they are not remiss in adopting all necessary precautions.

If in the course of plague laboratory work any mishap occurs, adequate methods of disinfection of the clothes and persons of the workers concerned, as well as of the whole room, should be started at once. Any wounds on fingers or hands, whether pre-existent or contracted during the accident, should be attended to first, preferably by thorough soaking in alcohol.

If the risk of infection of workers involved in laboratory accidents seems appreciable, prophylactic administration of sulfonamides should be resorted to.

It is, on the other hand, necessary to make sure that laboratory workers, in order to protect themselves against imaginary dangers, do not use sulfonamides indiscriminately. Pollitzer saw an instance of serious kidney affection (haematuria and oliguria) in a technician detached for field work who, with the idea of protecting himself against plague, took 1-2 g of sulfadiazine daily for several weeks. Fortunately, no permanent harm resulted.

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