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RECOMMENDED LABORATORY METHODS FOR THE DIAGNOSIS OF PLAGUE *

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GENERAL METHODS

Smear Examination

Fixation

The air-dried smears or impression films made on slides from plague-suspect materials may be fixed either by heat or with the aid of alcohol.

For the former purpose the slides, films upwards, are passed three times through the middle of the upper half of a gas flame (in emergencies through the flame of a spirit burner), taking care to avoid charring of the films.

The fluids routinely used for the fixation of plague films are (*a*) 95% ethyl alcohol (ethanol); (*b*) chemically pure methyl alcohol (methanol); (*c*) commercial methylated spirit, as used as fuel for burners, which is as effective as, and much cheaper than, (*a*) and (*b*).

In order to use alcohol for fixation, the films are either covered with sufficient amounts of the fluid, dispensed from a drop-bottle or with the aid of a pipette, or the slides are immersed in a staining jar filled with the fixation fluid—a procedure preferable for the simultaneous handling of numerous slides. The alcohol must be allowed to act for 2-3 minutes; it is then poured off (or allowed to drip off) and the slides must be allowed to dry before staining (blotting or washing with water is unnecessary).

Though it is permissible to use heat fixation in emergencies, particularly for smears made from plague-suspect organs, alcohol fixation is preferable in general because apt to promote good bipolar staining of the pasteurellae. Moreover, heat fixation does not invariably kill the organisms.

Recommended method: Fixation for 2-3 minutes in methylated spirit.

Simple staining

Though the simple stains, ordinarily available in bacteriological laboratories—particularly Loeffler's methylene blue, toluidin blue or phenol

methylene blue—may be used, phenol thionin or Wayson's stain are particularly suitable for the staining of plague smears.

Phenol thionin is prepared by adding 10 ml of a saturated solution of thionin in 50% ethanol to 100 ml of a 2% solution of carbolic acid in distilled water. Apply for 1 minute and wash off.

Wayson's stain is prepared by (i) dissolving 0.2 g fuchsin and 0.75 g methylene blue respectively in 10 ml amounts of absolute ethanol ; (ii) adding the combined solutions to 200 ml of 5% carbolic acid in distilled water. Though it is not necessary to fear overstaining, application of this stain for a few seconds, followed by thorough washing with water, gives satisfactory preparations.

In place of these simple stains, which are recommended for field work in particular, advantage may be taken of stains of the Leishman or Romanowski type, prepared and applied in accordance with the locally available ingredients.

Gram staining

Since the proper application of Gram's method of staining depends largely upon familiarity with the reagents and procedures used, insistence upon altered techniques might cause harm rather than proving beneficial. Still, attention has to be drawn to the following modifications of Gram's original method, which are now used in some of the leading laboratories.

(*a*) *Jensen's modification :* (i) Stain for 15-30 seconds with a 0.5% aqueous solution of methyl violet; (ii) rinse off stain with Lugol's iodine solution (prepared by mixing in a mortar 1 g of iodine and 2 g of potassium iodide with 5 ml distilled water, washing this into a bottle, and making up to 100 ml with distilled water) ; (iii) flood with fresh iodine for 30-60 seconds ; (iv) drain off iodine solution and wash with absolute ethanol until stain ceases to come out of film; (v) wash with water and counterstain for 15-30 seconds with neutral red solution (made up from 1 g neutral red, 2 ml glacial acetic acid, and 1 litre of water) or for 1 minute with a 0.5% aqueous solution of safranin.

(*b*) *Burke's modification :* (i) Cover fixed smear with 1% aqueous solution of gentian violet or crystal violet; (ii) immediately admix 3-8 drops of a 5% aqueous solution of sodium bicarbonate and stain with mixture for 1 minute; (iii) wash quickly with water and cover slide with iodine solution (as for Jensen's modification) for 1 minute; (iv) wash quickly with water and decolourize by dropping a mixture of acetone and ether (1 part of ether to 3 parts of acetone) on tilted slide until decolourizer flows off practically uncoloured (which usually requires less than 10 seconds); (v) drain decolourizer from slide and wash thoroughly with water; (vi) counterstain with a 0.5%-2% aqueous safranin solution for 10 seconds; (vii) wash with water, blot, and dry.

(*c*) *Modified Gram stain :* [a] This useful modification of Gram's original method is carried out as follows : (i) Air-dry a thinly spread film and fix by heat; (ii) stain with ammonium oxalate gentian violet solution (saturated alcoholic solution of gentian violet, 10 ml; 1% aqueous solution of ammonium oxalate, 40 ml) for 1 minute; (iii) wash in water; (iv) apply Lugol's solution for 1 minute; (v) wash in water; (vi) decolourize in 95% or absolute ethanol for 30 seconds with gentle agitation; (vii) counterstain with dilute safranin (1: 10 dilution of saturated solution).

[a] Hucker, G. J. & Conn, H. J. (1923) *Techn. Bull. N. Y. St. agric. Exp. Sta.* p. 93

(*d*) *Modification of method recommended by de Smidt :* [b] (i) Stain fixed smear for ½ minute with a 0.5% solution of crystal violet in distilled water; (ii) wash off in distilled water; (iii) apply iodine solution (as for Jensen's modification) for 1 minute; (iv) wash off with water and decolourize for 10 seconds with absolute acetone; (v) wash off and counterstain for 25-35 seconds with diluted carbol fuchsin (Ziehl's carbol fuchsin 1 part, distilled water 9 parts) or with 1% neutral red solution; (vi) wash off and dry in air.

Recommended method : Modifications (*b*) or (*d*) are preferable for workers not familiar with another modification of Gram's staining method.

Special stains

In the opinion of most experts no special stains are necessary in plague laboratory work. It deserves note, however, that:

(1) A stain similar to that of Wayson may be prepared *extempore* by admixing 2-3 drops of a saturated (i.e., 13%-15%) solution of methylene blue and 4-6 drops of Ziehl's carbol fuchsin to 15 ml of tap water. Smears fixed for 2 minutes in methanol are exposed to this stain for 15-25 seconds, then washed and air-dried.

(2) Concentrated carbol-fuchsin (Ziehl) may be used for instantaneous staining by covering the films with this stain and immediately pouring it off. The slides are then washed with water and dried.

Note. Ziehl's carbol fuchsin is prepared by admixing 10 ml of a saturated solution of basic fuchsin in 95% ethanol to 90 ml of a 5% aqueous solution of carbolic acid (phenol).

Cultivation

General indications

Reaction of the media

Though slightly acid as well as moderately alkaline media may be used for the cultivation of *Past. pestis* (pH range from 6.8 to 7.6), a pH of 7.2, as used by workers in India for instance, might be adopted as standard for plague diagnostic work with solid media, and one of pH 7.4 for the peptone-water media used for biochemical tests.

Incubation temperature

While some workers incubate their plague growths at the generally used temperature of 37° C, most recommend incubation temperatures ranging from 25° C to 30° C, with a preferable optimum of 27° C- 28° C for plague-diagnostic work in general.

[b] Smidt, F. P. G. de (1912) *E. Afr. med. J.* **19**, 15

Period of observation

Cultures seeded with plague-suspect material should be inspected after they have been incubated for 24 hours, but in view of the slow growth of *Past. pestis* they should be re-incubated for a further period of at least 24 hours, preferably for an additional period of 2 or 3 days. Observation for 6-7 days is advisable if, under field conditions, the cultures are exposed to temperatures below 25° C.

Solid media

The solid media used for the cultivation of plague bacilli in diagnostic work fall into three groups, namely, (*a*) plain agar media, (*b*) blood agar media, and (*c*) special media.

(*a*) *Plain agar media*

In view of the fact that owing to local conditions different ingredients, particularly different brands of peptone and different kinds of meat, have to be used for the preparation of nutrient media, it is impossible to give generally valid indications for the agar media to be used in plague-diagnostic work. Actually, however, such specifications are not urgently called for, because the standard agar media used in the various laboratories for general purposes are suitable for the cultivation of *Past. pestis*, if adjusted to a suitable pH.

Most plague experts use a broth prepared from the locally available kinds of meat or with meat extract as base for their agar media. However, the workers in the Institut Pasteur, Paris, maintaining that meat broth exerts to some extent an inhibitory action on the growth of *Past. pestis*, recommend for its cultivation a solid medium prepared by adding 2% agar to 2.5% peptone water and adjusting the pH to about 7.0. It is essential for the preparation of such media to use a suitable brand of peptone rich in amino-acids.[a]

(*b*) *Blood agar*

A suitable blood agar for plague-diagnostic work may be prepared by:

(1) melting 1 litre or smaller amounts of a suitable agar medium;
(2) cooling to a temperature of about 45° C;
(3) adding aseptically 5% sterile defibrinated sheep's blood;
(4) shaking carefully and pouring into sterile Petri dishes.

While, in view of its usual availability, the use of sheep's blood is generally recommended, other sorts of blood, including, in emergencies, human

[a] Girard, G. (1944 *Bull. Soc. Path. exot.* 37, 228

blood, can be substituted. Horse's blood in particular, also often available in laboratories, can be used for the preparation of blood agar in a concentration of 7.0% to 7.5%.

Workers in the Haffkine Institute, Bombay, found a superimposed blood agar economical and, in view of its transparency, also particularly suitable for plague-diagnostic work. This is simply prepared by:

(1) melting 100 ml of a suitable agar in a flask;
(2) cooling to a temperature of 45° C-50° C;
(3) adding 5 ml of sterile defibrinated rabbit's blood;
(4) superimposing a few ml of the mixture on agar slopes, which are then allowed to set in a slanting position.

(*c*) *Special media*

Hyper-salted agar. In the past great differential diagnostic importance was laid upon the use of agar containing, in place of the usual content of 0.5%, 3% of sodium chloride, because the appearance of marked involution forms on this medium was considered to be characteristic of *Past. pestis*. This claim has not been fully substantiated as far as *Past. pseudotuberculosis* is concerned, though it has to be admitted that the involution forms shown by the latter on salt agar develop more slowly and are morphologically distinct from those of *Past. pestis*. Nevertheless, the use of hyper-salted agar for plague-diagnostic work has been largely given up in favour of other tests more clearly distinguishing the plague bacillus from other pasteurellae, particularly the pseudotuberculosis bacillus.

Sodium-sulfite/gentian-violet agar. Certain agar media, e.g., those prepared with Huntoon's hormone broth or veal infusion broth, may be rendered eminently suitable for the isolation of *Past. pestis* by the addition of (*a*) 0.025% sodium sulfite (i.e., 0.25 ml of a freshly prepared 10% solution of sodium sulfite), and (*b*) 1/700%-1/400% of gentian violet (i.e., 1.5 to 2.5 ml of an accurately prepared 1 : 1000 solution of gentian violet) per 100 ml. The proper amount of gentian violet to be added must be experimentally determined for each agar batch, because sometimes a gentian-violet concentration of 1/400% may inhibit all bacterial growth. Apart from this drawback, however, the medium is eminently suitable because it inhibits the most troublesome contaminants met with in plague-diagnostic work, and also because the characteristic large colonies of *Past. pestis* developing in 36 to 48 hours may be easily picked out and used for slide agglutination tests.

Copper sulfate agar. Addition of 0.05% copper sulfate to agar is another means of checking the growth of contaminating organisms, particularly those of the proteus group of organisms, encountered in diagnostic work with the organs of plague-suspect rodents or human victims.

Fluid media

Broth

The peculiar crumbly and " stalactite " growth of *Past. pestis* in nutrient broth can no longer be considered of differential diagnostic importance, because such features, while not invariably present if this 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 that absence of turbidity renders it likely that the organisms are present in pure culture.

Peptone water

A convenient method of preparing peptone water for plague-diagnostic work is as follows:

(1) Add 10 g of a suitable brand of peptone and 5 g of sodium chloride to 1000 ml of distilled water.
(2) Steam the mixture for 20 minutes to dissolve the solids.
(3) Adjust the pH, and steam for a further 30 minutes.
(4) Filter through paper and dispense in 7-ml quantities into tubes.
(5) Autoclave at a pressure of 15 pounds per square inch (1.05 kg per cm²) for 20 minutes.

The pH for peptone water recommended by different workers varies from 7.4 to 7.6. It would be most desirable to adopt the first mentioned value (7.4), found satisfactory in recent studies on the biochemical properties of *Past. pestis*, as standard.

Biochemical Methods

Reactions produced in carbohydrate-containing media

Preparation of media

Most workers recommend peptone water prepared according to the formula given above as basic medium to which the various carbohydrates to be tested are added in a strength of 1%. Andrade's indicator added in a concentration of 1% is almost unanimously recommended. This is prepared by:

(1) dissolving 0.5 g of acid fuchsin in 100 ml of distilled water;
(2) adding normal sodium hydroxide solution (N/1 NaOH) until the colour changes to pink, then to brownish-red, and finally to yellow, shaking the reagent after each addition of alkali (the total amount of which required is usually about 17 ml);
(3) letting the thoroughly mixed fluid stand for 1-2 hours and then filtering it and dispensing it into brown glass bottles.

Details for preparing carbohydrate-containing media for plague-diagnostic work are as follows:

(1) The various carbohydrates used, with the exception of glycerol, may be kept in stock solutions of a strength of 10% or preferably 20% prepared by:
(i) dissolving the requisite amounts of the carbohydrates in proportionate amounts of distilled water;

(ii) filtering the solutions through sterilized Seitz filters into sterile containers;
(iii) exposing the latter after plugging and sealing with paper for 30 minutes to a temperature of 100° C in a steam sterilizer. (This process of sterilization must be repeated on two subsequent days if, owing to the lack of Seitz filters, sterilization by filtration is impossible.)

Note. Glycerol may be sterilized in the autoclave at a low pressure (10 pounds per square inch, or 0.7 kg per cm²) for 15 minutes.

(2) Actually to prepare the media:
(i) add Andrade's indicator in a strength of 1% to the peptone water base (e.g., 2 ml to 200 ml of peptone water); [a]
(ii) mix well and sterilize in the autoclave at a pressure of 15 pounds per square inch (1.05 kg per cm²) for 20 minutes;
(iii) after cooling, add under aseptic precautions sufficient amounts of the different carbohydrate stock solutions to proportionate amounts of peptone water to obtain concentrations of 1%;
(iv) dispense into sterile tubes (provided with differently coloured cotton plugs for easy distinction of the various carbohydrate media) and steam these in a steam sterilizer for 30 minutes;
(v) incubate the tubes at 37° C for 24 hours to ensure their sterility.

The action of the various pasteurellae on the carbohydrate substances which have been considered essential or desirable for inclusion in the series of tests now under review is illustrated below:

Carbohydrate substance	*Past. pestis*	*Past. pseudo-tuberculosis*	*Pasteurellae sensu stricto*
Glucose	+	+	+
Glycerol	±	+	−
Glycogen	+ [a]	− [b]	−
Lactose	− [b]	−	−
Laevulose	+	+	+ [a]
Maltose	±	+	−
Mannitol	+ [a]	+	+ [a]
Melibiose	−	+	−
Rhamnose	− [a, b]	+	−
Saccharose	− [b]	±	+ [a]
Salicin	±	+ [a]	− [a]
Xylose	±	+	±

Explanation of signs: + = Acidification without gas formation
− = No acidification
± = Acidification present or absent (variable results)
[a] Exceptions noted
[b] Occasional occurrence of late acidification

As shown by this tabulation:

(a) It is not possible with any of the enumerated carbohydrates to make a fully reliable distinction between plague and pseudotuberculosis bacilli

[a] Care must be taken to use for the preparation of the basic medium a brand of peptone which does not contain carbohydrate fractions or other substances apt to give false positives when making tests with plague bacilli or other organisms.

on the one hand and the pasteurellae in the strict sense (*Past. multocida* and allied species) on the other hand. Tests with saccharose sometimes recommended for this purpose are unsatisfactory in that, in contrast to the invariably negative reactions given by *Past. pestis*, pseudotuberculosis bacilli cause variable reactions, while pasteurellae in the strict sense sometimes fail to acidify saccharose-containing media.

(*b*) A distinction between plague and pseudotuberculosis bacilli may be made with quite rare exceptions through tests with rhamnose-containing media and, as far as indicated by still limited experiences, even more exactly with the aid of melibiose. Glycogen, which has also been recommended for this purpose, gave in the experience of one worker inconstant results with *Past. pestis*, but confirmation of these discrepant findings is still lacking.

(*c*) The reactions produced by *Past. pestis* in glycerol-containing media are variable, but since the differences met with in this respect are of a regional instead of an individual character, tests with glycerol are important for epidemiological studies.

For practical purposes it is recommended that tests be made with media containing the following carbohydrates:

Carbohydrate	*Rationale or test*
Rhamnose and, preferably, melibiose	Differentiation of plague and pseudotuberculosis bacilli.
Saccharose	Of limited value for the differentiation of plague bacilli from the pasteurellae *sensu stricto*.
Glycerol	Epidemiological classification of strains.
Glucose, lactose, maltose, and mannitol	Tests with these media, which are usually kept in laboratories, are useful for a generic distinction of the pasteurellae (including plague and pseudotuberculosis bacilli) from contaminants belonging to the *E. coli* group which, in contrast to the pasteurellae, produce in these media gas as well as acidification.

It is best to use double fermentation sets (Durham tubes) not only in the case of the last mentioned sugars, but also in that of all the carbohydrate media enumerated above, so as to facilitate recognition of the presence or absence of gas formation.

Such fermentation sets are prepared by dropping small test-tubes (6 mm × 25 mm) with their open ends downwards into larger test-tubes (10 mm × 125 mm) filled with uniform amounts of the media in question. Differently coloured cotton plugs should be used to close the larger tubes so as to facilitate the recognition of the various media used. Immediately after sterilization (to be done preferably in a steam sterilizer), the fluids completely fill the smaller tubes. If gas forms after inoculation and incubation of the media, it accumulates first in the upper part of the small inverted tubes.

For the actual performance of the tests, sets of the above-mentioned carbohydrate media are inoculated with uniformly large amounts of each of the strains to be examined. In order to obtain adequately heavy inocula, platinum loops with a diameter of 2 mm should be used.

Opinions differ on the question at what temperature the inoculated carbohydrate media should be incubated. Several workers, who recently studied the biochemical reactions produced by *Past. pestis*, recommended an incubation at 37° C in view of the fact that incubators constantly kept at this temperature are available in all laboratories, whereas it would be difficult, if at all possible, to provide for the universal adoption of a lower standard incubation temperature. Nevertheless, some experts insisted that a lower incubation temperature be adopted, because the optimum temperature for the growth of *Past. pestis* was about 27°-28° C. Since, however, the first-mentioned observers obtained consistently good results in tests with carbohydrate media kept in the usual incubators, it seems legitimate to overrule this objection and to recommend the easily applicable incubation temperature of 37° C as standard for these tests.

Some dissension also exists regarding the length of time during which the carbohydrate cultures under test should be observed. It is undeniable that positive results (i.e., acidification, in the case of pasteurellae) become as a rule manifest or at least incipient within a few days. Nevertheless, in order not to overlook late acidifications, it has been recommended by most workers that the growths be observed for longer periods, ranging from one week to three weeks. An observation period of two weeks might be adopted as standard.

Carrying out the tests, it is essential (*a*) to keep the growths aerated by shaking the tubes after their daily inspection; and (*b*) in order to ascertain that this procedure has kept the organisms alive, to make subcultures on blood agar plates once weekly from each of the tubes under test. In this way it will also be ensured that positive results are due solely to the presence of the pasteurellae tested and not to that of contaminating organisms.

Reactions produced in urea-containing media

It deserves great attention that, according to recent experiences, differential diagnostic advantage may be taken of urea-containing media, because this substance is broken down, with the formation of alkali, by the pseudotuberculosis bacilli but not by *Past. pestis*. To carry out such differential tests, use may conveniently be made of a medium composed as follows:

Distilled water	380.0 ml
Monopotassium phosphate (KH_2PO_4), anhydrous	3.64 g
Disodium phosphate (NA_2HPO_4), anhydrous	3.80 g
Urea	8.00 g
Yeast extract	40.00 mg
0.02% aqueous solution of phenol red	20.00 ml

Dissolve the solids in the distilled water with as little heat as possible and sterilize by filtration through Berkefeld or Seitz filters. Dispense under aseptic precautions in 3 ml volumes into sterile tubes, which must be incubated overnight to ensure their sterility.

Micro-organisms which, like *Past. pseudotuberculosis*, are capable of splitting urea cause a change of the reaction of this medium from its original slight acidity (pH 6.8) to alkalinity. This change in reaction is manifested by a turning of the originally yellow colour of the medium into red. To observe this change in reaction clearly it is well also to inoculate control tubes which have been filled with urea-free portions of the above-mentioned medium. The growth of pseudotuberculosis bacilli produces no colour change in the control tubes, whereas that of *Past. pestis* leads to no colour change in the urea-containing tubes either.

Tests with desoxycholate citrate agar

As recently established, plague bacilli, if cultivated on desoxycholate citrate agar for 48 hours at 37° C, grow rather scantily in the form of reddish pin-point colonies, the medium retaining its original pinkness; whereas during the same period pseudotuberculosis bacilli grow abundantly in large opaque colonies, which, like the medium in general, show a yellow colour.

The medium used for this new differential test consists of:

Water	1000 ml
Peptone	10 g
Agar	12-17 g
Sodium chloride	5 g
Lactose	10 g
Ferric ammonium citrate	2 g
Dipotassium phosphate	2 g
Sodium desoxycholate	1 g
1% aqueous solution of neutral red	3 ml

Dissolve the peptone in the water and add sufficient sodium hydroxide to bring the pH to 7.3-7.5; boil for a few minutes and filter through paper. Let the agar soak in the peptone water for at least 15 minutes, then melt it by boiling or in the autoclave. Add about 6 ml of sodium hydroxide and then the above-mentioned ingredients except the neutral red solution as rapidly as possible. When the ingredients are dissolved, titrate to a pH of 7.3 or 7.5 and add the neutral red solution. Fill 10-15 ml quantities into tubes and sterilize for 15 minutes in the steam sterilizer.

Test for hydrogen sulfide and indole production, methylene-blue reduction test, and methyl-red test

The differential diagnostic value of these tests is illustrated by the following tabulation:

Test	*Past. pestis*	*Past. pseudo-tuberculosis*	*Pasteurellae sensu stricto*
Hydrogen sulfide	–	+ [a]	+
Indole	–	–	+
Methylene-blue reduction	–	+	+
Methyl red	+	+	–

[a] As recently confirmed through a study of 186 strains.

It will be noted that with the exception of the methylene-blue reduction tests, in regard to which no ample experiences seem to have been made, these methods of examination are of importance for a distinction of the pasteurellae in the strict sense from both *Past. pestis* and *Past. pseudo-tuberculosis*.

Suitable techniques for carrying out these tests are as follows:

Hydrogen sulfide production. Though solid lead-acetate-containing media may be used for demonstrating the production of hydrogen sulfide in the course of incubation, a simple test may be carried out by utilizing sterilized 5 mm × 50 mm filter-paper strips soaked in a saturated solution of lead acetate as follows:

(1) Dry the soaked paper strips in a Petri dish placed in the hot air oven for 1 hour.

(2) Inoculate a peptone water tube with the pasteurellae in question.

(3) Immediately after inoculation insert one of the strips into the tube so that one half projects below the plug.

(4) Watch for blackening of the strip during incubation.

Another simple test used in the Haffkine Institute, Bombay, is carried out as follows:

(1) Standard 1% peptone water containing 0.5% sodium chloride is filled in about 4-ml quantities into small test-tubes plugged with white absorbent cotton wool.

(2) After inoculation with the organisms under test the under-surface of the cotton wool plug is moistened with 1 or 2 drops of a 5% lead acetate solution.

(3) The tubes are then kept overnight in the incubator at 37° C. If hydrogen sulfide is produced, the under-surface of the plug will be found browned or blackened, while in negative cases no such decolouration will become manifest.

Note. As noted below, further biochemical tests may be made with the same cultures.

Indole production. A simple test may be performed by:

(1) Folding up the rough outer projections of the plugs of the cultures used to demonstrate hydrogen sulfide production (see above) and putting on these smoothened surfaces 1 or 2 drops of a 1% potassium persulfate solution in distilled water and then 1 or 2 drops of Ehrlich's rosindole reagent; and

(2) inverting the plugs so that their moistened surface faces the cultures and incubating the latter for a further 24 hours.

If indole is present, a pink colour will become visible at the lower end of the cotton plugs.

Note. Ehrlich's rosindole reagent is prepared for these tests by dissolving 1 g of para-dimethylamino-benzaldehyde in 95 ml of absolute ethanol and 20 ml of concentrated hydrochloric acid and mixing the whole with equal amounts of what is called "rectified spirit" in the original description of this test. The use of potassium persulfate solution though advisable, is not indispensable.

An alternative simple method, depending on the volatility of indole at 37° C, consists of hanging strips of filter paper, which after sterilization have been soaked in a saturated watery solution of oxalic acid and have been subsequently dried, over the inoculated media by securing the ends of the strips between the cotton plugs and the mouth of the tubes. If indole is produced, a pink colour will become visible on the exposed portions of the paper strips in the course of incubation at 37° C.

For an elaborate test, which has been recommended recently for plague-diagnostic work, the following reagent is used:

Para-dimethylamino-benzaldehyde	5 g
Amyl or butyl alcohol	75 ml
Concentrated hydrochloric acid	25 ml

It was originally recommended that 25-30 drops of this reagent be added to the cultures of the organisms under test grown in peptone water or broth, and the tubes then swung to and fro a few times but not shaken vigorously. In positive cases a supernatant layer, showing a red-violet colour, was formed within a few minutes. A modern modification is (i) to add 1 ml of ether to the cultures and to shake the tubes; (ii) to allow the ether to rise to the top and to form a layer; (iii) to add 0.5 ml of the reagent in such a manner that it forms a layer between the medium and the ether. In positive cases a red colouration becomes manifest at the junction of the reagent and the ether layer and spreads into the latter.

Methylene-blue reduction tests are carried out with 24-hours-old broth cultures, which have been incubated at 37° C, by (i) adding 1 drop of a 1% aqueous methylene-blue solution and (ii) incubating at 37° C for 24 hours. A strongly positive reaction is manifested by complete decolourization, while a green colouration indicates a weakly positive and absence of decolourization a negative reaction.

Methyl-red tests are performed with cultures grown for 5 days at 30° C, or for 3 days at 37° C, in a glucose-phosphate medium (peptone, 0.5 g; K_2HPO_4, 0.5 g; glucose, 0.5 g; water, 100 ml; pH 7.5). Tests are made by adding 5 drops of an 0.02% solution of methyl red in 95% ethanol, the appearance of a red colour indicating a positive acid reaction.

Tests for reduction of nitrates and production of nitrous acid

Tests to demonstrate the reduction of nitrates to nitrites and the production of nitrous acid in the course of cultivation are of no general differential diagnostic value in the case of the pasteurellae, which have been found to react either positively or negatively. They are, however, of interest in that the variability shown in such tests by *Past. pestis* strains has been found to be of a regional and not of an individual character. It has been stated in this connexion

that by combining these tests with tests utilizing glycerol-containing media, the plague strains may be classed into three groups, namely:

(1) an "oriental variety", not acidifying glycerol, but capable of reducing nitrates and producing nitrous acid in nitrate-free media;

(2) a *variatio antiqua*, proving positive in all these tests; and

(3) a *variatio mediaevalis*, acidifying glycerol, but giving negative results in regard to nitrate reduction and nitrous acid production.

To show the reduction of nitrates to nitrites by *Past. pestis*, workers in the Haffkine Institute, Bombay, have recommended a simple test, for which the cultures incubated overnight to demonstrate the production of hydrogen sulfide (see page 468) could be utilized.

A 5-mm loopful of such growths is placed on a white opal glass and mixed with a 2-mm loopful of Ilosvay's reagent. A positive result was indicated by the appearance of a pink colour within a minute.

Ilosvay's reagent is prepared by mixing *extempore* in a small test-tube an equal number of drops of (*a*) a solution of 1 g of sulfanilic acid and 14.7 ml of glacial acetic acid in 285 ml of distilled water, and (*b*) a solution of 0.2 g naphthylamine and 14.7 ml of glacial acetic acid in 325 ml of distilled water.

In general, for a demonstration of nitrate reduction, the use is advocated of broth or peptone water media of a pH of 7.2-7.5, to which 1 g potassium nitrate per 1000 ml or even per 100 ml has been added and which after sterilization have been filled in 10-ml amounts into tubes. The two reagents used consist of:

A	Sulfanilic acid	4 g	and	B	α-naphthylamine	2.5 g.
	5 N acetic acid [a]	500 ml			5 N acetic acid [a]	500 ml

[a] One part of concentrated acetic acid diluted with 2.5 parts of distilled water.

To carry out the tests, 0.2-ml amounts of solution A are added to the culture fluids after an incubation for 24 hours, and then solution B is put in drop by drop. Additions of 0.2-0.3 ml of the latter solution made in this manner suffice to produce a positive reaction, manifested by the appearance of a pink colour.

The same media without addition of potassium nitrate may be utilized to demonstrate the production of nitrous acid by the organisms under test, provided that they are free from nitrates. For practical purposes the absence of nitrates from the media may be ascertained by testing samples before inoculation with the above reagents. No red colour should appear even after a pinch of zinc dust has been added.

In actual practice it is advisable to make parallel tests with nitrate-free batches of the medium used and with batches to which potassium nitrate has been added. Non-inoculated tubes of both types of media should be incubated and tested at the same time.

Supplementary methods

In addition to the above-mentioned methods for the differentiation of plague and pseudotuberculosis bacilli (among which, according to the present state of knowledge, tests with rhamnose, urea-containing media, and with desoxycholate citrate agar are the most important) and the procedures recommended in the sections which follow, some stress has been laid upon the use of motility tests for a distinction between the invariably immotile *Past. pestis* and the typically motile *Past. pseudotuberculosis*.

However, important as this difference appears at first glance, the usefulness of motility tests in practical laboratory work is limited for various reasons. Motility is shown by the pseudotuberculosis bacilli only so long as they are cultivated at comparatively low temperatures, in actual practice at 20°-23° C. Far more important still, there can be no doubt that pseudotuberculosis strains exist which, temporarily at least, show no motility. For instance, in a recent report on the examination of 186 such strains it was stated that often repeated subcultivation had to be resorted to in order to demonstrate motility of the organisms. On the other hand, plague bacilli may show such marked Brownian movement that even experienced workers may find it difficult to decide whether a given organism is immotile or motile.

To facilitate distinction between active Brownian movement and true motility, it has been recommended that the normal saline used for hanging-drop preparations be replaced by a 1 per mille mercuric bichloride solution which, while not interfering with the Brownian movement, abolishes the active motility of the organism.

It has also been recommended that stab cultures be made in semi-solid agar (0.5% agar in a broth base), so as to demonstrate the more diffuse growth beyond the line of the stab which is shown by motile species in contrast to immotile organisms. A prolonged incubation (preferably for 4 or even more days) is essential for bringing out this feature.

Another simple method for the differentiation of plague from pseudotuberculosis bacilli, which has been successfully used in the Haffkine Institute, Bombay, may be carried out as follows:

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

Phage Identification Tests

As shown by recent studies, a clear-cut distinction may be made between plague and pseudotuberculosis bacilli by properly conducted tests with

plague bacteriophages. The following two methods have been recommended for this purpose:

Method A

(1) The strains to be tested are grown for 18-24 hours in broth at a temperature of 18°-20° C.

(2) The growths are then implanted on dry agar plates, each growth being spread over a circular area 2 cm in diameter, so that one dish can accommodate 10 different growths.

(3) After these implants become dry, they are touched with a suspension of bacteriophage which has been adapted to an avirulent plague strain.

(4) The plates are then incubated in an inverted position for 48 hours at a temperature of 20° C.

It has been established that at this temperature the plague-phage exerts a lytic action solely on *Past. pestis* strains, and not on pseudotuberculosis bacilli. Tests made at 37° C, on the other hand, have given unreliable results.

Before carrying out the tests, the plague-phage is titrated against the culture to which it has become adapted by testing serial tenfold dilutions of the phage with the aid of the technique described above. The highest dilutions giving confluent lysis are noted, and for the diagnostic tests ten times this amount, the " critical test dilution ", is used.

Method B

(1) 100 ml of broth in a 500-ml Erlenmeyer flask are seeded with an 18-hour broth culture of an avirulent plague strain and this culture is incubated with shaking for 6 hours at 30° C.

(2) 5 ml of a plague-phage filtrate with a titre of 10^{-4}, determined according to the technique described above, are then added to the culture and this is allowed to stand at room temperature for 18 hours.

(3) The organisms are then removed by Seitz filtration and a determination is made of the phage titre of the filtrate (which is usually at least 10^{-6}).

(4) Strips of filter paper 0.5 cm × 3 cm, previously sterilized in the autoclave, are then placed in 0.5-ml quantities of the filtrates (sufficient to soak 3 strips) and allowed to stand at room temperature for 2 hours.

(5) The strips are then removed under aseptic conditions and either shell-frozen and lyophilized or desiccated in vacuo.

(6) After they have become dry, the strips are sealed under nitrogen and stored at room temperature.

(7) To carry out the tests, 18-hour broth cultures of the organisms under test are heavily seeded over the dry surfaces of blood-agar plates and, while the surface is still wet, bacteriophage-coated strips are applied with sterile forceps to the cultures and gently pressed into place, non-coated sterilized strips of filter paper serving as controls.

(8) The dishes are then incubated at 20° C.

If plague cultures are dealt with in this manner, 1-mm wide zones of lysis, surrounding the bacteriophage-coated strips, usually become visible in 18-24 hours and the lysis extends into the surrounding parts of the cultures on prolonged incubation. Control tests with pseudotuberculosis

strains as well as with *Shigella* and *Salmonella* strains have invariably given negative results.

It has been found that the bacteriophage-coated strips preserved by lyophilization remain capable of producing lysis of *Past. pestis* growths for at least three months, if sealed under nitrogen and stored at room temperature. The strips desiccated in vacuo do not display such good keeping qualities.

There can be no doubt that these tests, particularly those performed according to the expedient second method, are of great differential diagnostic value. In fact, some experts are inclined to ascribe much greater value to them than to the biochemical tests outlined earlier, which they consider rather outmoded. It should be noted in this connexion that potent plague bacteriophages are maintained in some of the leading laboratories, such as the George Williams Hooper Foundation, San Francisco; the Haffkine Institute, Bombay; the Institut Pasteur, Paris; and the San Francisco Field Station of the Communicable Disease Center, US Dept. of Health, Welfare, and Education.

Serological Tests

Agglutination tests with known sera

Preparation of agglutinating sera

While the use of rabbits is unanimously recommended for the production of agglutinating sera for plague-diagnostic work, different antigens, administered by various routes, have been used for this purpose.

Workers in India recommend beginning the immunization of the rabbits with killed plague bacilli by (i) growing the organisms in broth for 48 hours; (ii) using exposure to a temperature of 55° C for 15 minutes to sterilize the growths; and (iii) giving an initial dose of 0.5 ml of the killed suspension intravenously, followed by further weekly doses, which are increased each time by 0.5 ml until a dosage of 5 ml is reached. Then further doses of live plague bacilli are administered by the intravenous route, until, as shown by the examination of samples obtained from the marginal ear vein of the animals, a satisfactory agglutination titre has been reached.

An alternative method is to grow plague bacilli for 3 days at 37° C on a suitable high-quality agar in Roux bottles which have been seeded with 2-3 ml of an 18-24-hours-old broth culture of *Past. pestis*. The growths are then harvested with the aid of 20 ml of normal (0.85%) saline, and 10 ml of a 1.5% formol solution are added to the suspension.

In place of this specially prepared antigen which, if kept in the refrigerator, remains stable for a period of 18 months at least, successful use has been made of the formol-killed plague vaccine produced with a titre of 2000 million per ml during the Second World War in the United States of America.

As shown by large-scale tests, comparatively better results were obtained by the intravenous than by the subcutaneous administration of these formol-killed antigens. The dosages used varied from 1000 to 4000 million organisms administered at weekly intervals or on alternate days for two or three weeks. Even at best the agglutinating titres remained almost invariably so low that it was found to be necessary either to continue immunization with live avirulent or virulent plague bacilli or to administer six months after the initial immunization a booster dose of 2000 million formol-killed organisms. It was essential to use batches of several rabbits for serum manufacture because considerable individual variations in the agglutinin response of different animals were found to exist.

Immunization of rabbits with live avirulent plague bacilli, preferably strains which have lost their virulence spontaneously, has been recommended by several workers, some of whom, as noted above, used such live growths after they had produced a basic immunity in their animals with the aid of killed antigens. The technique adopted in the Institut Pasteur, Paris, which, since it has been followed without considerable modification by other workers, might serve as standard, was (i) to grow the avirulent EV strain (isolated in Madagascar) for 48 hours at a temperature of 30°-35° C; (ii) to prepare from this growth suspensions in normal saline containing 1000 million organisms per ml; (iii) to inject rabbits intravenously at intervals of three days with six doses of such suspensions, amounting to 0.1, 0.5, 1, 2, 3, and 3 ml respectively; (iv) to bleed the animals eight days after the last injection.

There is no doubt that sera with a higher agglutination titre may be produced if the administration of live avirulent bacilli is followed by that of virulent organisms. However, there seems no need to resort to this somewhat risky method in order to manufacture agglutinating sera for routine purposes.

It is a great drawback of the agglutinating sera prepared by any of the above methods that they give positive results with pseudotuberculosis as well as with plague bacilli. However, as suggested by recent experiences, preliminary absorption with pseudotuberculosis bacilli renders the sera strictly specific for *Past. pestis*. Parallel tests with such absorbed sera should be made, therefore, whenever there is reason to suspect the possible presence of *Past. pseudotuberculosis*. However, the availability of sera raised with fractions containing the envelope antigen of *Past. pestis* in purified form would obviate the necessity of performing such tedious tests.

Technique of tests

For orientative purposes rapid slide tests may be performed by placing 5-mm loopfuls of the bacterial suspensions to be tested, which should have a content of about 2000 million organisms per ml, on a slide and admixing

to these drops loopfuls of suitable dilutions of the agglutinating serum, such as 1 : 50, so as to obtain a final titre of 1 : 100. Results may be read after a few minutes' standing at room temperature (27°-28° C) with the aid of a magnifying lens or under the low power of the microscope. It is essential to use drops of normal saline as control so as to exclude the presence of a spontaneous agglutination of the test organisms.

For more elaborate tests a series of serum dilutions may be prepared in the manner described below. Squares are then marked off with the aid of a wax pencil on a slide, loopfuls of the various serum dilutions are placed in these, and equal amounts of the test suspensions are admixed to them.

To carry out classical agglutination tests, series of 10 test-tubes of a suitable size (e.g., 100 mm × 13 mm) are placed in racks. An amount of 0.9 ml normal saline is placed into the first tube of each series, 0.5 ml in each of the other tubes. Then 0.1 ml of the agglutinating serum is added to the first tube, and after thorough shaking 0.5 ml of the serum-saline mixture is transferred to the second tube, 0.5 ml from this to the third, and so on up to the ninth tube, the tenth serving as saline control and therefore receiving no serum. Amounts of 0.5 ml of live or formalin-killed suspensions of the test organisms, which ought to have grown for 24 or 48 hours at 37° C, are then added. After the contents of each tube have been thoroughly mixed once more, the racks are kept in the incubator at 37° C for 1-4 hours and then for about 20 hours at room temperature, when readings are taken. As will be gathered, the final serum titres used in these tests are 1 : 20, 1 : 40, and so on up to 1 : 5120.

In place of saline suspensions prepared from agar or blood-agar growths of the test organisms, the live or formol-killed supernatants of blood cultures made from plague-suspect patients or rodents in broth (100-ml amounts in flasks) may be used with advantage for tube agglutination tests. Addition of 0.25% formol has been found to effect sterilization of such growths after 3-8 hours' standing at room temperature, depending upon concentration.

Agglutination tests with known antigens

While in the opinion of most workers agglutination tests with the sera of plague-affected rodents are not useful for diagnostic purposes,[a] increasing stress has been laid upon such tests performed with the sera of plague patients or, rather, convalescents. In fact, in the case of sufferers who have received early and thorough specific treatment by the methods now available, particularly with antibiotics, such tests form a most important, sometimes even the sole, means of finally confirming the diagnosis of plague.

[a] However, as has been noted before, agglutination tests with the sera of rabbits undergoing immunization are of great importance for assessing the agglutination titres reached and consequently the need for further antigen administrations.

The method is also of outstanding importance in making a certain diagnosis in the case of individuals seen for the first time after they have recovered from an illness clinically resembling plague.

In order to perform such tests, 10-15 ml amounts of blood are obtained by puncture of a suitable vein and the serum, separated off preferably by centrifugation, is used according to either the rapid slide method or the classical tube method.

Rapid slide tests with the sera of patients or convalescents are performed in a manner analogous to that described above with live or formol-killed *Past. pestis* cultures which have been grown for 1-2 days at 37° C. In view of the low agglutination titres to be expected, it is sufficient to use serum dilutions of up to 1 : 320; in fact, the proponents of the method have advocated simply serum dilutions of 1 : 3 or 1 : 4. Controls in normal saline are indispensable to guard against spontaneous agglutination of the test organisms. It has also been advocated that their agglutinability should be demonstrated by including controls made with suitable dilutions of a known plague-immune serum.

Tube agglutination tests may also be carried out with live or formol-killed suspensions of plague bacilli grown at 37° C. In a series of recent tests, particularly good results were obtained with formol-killed suspensions of virulent plague bacilli, grown for two days at 37° C, with the following procedures:

(1) 10 ml of normal saline were added in centrifuge tubes to 5 ml of formol-killed suspensions of *Past. pestis* prepared according to the method described above (see page 474) and the tubes were subjected to centrifugation at 10 000 revolutions per minute (r.p.m.) for 20 minutes.

(2) According to the period of storage of the suspensions (which were used for three months), the deposit was re-suspended once or twice in fresh normal saline and centrifugation was repeated.

(3) The final deposit was re-suspended in 5 ml normal saline and centrifuged at slow speed (1600 r.p.m.) for one minute to remove any gross particles.

(4) The supernatant was added to normal saline in a 500-ml flask and served after standardization with the aid of opacity tests to a titre of 2000 million per ml as antigen for agglutination tests with unknown sera.

(5) The latter were carried out by mixing in agglutination tubes 0.5-ml amounts of the antigen with equal amounts of serum dilutions ranging from 1 : 10 to 1 : 640. Results were read after an incubation of the tubes at 37° C for four hours, followed by exposure to room temperature for a further 24 hours.

Occasionally, positive results were obtained in rapid slide tests performed with low serum dilutions as early as the fourth day from the onset of plague. As a rule, however, the sera of persons who had been attacked by plague reacted positively in agglutination tests not earlier than a week after onset of the disease. On the other hand, positive results at the comparatively high titres of 1 : 640 and 1 : 160 were obtained in tube tests with convalescent

sera examined on the 36th and 44th day, respectively, after the individuals concerned had fallen ill with bubonic plague. There can be no doubt, therefore, that such tests are suitable for a retrospective diagnosis of the disease.

Precipitin tests

A recently devised modified precipitin test with soluble antigens obtained from the tissues of animals which had succumbed to plague was found to be valuable in laboratory trials even in instances where the materials had been stored at 37° C for periods of over three months. This test might, therefore, deserve attention in actual practice when dealing with decayed or mummified carcasses of rodents, which cannot be examined satisfactorily with the aid of the usual methods.

The technique used for this precipitin test is as follows:

(1) Organs removed from plague-suspect carcasses, preferably the liver, spleen, lymph nodes, and lungs, if necessary moistened with normal saline solution, are ground in a mortar with sterile sand and suspensions are prepared by the addition of 3-5 volumes of normal (0.85%) saline.

(2) After the suspensions have been transferred to test-tubes or Erlenmeyer flasks fitted with cork stoppers, approximately 2 volumes of diethyl ether are added and the containers are gently but thoroughly agitated.

(3) After the mixtures have been allowed to stand at room temperature for 5-20 hours, 0.5-2 ml of the clear aqueous supernatant fluids are removed and centrifuged at 3500-4500 r.p.m. for 30 minutes.

(4) Precipitin tests can conveniently be made by drawing equal volumes (0.03-0.1 ml) of the centrifugates and serial dilutions of a plague-immune serum into capillary tubes having an inner diameter of 1 mm to 3 mm. As a rule cylindrical zones of precipitation form in positive cases within a few minutes. Otherwise, the tubes are incubated for three hours at 37° C and placed in the refrigerator overnight, readings then being taken once more.

It is important to note, however, that tests carried out in the manner described above are not fully specific, positive results having been obtained in laboratory tests with plague serum not only with the soluble antigens of *Past. pestis*, but also with those of the pseudotuberculosis bacillus and even of *Past. multocida*. However, fully specific reactions were obtained when plague sera absorbed with the heterologous antigens were used.

Haemagglutination tests

Haemagglutination tests with protein fractions of *Past. pestis*, because they are strictly specific for this organism to the exclusion of even *Past. pseudotuberculosis*, and because they are highly sensitive, no doubt form one of the most valuable methods for plague laboratory diagnosis, provided that the difficulty of preparing or procuring the antigens necessary for this work can be overcome.

The performance of haemagglutination tests may be outlined as follows:

(1) *Antigenic cells*: To a volume of 2.5% washed sheep erythrocytes in normal saline one adds an equal volume of a 1 : 20 000 solution of tannic acid in saline, which has been freshly prepared from a 1% stock solution. After the mixture has been incubated in the water-bath for 10 minutes at 37° C, it is centrifuged at low speed for 3 minutes; fresh saline is next added to the sediment and centrifugation is repeated. The tannic-acid-treated cells are then re-suspended in normal saline to obtain again a concentration of 2.5% and an equal volume of a solution of the water-soluble antigenic fraction 1B of *Past. pestis*,[a] containing 1 mg of this antigen per ml, is added. After the mixture has been allowed to stand at room temperature for 15 minutes, the cells are washed twice with a 1 : 250 saline dilution of normal rabbit serum. Finally, the cells are re-suspended in a sufficient amount of this saline dilution of rabbit serum to obtain a concentration of 2.5%.

(2) *Test serum*: The sera to be tested are inactivated by incubation at a temperature of 56° C in a water-bath for 30 minutes. Nine volumes of the sera under test are then mixed with one volume of washed and packed normal sheep erythrocytes and the mixtures are left standing for 30 minutes.

(3) *Test procedure*: Serial dilutions of the inactivated and absorbed test sera are made in a manner analogous to that adopted for agglutination tests, but using instead of normal saline a 1 : 100 dilution of inactivated and absorbed normal rabbit serum as diluent. One adds then to each serum dilution 0.05 ml of the antigen-containing suspension of sheep erythrocytes. After mixing, the tubes are incubated for 2 hours at room temperature in a water-bath, and readings are then taken. A positive result is indicated by the appearance of a uniformly thin layer of red cells covering the curved bottom of the tubes, the pattern showing irregular edges in the presence of a marked excess of antibody. A negative result is indicated by the formation of a firm button of red cells in the bottom of the tubes.

Complement-fixation tests

Complement-fixation tests have been used in plague laboratory work both in order to confirm the presence of plague antigen in plague-suspect bacilli or in tissue extracts of supposedly infected animals and to detect antibodies in the sera of human convalescents or immunized animals. However, modern workers are almost unanimous in stating that this method, though valuable for research purposes, is not suitable for routine diagnostic work in view of the difficulty of procuring fully suitable antigens and sera and the tediousness of carrying out such tests with the controls indispensable for obtaining fully reliable results.[b]

[a] As prepared in the George Williams Hooper Foundation, San Francisco, Calif. (see Baker et al. (1952) *J. Immunol.* 68, 131). Alternative methods of preparing antigens for these tests have been described by: Amies, C. R. (1951) *Brit. J. exp. Path.* 32, 259 (see also Landy, M. & Trapani, R. J. (1954) *Amer. J. Hyg.* 59, 150) and by Silverman, S. J. (1954) *J. Lab. clin. Med.* 44, 185.

[b] See Chen, T. H., Quan, S. F. & Meyer, K. F. (1952) *J. Immunol.* 68, 147, for a description of up-to-date methods of complement-fixation tests with plague-suspect antigens or antibodies, which give results strictly specific for *Past. pestis*.

Animal Experiments

Choice of experimental animals

No uniform system has been adopted in regard to the choice of experimental animals for plague-diagnostic work because in some areas it has been found advantageous to substitute for the usual laboratory animals locally available species of wild or semi-domestic rodents, such as *Rattus* (*Mastomys*) *natalensis* and the white-tailed rat *Mystromys albicaudatus* in South Africa, or the *Bandicota bengalensis kok* (formerly called *Gunomys kok*) in India.

In place of these or other wild rodent species large-scale use has occasionally been made of cosmopolitan rats (particularly *Rattus rattus* subspecies) for plague-diagnostic work. However, it is rather difficult (i) to ascertain in areas where plague is endemic or apt to occur that the animals trapped for this purpose are free from the infection or are not resistant to it on account of preceding epizootics; (ii) to free the animals from their ectoparasites before they are transported to the laboratories or stabled—a procedure which, though indispensable, may be the cause of a considerable mortality; (iii) to keep the animals alive in captivity. In view of these obstacles as well as of the practical impossibility of breeding these rats in captivity, it seems rather questionable whether their large-scale use for plague-diagnostic work should be countenanced. In the opinion of most plague workers preference ought to be given to the rodent species generally used for laboratory purposes. It is, however, unanimously agreed that *rabbits*, though indispensable for the production of plague-immune sera and for immunological work in general, are not suitable for diagnostic tests, not merely because these animals are fully susceptible to pseudotuberculosis infection, but mainly because many of the races of this species are not uniformly susceptible, and some are even rather resistant, to infection with *Past. pestis*.

The use of *white rats* in plague laboratory work is of considerable importance in that, in contrast to the other universally utilized rodent species, these animals are practically insusceptible to infection with *Past. pseudotuberculosis*.

It would be quite legitimate to utilize white rats not only for the purpose of differentiating *Past. pestis* and pseudotuberculosis bacilli, but for other plague-diagnostic work as well—the more so because, as will be described below, these animals may be inoculated conveniently and safely with plague-suspect material by a special method. There can be no doubt, however, that *guinea-pigs* are of primary importance for plague laboratory work,

while as second choice *white mice* rather than white rats are preferable for reasons of economy. It is, however, important to note that not all, but only certain inbred strains of white mice are uniformly and highly sensitive to plague infection, and that consequently care must be taken to procure and maintain such suitable strains in the plague laboratories. No such racial differences have been found to exist in the case of guinea-pigs. However, experiences in California have shown the occurrence of some seasonal variation in the susceptibility of these animals to plague infection. During such periods emphasis has to be laid in plague-diagnostic work upon tests with white mice and, as far as necessary, also with white rats.

Methods of infection

Cutaneous infection

The applicability of the method of cutaneous infection in plague-diagnostic work is restricted in that among the usual laboratory animals guinea-pigs alone are suitable for such tests, which cannot be made conveniently with the small and rather fragile white mice and have been found to give not fully constant results in white rats. However, the method of cutaneous infection is of outstanding value because with its aid clear-cut results may be obtained with heavily contaminated materials such as putrefied rodent organs—i.e., under circumstances where cultural methods often fail altogether—while animal inoculation by other routes is apt to produce mixed infections or may even lead to rapidly evolving and fatal infections by concomitant bacteria before signs of plague become manifest.

Still, eminently useful as the method of cutaneous infection is in this respect, it must be realized that it does not give strictly specific results because, as confirmed by recent experiences, guinea-pigs may be infected with *Past. pseudotuberculosis* by the cutaneous as well as by the subcutaneous route, while according to earlier observations skin tests proved occasionally positive even with pasteurellae in the strict sense.

It must be realized also that the method of cutaneous inoculation, which usually does not cause death of the experimental animals until after 4-5 days, is less expedient than infection by the subcutaneous and particularly by the intraperitoneal route. However, since buboes become apparent in cutaneously infected guinea-pigs within 24-48 hours after inoculation, an early diagnosis may be made by the examination of material aspirated from these incipient swellings with the aid of a syringe, using the technique recommended below for bubo puncture in human plague. The material obtained in this manner may be utilized not only for smear examination and culture tests, but also for animal inoculation, particularly intraperitoneal infection of white mice.

To arrive at an earlier diagnosis it has also been recommended that the plague-infected animals be killed as soon as they become moribund (i.e., when they lie on their sides), with the aid of ether or chloroform or by other methods, and dissected forthwith. This method, which may be applied also in the case of animals infected by other routes, not only saves time, but also increases the chances of isolating pure cultures, particularly in a tropical climate, where decomposition of naturally succumbing animals is apt to be rapid.

In order to produce groin buboes, which are most easily manipulated, most workers are in favour of using either the inside of the thigh or the skin of the lower abdomen for cutaneous inoculation. To carry out the tests in a reliable manner, it is necessary to remove the fur of the animals at the chosen site over an area measuring 2-3 cm^2 or about one square inch. Various procedures are recommended for this purpose, some workers using a depilatory (e.g., barium sulfide paste) which is afterwards washed off, others a shaving blade or a scalpel. In the case of long-haired races, the hairs may be simply plucked off. Some workers advocate lightly scarifying the areas thus bared with a scalpel before inoculations are made, but since plague bacilli are capable of entering even through the intact skin, the minor skin defects produced by shaving or plucking the hairs fully permit an entry of the organisms.

Various methods have also been recommended for rubbing in the infective material, some workers utilizing the stout platinum or chromium-nickel wires used for culture work for this purpose or depositing the material with these and distributing it with the aid of sterile spatulas or other suitable instruments, others preferring sterile, mounted, cotton plugs, which have been dipped into suspensions of the test materials. The organs of plague-suspect animals to be tested may be applied directly with the aid of forceps or artery clamps. Applying suspended infective material, one might use the multiple pressure method recommended for smallpox inoculation.

Inoculation by pricking the tail of rats

The method of pricking the root of the tail of white rats with an injection needle which has been dipped into the plague-suspect material to be tested possesses the following advantages:

(1) The procedure may be easily and safely carried out in the glass jars in which the rats are kept individually before and after infection, by temporarily replacing the ordinary cover of the jars by a wooden one with a hole in the centre, through which the tail of the animal alone is drawn out with the aid of a long forceps or clamp for making the inoculation.

(2) The method, besides giving positive results with plague only and not with pseudo-tuberculosis bacilli, has also been found useful with contaminated materials and when dealing with some rat-pathogenic organisms which produce fatal infections when administered subcutaneously.

Subcutaneous inoculation

The method of subcutaneous inoculation of experimental animals with plague-suspect material is the most universally applicable of the procedures under review, both because such tests can be made with white rats and mice as well as with guinea-pigs and because it permits the testing of suspensions of the pooled organs of plague-suspect rodents or of pooled fleas as well as of all other suspended plague materials. Further, direct use may be made of fluid cultures, of the blood of plague-suspect rodents or human sufferers, and of the sputum of persons suspected of suffering from pneumonic plague. However, in view of their great sensitivity to infection with pneumococci, white mice should not be used for tests with sputum.

As in the case of cutaneous infection, most workers recommend either the inner side of the thigh or the lower part of the abdomen as the sites for subcutaneous inoculation in guinea-pigs or rats and the lower part of the abdomen near the groin region in the case of white mice. Not more than 0.2 ml to 0.5 ml at most of the test fluids should be used for the subcutaneous inoculation of these animals, or amounts of 0.5 ml or slightly more up to 1 ml at most in the case of guinea-pigs and white rats. The test materials must be prepared and the injections must be made under aseptic conditions.

Subcutaneously infected laboratory animals usually succumb to typical plague within intervals of three to four days, provided that test materials containing virulent plague bacilli but free from any considerable contamination have been used.

Intraperitoneal inoculation

The diagnostic importance of intraperitoneal inoculation of plague-suspect materials is due to the rapidity with which the test animals succumb after the administration of even limited amounts of virulent plague bacilli by this route. Death of the animals may take place as early as 24-36 hours after infection and is as a rule not delayed beyond three days. Intraperitoneal inoculation is therefore particularly indicated for the rapid establishment of the diagnosis in instances of previously unobserved rodent plague—e.g., in the case of rats found sick or freshly dead on board ship or in ports—and in human patients, especially in the early stages of suspected pneumonic or septicaemic plague, when only signs of a severe unlocalized infection are manifest. However, as has been noted above, white mice should not be used for tests with the sputum of plague suspects.

Even more rapid results may be obtained by taking, with the aid of a syringe, material for microscopic and culture examination from the peritoneal cavity during the life of the intraperitoneally infected animals. If virulent plague bacilli are present, they can be demonstrated in this

manner in the peritoneal exudate aspirated about 24 hours after inoculation of the animals.

Carrying out intraperitoneal inoculation tests with plague-suspect materials, these rules must be followed:

(1) It is necessary to restrict the use of this method to non-contaminated materials, because, otherwise, mixed infections are bound to result or, worse still, the animals are apt to succumb quickly to infections caused by contaminating bacteria without presenting evidence of plague infection.

(2) The injection of too large amounts of virulent materials should be avoided, because otherwise the animals, particularly mice, may rapidly succumb to toxaemia without showing macroscopic evidence of plague and yielding unsatisfactory or even no growths of *Past. pestis*. This precaution is particularly necessary when intraperitoneal inoculation is used to confirm the plague nature of suspicious cultures.

(3) The inoculations must be carried out under aseptic conditions. It is best to free the site of the infection from hairs in an area of about 1 cm² and to disinfect the bared skin with alcohol before the injection is made.

(4) Great care must be taken not to puncture intraperitoneal organs. To avoid this, it is best to hold the animals before injection with their heads downwards and to introduce a short bevelled needle, with the syringe held as far as possible parallel to the skin, in the opposite direction (i.e., upwards) into the lower half of the abdomen. The syringe should then be gently moved to and fro in order to ensure that the point of the needle has not become fixed in an organ but lies free in the peritoneal cavity.

(5) The amounts injected should not exceed ½-1 ml in the case of mice, or 1-2 ml in the case of guinea-pigs and white rats.

Other methods of inoculation

In the almost unanimous opinion of the experts consulted, inoculations by other methods, e.g., by the conjunctival route, are not necessary for plague-diagnostic purposes. It should also be noted that this method as well as that of nasal instillation, because apt to lead to infective excretions, is fraught with greater risks than the procedures described above.

Methods of examination

All experimental animals succumbing after inoculation with plague-suspect materials must be carefully dissected under aseptic conditions in order to obtain macroscopic evidence of the presence of the infection as well as material for bacteriological examination and, where necessary, for repeated animal experimentation.

While, as a rule, white mice merely show more or less marked spleen enlargement and other non-specific signs of a generalized infection (in the case of intraperitoneal infection a usually scanty peritoneal exudate as well), the macroscopic findings made in white rats and more still in guinea-pigs which have been infected percutaneously or subcutaneously with virulent materials, though not fully pathognomonic, are usually quite characteristic of the presence of plague. The most important features

met with, in addition to subcutaneous congestion, in guinea-pigs are (*a*) the presence of a marked infiltration, and often of ulceration at the site of infection, or a bubo in the corresponding regional lymph-nodes, or both; (*b*) enlargement of the spleen and liver, which often show small or relatively large necrotic nodules, in addition to engorgement of the former organ and parenchymatous or early fatty degeneration of the latter; (*c*) the presence of similar nodules or, more frequently, of variably sized pneumonic foci in the lungs. In intraperitoneally inoculated animals an exudate is found in the abdominal cavity which as a rule is rather scanty and serous in the case of white rats, and more abundant and purulent in guinea-pigs. Owing to the usually rapid death of animals infected by this route, marked characteristic changes in the internal organs are likely to be absent.

Material for bacteriological examinations should be taken under aseptic precautions from the site of infection or the bubo, or both; from the spleen, liver, and heart; and—particularly in the case of animals which succumbed with some delay—from the lungs as well. Cultivations from the bone marrow, which are indispensable when examining decomposed carcasses, are unnecessary in the case of experimentally infected animals, if, as ought to be the invariable rule, they are dissected within a few hours after natural death or are killed in the moribund state.

Various methods may be used to make cultivations from the organs of the experimentally infected animals. Pieces of the organs, removed with the aid of freshly sterilized instruments, may be utilized directly for the inoculation of agar, or preferably blood-agar, plates, segments of which may be inoculated for the sake of economy with different organs of one and the same animal. Alternatively the surface of the organs may be seared with a hot spatula and material taken with a platinum or chromium-nickel loop. To obtain heart blood it is best to use a sterile glass capillary provided with a rubber bulb, which is plunged in, if necessary after the surface of the heart has been seared. Such small glass pipettes may also be used conveniently for the collection of peritoneal exudates.

After material for cultivation has been taken, smears or impression films are made from the same organs. For the sake of expediency and economy one and the same slide may be used for several or all the organs in question, but it is well to prepare two such sets so that, in addition to colouration with Wayson's or another suitable simple stain, Gram's method may be used if necessary.

It is well to keep pieces of suitable organs of the dissected animals —e.g., of the bubo, the spleen, the liver, and the lungs— in a sterile dish or tube so that, unless smear examinations give satisfactory preliminary results, direct animal experiments can be made. It should be noted that in this way clear-cut results may ultimately be obtained with materials containing plague bacilli of low virulence which at first produce "inapparent" infections with scanty or even negative macroscopic and bacterio-

logical findings. Direct passages by the intraperitoneal route are apt to prove particularly useful in such cases.

To what extent confirmatory tests must be made with the cultures isolated from the experimental animals depends upon the nature of the test materials. Full confirmation of the diagnosis with the aid of serological methods and by phage or biochemical tests is indispensable in instances of incipient rodent or human plague as well as in sporadic or clinically atypical cases of plague in man. During previously confirmed outbreaks it is permissible to consider fully typical results of macroscopic and microscopic examination and of cultural tests as conclusive. Smear examination alone is in the opinion of most experts not sufficient to establish the diagnosis in experimental animals.

LABORATORY DIAGNOSIS OF RODENT PLAGUE

Methods of Collection

Commensal (domestic) rodents

Though different methods, including, for instance, killing of the rodents by the public with the aid of primitive means and opening of the burrows followed by catching or killing the escaping animals, have been used, only two procedures are utilized on a large scale to procure plague-suspect rats and mice for laboratory examination—namely, collection of animals found dead, and trapping.

While some workers advocate the use of either one or the other of these principal methods, others maintain with much reason that these two procedures, because they supplement each other, should be applied in combination. Collection of dead rats and mice is according to these workers the method of choice during epizootics, because one may expect to find far more frequent and conspicuous evidence of plague infection in the carcasses than in the animals trapped alive. Trapping, on the other hand, ought to be used on the widest possible scale (i) in the localities round the epizootic foci, so as to delimit the latter and to detect whether and in which direction the infection spreads; and (ii) during the off-seasons as well in the plague-affected localities, because then rat-falls are few and far between, but evidence of the persistence of the infection may be found by an examination of trapped animals or their fleas with the aid of pooling tests.

Different methods of collecting the carcasses of plague-suspect rats and mice have been used in accordance with the local conditions in the various plague areas. Generally speaking, it is most desirable that such

collections be made by properly equipped members or squads of the plague staff so as to keep the public as much as possible away from the dead animals or their fleas.

If such workers can be employed, they should make systematic rounds to detect and collect carcasses not only outside but also inside houses and compounds. Such rounds should be made in particular early in the morning, preferably, as has been recommended for India, before sunrise. The dead animals found should be gathered with long tongs and, after they have been tagged, should be placed in metal containers with well-fitting lids for transport to the laboratory.

If collection of the carcasses has to be left to the public, they should be advised not to touch the animals, but to handle them with tongs or similar implements. The carcasses should be enclosed forthwith in paper bags or in tins or other containers available in the households and, pending delivery to the laboratory, should be stored outside the houses. During severe outbreaks in China it was found suitable to establish collecting posts for the depositing of rat carcasses found by the public by affixing solid containers with lids on the outside walls of houses and compounds, telephone poles, or the like, or by placing large tins or earthenware jars with well-fitting lids at strategic points in the thoroughfares. Staff members visited these posts at least twice daily to tag and collect the rodents deposited by the public.

In the opinion of most workers cage-type traps should be used in preference to snap traps to catch rats and mice for plague-diagnostic work, employing baits attractive to these animals in accordance with the local conditions. The traps used, which should be as numerous as possible, ought to be inspected at least once daily, preferably early in the morning. If captures have been made, the traps should be enclosed in canvas or nylon bags for transport to the laboratory so as to prevent an escape of the fleas on the trapped animals. If cage-type traps are handled in this manner, satisfactory numbers of fleas can almost invariably be recovered for examination.

Wild rodents

No generally valid indications can be given for the collection of plague-suspected wild rodents in view of the profound differences in the ecology of the numerous species involved in various parts of the world or even in individual areas, so that attention has to be given to animals with either diurnal or nocturnal habits and to species given to hibernation or aestivation.

Collection of the plague-suspect carcasses of wild rodents may be rendered difficult by the often large extent of the affected areas and the presence of carrion-eating beasts or birds of prey, but may be used to great advantage in the case of localized severe epizootics. If no carcasses are

found, satisfactory results may be obtained by the use of traps suited to the size of the species involved. Shooting diurnal rodents with 0.22 calibre rifles or shotguns by trained marksmen has been found eminently suitable in the USA. Good results have been obtained also by stirring in the rodent burrows with long metal rods or by flooding the burrows so as to force the animals to the surface where they may be killed by mechanical means. If other methods fail, it is necessary to open inhabited burrows by digging. This procedure, besides offering good chances for the capture of live rodents or the recovery of carcasses, is advantageous in that numerous ectoparasites are almost invariably found in the nests of the animals.

Observation of Captured Animals

Opinions on whether plague-suspect rodents which have been captured alive should be kept under observation in the laboratory instead of being killed forthwith are divided, some experts recommending this procedure, while others are against it. Since, in view of the cannibalistic tendencies often shown by rodents kept in captivity, it is necessary to confine them individually, it would certainly be difficult to keep numerous plague-suspect rodents under observation. It has also been pointed out with much reason that animal experiments with the organs of individual rodents captured alive and killed forthwith or experiments with the pooled organs of such animals are bound to give positive results if the animals are incubating plague or are in the incipient stages of the disease.

Since, however, some workers have had favourable experiences with animals succumbing to manifest plague after an observation period in the laboratory, no hard and fast rules should be laid down. If rodents captured alive are subjected to observation, they must be kept under conditions strictly preventing an escape of their fleas. To extend the period of observation beyond about a week seems unnecessary. If not succumbing within this time, the animals must be sacrificed, dissected, and tested with the aid of microscopic and culture methods, and as far as necessary, by animal experiment as well. Identical tests must be made, of course, in case of the animals spontaneously succumbing during the observation period.

Methods of Killing Captured Animals

The following methods of killing captured rodents have been recommended by different workers:

(1) Killing by mechanical means, e.g., by strangulation, by crushing the skull with a stout clamp, or by administering a blow to the head;

(2) immersion of the animals;

(3) administration of volatile fluids, such as ether, chloroform, chloropicrine (trichloronitromethane), or gasoline; and

(4) gassing with calcium cyanide.

Except in emergencies the methods mentioned under (3) and (4) should be chosen, both because they are more humane and because they ensure the killing or at least the stunning of the fleas parasitizing the rodents, besides killing the latter. Both these methods can be implemented in the case of rodents confined or enclosed in any solid container with a well-fitting lid. To handle rodents captured in cage traps which have been afterwards enclosed in canvas or nylon bags, the following procedures should be followed:

(1) Volatile fluids, preferably gasoline, may be simply and effectively applied under these circumstances by (i) putting each bag with the trap inside into a suitably sized box or tin; (ii) partly opening the bag and pushing in a cotton pad soaked in the volatile fluid in question; (iii) keeping the box closed for about 20 minutes, a time fully sufficient to kill the rodents and at least to stun their fleas;

(2) If calcium cyanide is available, the cage traps in their bags, which should first be opened up, are put into a tin or box provided with a well-fitting cover and an opening suitable for pumping in calcium cyanide after the receptacle has been closed. Several traps may be handled at the same time, provided that containers of an adequate size are available. A few strokes of a pump delivering $^1/_{10}$ ounce (2.4 g) of calcium cyanide per stroke ought to be sufficient to deal with a receptacle having a cubic content of 10 feet (0.3 m).

Forwarding Materials

No generally valid recommendations can be made on how to arrange for an examination of plague-suspect rodents if, as is often the case, no or incomplete facilities for such work are available in the field or in nearby laboratories. The choice of suitable methods to be adopted under such circumstances depends upon the presence or absence of facilities for dissecting the rodents at or near the places where they have been found.

It is generally agreed that, if no local facilities for dissection are available, the rodent carcasses must be forwarded to distant laboratories in solid, well-sealed containers (preferably metal containers or stout glass or earthenware jars well packed in wooden or metal boxes), and that, if hot weather prevails, the tins or boxes should, if possible, be kept under refrigeration pending transport. However, different methods of treating the carcasses before packing have been recommended. Some workers advocate dipping the carcasses in solutions of antiseptics, e.g., a 5% compound solution of cresol as available ready-made in pharmacies, while recently others have advised dusting the carcasses before packing with an insecticide, particularly 10% DDT. A method recently recommended is to place the carcasses after dusting with DDT into screw-capped $^1/_2$-lb (250-g) jars

containing a layer of about 50 g of commercial sodium chloride. If larger jars are used to accommodate several carcasses, 2-3 ounces (56-84 g) of salt are allowed per animal.

If local facilities for dissection are available, the usual practice is to forward to the laboratories the lymph-nodes or pieces of the internal organs, or both, of the dissected animals (especially the spleen and liver) in a preserving fluid. For this purpose Broquet's fluid is often recommended.

According to a recently given formula Broquet's fluid may be prepared by admixing 2 g of calcium carbonate and 20 ml of pure neutral glycerol to 100 ml of distilled water. To prevent acidification of the fluid it was found advisable to buffer it with 1.1046 g of bisodium phosphate and 0.0230 g of citric acid per 100 ml. The fluid was steamed for 10 minutes at 100° C and then kept in bottles, the corks of which had been sterilized by dry heat and afterwards coated with sterile melted paraffin.

To remove the excess of glycerol before the pieces thus preserved were used for laboratory examination, particularly animal experiments, it was recommended that they be placed on sterile filter paper, but this should preferably be followed by washing the pieces in sterile normal saline.

It was found that pieces of plague tissue preserved in Broquet's fluid continued to harbour virulent *Past. pestis* for periods of at least two weeks, provided that they were kept at room temperature or in the refrigerator. Positive experimental results were also obtained with plague fleas kept in the fluid for periods up to six days.

As shown by experiences in India and Madagascar, it is also satisfactory to preserve material obtained by puncture from the heart or the liver and lungs of plague carcasses in normal saline.

According to the findings made in this respect in Madagascar, normal saline suspensions of materials from the liver and lungs of experimentally infected animals, obtained by puncture 3-10 hours after death, remained virulent for periods up to six days at a temperature of 16°-26° C, but were no longer virulent after having been kept for three days at 37° C. Suspensions made from carcasses which had been kept for 48 hours at 21° C remained virulent for 24 hours only, though the organs of the putrefied animals were still infective after three days.

If material from several or numerous carcasses has to be forwarded at one and the same time, pools may be made from the lymph-nodes or the spleen and liver, or both, of the rodents in saline solution or in Broquet's fluid; or, as has been recommended by some workers, such pools may be prepared instead from the bone marrow of the carcasses, collected from the femoral bones of the animals with the aid of a syringe provided with a suitably thin and short bevelled needle. Whatever organs are chosen, it is essential to use exclusively materials collected from rodents belonging to one and the same species for the preparation of individual pools, because otherwise, in view of the great sensitivity of the animal tests performed with these collected materials, rodent species which are not actually suffering from plague might be incriminated.

Methods of Examination

Two fundamentally different methods are used for examination of the carcasses of plague-suspect rodents: either complete autopsies are made and the material thus obtained is used for microscopic and culture examination followed by the necessary tests to confirm the plague nature of the growths; or dissection is carried out only far enough to obtain fragments of the lymph-nodes or the spleen and liver, or both, or, as is advocated by some workers, of the bone marrow of the animals, which are pooled and used for the inoculation of test animals, usually for subcutaneous inoculation of guinea-pigs.

The methods suitable for individual examination of the rodent carcasses have already been described (see page 484), but it is necessary to add that when dealing with mutilated specimens or with carcasses which have undergone putrefaction, satisfactory results may be obtained by opening the femurs of the animals with a bone forceps or strong scissors and collecting particles of the bone marrow with the aid of a needle or a syringe.

The methods adopted by different workers for preparing organ pools vary considerably. Most workers use normal saline solution to prepare suspensions from this material for animal inoculation, but some advocate the utilization of more concentrated sodium chloride solutions, of as much as 10%-20%. The usual method of making the suspensions is to grind up the collected small organ pieces with the aid of a little saline in a sterile mortar. An alternative expedient method recommended by other workers consists of (i) putting the pieces of organs into a thick-walled tube into which some coarse sand or glass powder had been inserted before sterilization, and (ii) to break the organs into fragments, after a little sterile normal saline had been added, by vigorous shaking of the tube.

Though successful use has been made of the bone marrow pools collected from large numbers of animals, it seems advisable to prepare pools of other tissues from not more than 10 plague-suspect rodents.

It has been maintained by some workers that pooling tests, made either with organ pools or, as will be described below, with flea pools, render the method of individually examining the rodent carcasses superfluous. However, not all experts are in agreement with this opinion, many pointing out that the two methods, as they supplement each other, ought to be used in combination. Pooling tests are of paramount value for survey purposes, i.e., for establishing whether plague is present in a given area or locality, a type of work in which the method of individual rodent examination is apt to give disappointing results. The pooling methods are likewise invaluable for ascertaining whether the infection persists in areas or localities where it has previously been manifest. It must be realized, on the other hand, that pooling tests, because they are apt to prove positive in the case of pools containing merely a few, or possibly even single, virulent

plague bacilli derived from one carcass or flea, do not indicate the extent to which the infection is present. This gap can be filled only by a careful individual examination of an adequate number of rodents; and this is therefore of paramount importance for watching the trend of the infection during actual plague outbreaks and thus obtaining information indispensable for a proper conduct of the anti-epidemic campaigns.

LABORATORY DIAGNOSIS OF PLAGUE IN FLEAS (OR OTHER INSECT VECTORS)

Methods of Collection

From rodents found dead or killed in the laboratory

Since it is unnecessary to examine live fleas or other insect vectors for the purposes of plague diagnosis, the usual practice is to kill these ectoparasites before their removal from the plague-suspect rodents. In this respect the methods of handling animals caught in cage traps have been described above (see page 488). In carrying out these procedures it is essential to examine the inside of the bags or boxes in which the traps have been enclosed, because the fleas often leave the fur of the rodents before becoming killed or stunned.

A simple and efficient method for dealing with rodents which have been found dead or have been killed by snap traps or by other means at their place of capture is as follows:

(1) As noted already, the rodents collected dead or killed at the spot must be taken to the laboratory in tightly closed tins or boxes, or in well-closed paper or nylon bags.

(2) To kill the ectoparasites in the laboratory, a 15-gallon (or 65-litre) garbage tin provided with a well-fitting lid is used, into which a piece of hardware cloth (strong wire netting) has been placed on three or four about 8-10 cm high wooden blocks.

(3) After about 2 tablespoonsful of calcium cyanide have been placed under the hardware screen, the boxes or tins containing the carcasses are opened and the latter are quickly poured in; then, before the lid of the garbage tin is closed, the boxes or tins are held open over its mouth and shaken or knocked so as to remove any ectoparasites which had left the carcasses.

(4) Paper or nylon bags containing carcasses may simply be opened and quickly put into the garbage tin, the lid of which is then closed.

(5) It is preferable to let the calcium cyanide act for 1-2 hours, but if necessary, the garbage tins may be opened after as little as 20-30 minutes.

(6) The opened garbage tins are left standing for a few minutes to air them (an operation best performed in the open and not in a room) and the carcasses are then removed with the aid of tongs to be handled as described below.

Note. In emergencies this method may also be used to kill rodents enclosed in cage traps.

To collect killed or stunned fleas and other ectoparasites from carcasses treated according to any of the above methods, it is sufficient to hold the carcasses by their tails over an empty white enamel basin and to comb their fur with the aid of a fine-toothed comb, or to brush the fur with a stiff brush or a forceps, or simply to strike the carcasses sharply with a three-cornered file or another suitable instrument. However, many workers prefer to fill the white enamel basins used for ectoparasite collection with water and to immerse the carcasses before they are deparasitized. Some workers keep the carcasses immersed while combing or brushing them—a procedure which is desirable when handling carcasses the ectoparasites of which had not been killed beforehand.

The ectoparasites removed from the carcasses by these procedures are picked up with small forceps, preferably with curved ends, and put into empty vials or, better still, vials filled with normal saline; or, if the ectoparasites cannot be tested forthwith, into vials filled with 2%-3% saline solution. Fleas, ticks, lice, and mites from individual rodents or from batches of carcasses belonging to one rodent species must be collected in separate vials.

In flea-infested premises

The three principal methods for collecting "free-living" fleas in infested premises are (1) exposure of guinea-pigs on the floors, or of guinea-pigs or other rodents (e.g., rats), which are free or have been freed of fleas, in cages placed on the ground or not more than 6 inches (15 cm) above the floors in the premises in question; (2) the use of tanglefoot paper or, preferably, of more elaborate artificial flea-traps; and (3) the collection of fleas from floor sweepings, using a small dustpan and brush and a flea-sieve.

Details of these three methods are as follows:

(1) The test animals, after being exposed in the infected premises for one night or for periods of a few days, must be taken to the laboratory in flea-proof containers. If it is desired to spare their lives, the animals may be anaesthetized with ether or chloroform and may be brushed or combed while under anaesthesia in empty white enamel basins, from which the fleas can be easily and safely collected. Otherwise, the animals are killed and examined according to the methods described above (see pages 488 and 492).

(2) A simple and efficient flea-trap consists, as originally recommended, of a dish filled with lamp oil and provided with a floating wick, which is lighted at night to attract the fleas. Actually, however, it is often sufficient simply to fill the dishes with water, preferably adding a little ethanol or chloroform or, better still, DDT or some other suitable insecticide.

If used in houses with soft floors, it will be found advantageous to set the rim of the dishes level with the ground. However, fully satisfactory catches can be made by placing the traps (preferably lighted traps) on solid floors.

After the traps have been exposed overnight, they are inspected and the fleas are collected in ampoules in the manner described above.

Tanglefoot paper has been found eminently useful for the collection of standard samples of fleas from the floors of human habitations in French West Africa.

(3) Fleas from floors may be collected on the spot by sweeping up dust and other debris by means of a brush and pan, and transferring the material to a double flea-sieve, which is shaken over a deep white enamel basin. The fleas are readily collected in a

bulb-suction tube. If water is used in the basin, the fleas are picked up with forceps as described above.

From rodent burrows and nests

In order to collect fleas and other ectoparasites from rodent burrows, the burrows are gradually opened and the soil covering portions of about 20 cm or 1 foot of the corridors is scraped off at a time. These materials as well as the nests eventually reached are placed in flea-proof bags or other containers for transport to the laboratory, unless facilities for flea collection are available on the spot. Working in manifestly plague-affected localities it is most desirable to treat the burrows with calcium cyanide or chloropicrine before digging operations are started. Otherwise, the materials removed from the burrows must be treated with calcium cyanide or chloropicrine.

In order to collect the ectoparasites from these materials large galvanized buckets may conveniently be used, which are open at the lower as well as at the upper end and contain two or three sets of sieves made of wire netting of decreasing gauge (e.g., of ¼-inch (6.35 mm) gauge and of a gauge corresponding to that of mosquito netting). Before the materials to be examined are sieved, the apparatus is placed in a large white enamel pan which may be used empty or filled with water to facilitate the collection of the ectoparasites.

Another suitable apparatus for collecting fleas from dust has been described by Estrade.[a] To collect fleas from the litter found in the nests of rodents, workers in the USA recently took advantage of Berlese's funnel, described by Baker & Wharton.[b] The principle of the modification is to force heat, with the aid of an electric fan, through a funnel system so as to drive fleas and other insects into a collecting pan.

In place of these tedious operations some simple procedures have been recommended for collecting fleas from the mouth or the entrances of wild rodent burrows. Thus it was found that considerable numbers of these insects may be collected by moving a piece of stout white material fastened to a stick over the ground near the burrow entrances and by pushing the cloth into the burrows to a depth of approximately 3 feet or 1 metre, and subsequently shaking it over a white enamel basin, preferably filled with water. It was also recommended that wild rodent fleas be caught with the aid of cotton wads inserted into the mouth of the burrows.

Forwarding of Fleas or Other Insect Vectors

It is generally agreed that, in order to transport fleas or other ectoparasites to distant laboratories, they must be placed in solid tubes or

[a] Estrade, F. (1934) *Bull. Soc. Path. exot.* 27, 458 (See also: Pollitzer, R. (1954) *Plague*, Geneva, p. 366

[b] Baker, E. W. & Wharton, G. W. (1952) *An introduction to acarology*, New York

ampoules, corresponding in size to that of Wassermann tubes, and preferably provided with screw caps. Failing these, cotton plugs coated in paraffin should be used to close the tubes. These must then be securely packed into solid tins or wooden boxes.

Most workers recommend the use of a preserving fluid for long-distance transport of plague-suspect fleas or other insect vectors. Some advocate the use of Broquet's fluid for this purpose, but fully satisfactory experiences have been made by utilizing instead of this 2% or 3% sodium chloride solutions.

Methods of Examination

Since bacteriological examination of plague-suspect fleas often gives disappointing results, inoculation of test animals with pools of such insects has to be resorted to in diagnostic work. The technique for such tests may be outlined as follows:

(1) Before preparing suspensions from the pools, the collected fleas must be examined with the aid of a dissecting microscope or under the low power of an ordinary microscope in order to determine the species to which they belong. Sterile instruments and sterile slides or dishes ought to be used for this purpose.

(2) Fleas belonging to a single species should preferably be used for the preparation of individual pools.

(3) It is advisable to restrict the maximum number of fleas to be used per pool to 50, but to reduce this number whenever possible to 25-30 when guinea-pigs are used as test animals and to not more than 20 when mice experiments are performed.

(4) Before using the fleas for the preparation of the test suspension, they must be thoroughly washed—preferably several times—in sterile normal saline.

(5) Sterile mortars and pestles or glass rods may be used to triturate the fleas after a few drops of sterile normal saline have been added. Another convenient method is to put the fleas into a larger thick-walled test-tube and to use, after a few drops of sterile normal saline have been added, a long narrower test-tube (which has previously been sterilized inside the larger test-tube) for crushing the fleas. Some sterile sand or glass powder or a small amount of ammonium oxalate may be added to facilitate trituration.

(6) After trituration is completed, sufficient sterile normal saline is added to prepare suspensions for injection; the specimens are then vigorously shaken and allowed to stand for a short time until the gross particles have become deposited.

(7) Either guinea-pigs or white mice may be used for subcutaneous inoculation with the supernatants. Tests with white mice are preferable as this facilitates the utilization of two test animals for each pool—a procedure which is desirable to guard against intercurrent death of the inoculated animals.

Comparative Value of Flea-pooling and Tissue-pooling Tests

In the opinion of some workers, flea-pooling tests, since they form an accurate means of ascertaining whether plague is present in the rodent herds concerned, can be used exclusively in preference to the less expedient and comparatively more dangerous method of testing the tissues of the

rodents. However, it is usually held that these two categories of tests, supplementing each other as they do, ought to be used in combination. Accordingly it is legitimate to give preference to flea-pooling tests to expedite large-scale surveys of areas where the presence of wild-rodent plague is suspected. Such tests are also of great value during the off-seasons of rat-caused plague, when rodent infection is apt to be quite inconspicuous, while the fleas play an important part in carrying over the infection. However, tissue-pooling tests, or rather, in view of what has been discussed above, individual examinations of the rodents, are of paramount importance during the plague seasons, when it is essential not merely to obtain information on whether the infection is present, but also to learn exactly with what intensity it has spread.

LABORATORY DIAGNOSIS OF PLAGUE IN PATIENTS

Bubonic Plague

According to the character of the disease and to the stage in which the patients are first seen, the following methods may be available for the laboratory examination of materials collected from bubonic plague patients.

Examination of pus from the site of infection

In instances in which a local reaction to the infection is manifested by the appearance of blisters at the site of the infective flea-bites, material for laboratory examination can easily be obtained by collection of the pus filling these thin-walled vesicles, which often break spontaneously. The diagnostic value of tests made with this material is considerable, because in positive cases it is invariably possible to arrive at a *prima facie* diagnosis by the examination of smears, which show the presence of characteristic bipolar-stained and gram-negative plague bacilli. However, both because such manifestations of primary skin plague are usually rare and because, if present, they can be detected only in patients seen in the earliest stage of the disease, it is exceptional that advantage can be taken of this method of examination.

Examination of material from the buboes

The usual method of obtaining material for laboratory examination from the buboes is to puncture them under aseptic conditions with the aid of a syringe mounted by a thin (20-22 gauge (0.90-0.70 mm)) and short-bevelled needle. Early in the disease only little fluid can be aspirated in this manner, as a rule not more than enough to fill the needle. However, this drawback can easily be overcome by washing the needle and syringe well after puncture with a little sterile normal saline and using the resulting dilutions of the material for examination.

Late in the disease when, as is often the case, suppuration of the buboes has taken place, not only is it possible to obtain ample material for laboratory examination by puncture, but pus may sometimes also be obtained directly when the buboes have opened spontaneously or when, to avoid this untoward fistula formation, they have been incised. However, the results of examination of such pus are rather disappointing, because as a rule plague bacilli are absent or, if present, are intermixed with numerous contaminating organisms. The presence of the latter interferes with examination by culture methods, while, owing to a lowering or loss of the virulence of *Past. pestis* in the pus, animal inoculation by the cutaneous route (which alone is practicable with this contaminated material) is apt to give negative results.

Laboratory examination of the bubo contents withdrawn by puncture during the early stages of the disease is, on the contrary, a method of paramount diagnostic importance. In fact there is no difficulty in arriving rapidly at a presumptive diagnosis because, if plague is present, large or at least fairly large numbers of characteristic bipolar-stained and gram-negative bacilli are invariably seen in smears prepared from the puncture dilutions. It is likewise easy to confirm the validity of these findings by examining and testing the agar or blood-agar cultures, which must be made with these materials. If a rapid confirmation of the diagnosis is essential, the material obtained by bubo puncture may be used for the intraperitoneal inoculation of test animals, particularly of pairs of white mice.

In spite of these advantages, most plague workers are rather hesitant in making large-scale use of bubo punctures. Some of them express the fear that use of this method might lead to a generalization of an infection which had hitherto been localized in the buboes. Though such misgivings are unwarranted if bubo punctures are made cautiously in the manner described and without any unnecessary manipulation of the buboes, this method of examination is rather undesirable on account of the great pain which it usually causes. In the opinion of most workers bubo puncture ought to be used, therefore, exclusively in cases of particular diagnostic importance, namely, (i) in early or sporadic cases and (ii) in atypical cases where it is impossible to arrive at a presumptive diagnosis on clinical grounds. During a previously confirmed outbreak it is as a rule not at all difficult to decide by clinical examination whether specific plague treatment should be injtiated forthwith. A confirmation of the diagnosis may be obtained by blood cultivation, which must be made before specific treatment is started or, should this method give negative results, by a retrospective serological diagnosis.

Blood examination

In order to appreciate the value of bacteriological blood examinations in bubonic plague it must be kept in mind that, though passing invasions of the blood stream by the causative organisms possibly take place more frequently than is often assumed, it is only in severe cases and later in the disease that a secondary bacteraemia becomes established, while in slight and milder cases the infection becomes localized in the buboes. It follows that as a rule bacteriological examination of the blood is of diagnostic value only in the case of patients who are severely affected and tested later in the disease. In such patients bacteraemia may become so conspicuous that plague bacilli may be found easily in smears made from the finger blood. However, except in the case of such moribund patients it is necessary to obtain blood for laboratory examination by puncture of a vein. An amount of 10-15 ml of blood should be withdrawn under strictly aseptic conditions and should be used for the inoculation of: (i) two agar slopes with 0.5-ml quantities of the blood each; and (ii) one or preferably two flasks, each containing 50 ml or, better yet, 100 ml of a suitable broth medium or of peptone water with 5-ml quantities of the blood. If it is possible to inoculate two such flasks, it is advisable to keep one of them at 37° C in the incubator and the other at about 28° C. Daily subcultures on agar or, preferably, on blood agar must be made from these flasks for 3-4 days. The plague nature of the growths appearing on these plates and on the primarily inoculated agar slants must be confirmed by appropriate tests. In the course of established epidemics it is legitimate to resort for this purpose solely to smear examination or rapid slide-agglutination tests, or both, provided that the growth appearances of the organisms are fully typical.

In localities remote from laboratories it may be impossible to utilize the method described above of enrichment in broth or peptone water flasks. However, there ought to be no difficulty under any circumstances in inoculating agar slopes at the bedside of the patient with 0.5-ml quantities of blood withdrawn from a vein. Such cultures will prove suitable for examination even if they do not reach the laboratories for some days. Further, autoclaved solutions of 10% sodium taurocholate in normal saline, filled in 50-ml quantities into previously sterilized screw-capped jars or large tubes and inoculated with 5-ml quantities of the patient's blood may be used to advantage for dispatch to even distant laboratories, where these fluids are used for subcultivation and, if desirable, for direct animal inoculation.

Septicaemic Plague

To arrive at a speedy presumptive laboratory diagnosis of incipient primary septicaemic plague is well-nigh impossible because it is only late in the disease that a demonstration of the causative organisms in blood smears is easy or at all possible.

Comparatively the most rapid means of confirming the diagnosis of septicaemic plague is intraperitoneal inoculation of test animals, preferably of pairs of white mice, with blood withdrawn from a vein of the patient. At the same time agar slopes and flasks with broth or peptone water ought to be inoculated with adequate quantities of the blood as recommended in the case of bubonic plague.

Since it would be disastrous to wait for the results even of direct animal inoculation tests before initiating specific treatment with antibiotics, a decision whether to start this therapy has to be reached on clinical grounds as soon as the patients are seen. In view of the non-pathognomonic, though severe, clinical signs of primary septicaemic plague this decision is rather difficult, especially in early or sporadic cases. However, the rapid deterioration of the condition of the patients, since it is rarely seen in diseases other than septicaemic plague, ought to attract attention.

Primary Pneumonic Plague

To arrive at a laboratory diagnosis of primary pneumonic plague in the manifest stage of the disease, commencing usually about 20-24 hours after onset and characterized by the expectoration of a peculiar blood-stained sputum, is easy: a presumptive diagnosis may be made rapidly by smear examination of the sputum, in which typical, bipolar-stained and gram-negative plague bacilli are invariably conspicuous and often abound. Cultivations made from the sputum on ordinary agar or blood-agar plates yield a positive result within 48 hours, and often after only 24 hours. Even if no pure or almost pure growths of *Past. pestis* are obtained—as is often the case later in the disease—isolated colonies of this organism may as a rule be found on properly inoculated plates, and these colonies may be used immediately for confirmatory tests, including in early or sporadic cases intraperitoneal inoculation of test animals, preferably of pairs of susceptible white mice.

Whether direct animal inoculation tests should be made with the blood-stained sputum of pneumonic plague patients is a moot question. Since in the earlier stages of the disease pneumococci, in addition to other contaminating organisms, may be present or even plentiful in the sputum, it is —as has been noted before—not advisable to use white mice for such inoculation tests. Many workers are also of the opinion that, when inoculating guinea-pigs with such sputum, the fully reliable but comparatively tedious method of cutaneous infection ought to be used. It may be held, therefore, that the procedure recommended above of intraperitoneally inoculating test animals with suspensions made from primarily isolated *Past. pestis* colonies is apt to give speedier and equally reliable results.

While in early, sporadic, or atypical cases of pneumonic plague a series of confirmatory tests must be made with the growths isolated directly from

the sputum or from the test animals, the diagnostic procedures may be considerably simplified when dealing with the sputa of patients who have become typically affected in the course of an established pneumonic plague epidemic. It is sufficient in such cases to make smear examinations or rapid slide tests with the cultures isolated from the sputum, provided that these growths show a typical gross appearance.

If pneumonic plague outbreaks occur in places without local laboratory facilities but not far distant from laboratories, it is permissible to forward the sputa of the patients, collected under aseptic conditions, to these laboratories in sterile, solid glass jars or tubes provided with tightly fitting screw-caps, adding preferably some sterile normal saline or broth to guard against exsiccation. If materials collected from pneumonic plague patients have to be dispatched to distant laboratories, the following procedure should be followed: (i) unless facilities for the staining of smears are locally available, unstained but alcohol-fixed sputum smears should be forwarded; (ii) a loopful of each sputum specimen should be used for the successive inoculation of two or three agar slopes which should be forwarded as well; (iii) in addition, it is advantageous to put 5 ml of blood, withdrawn from a vein of the patient's, into a screw-capped jar or tube containing 50 ml of 10% sodium taurocholate in normal saline for dispatch to the laboratory. It should be noted in this connexion that a secondary bacteraemia develops fairly rapidly in pneumonic plague patients who have not received early and energetic specific treatment.

In distressing contrast to the ease and rapidity with which a laboratory diagnosis may be made in cases of manifest pneumonic plague, it is most difficult to arrive at such a diagnosis or even a presumptive diagnosis in the earliest " closed " stage of this disease. For during this stage numerous organisms belonging to other bacterial species are present in the scanty and uncharacteristic sputum or in the saliva of the patients, while plague bacilli are rather inconspicuous, if visible at all. To make matters worse, some of the heterogeneous organisms, including gram-negative species, may more or less resemble *Past. pestis* in stained preparations.

To arrive at a presumptive laboratory diagnosis at the earliest possible moment it is therefore usually necessary to repeat smear examination of the sputa at frequent intervals (i.e., every 1-3 hours) and to resort to cultivation as soon as the presence of *Past. pestis* appears to be certain. However, particularly when dealing with patients known to have been in contact with plague sufferers, it seems advisable not to await a presumptive laboratory diagnosis, but to commence treatment with antibiotics as soon as the presence of pneumonic plague is suspected on clinical grounds. Similarly, one should not hesitate to commence specific treatment forthwith in cases in which, owing to the lack of local facilities, it is impossible to arrive at a speedy presumptive laboratory diagnosis later in the disease.

METHODS OF EXAMINATION OF PLAGUE-SUSPECT DEAD BODIES

The various methods available for collecting and examining materials from plague-suspect dead bodies may be described and evaluated as follows.

Simple Methods

Materials collected from nostrils or mouth

If the dead bodies are fresh, it is often possible to collect material for laboratory examination from their nostrils or mouth. This is true not only of victims of pneumonic plague, but also of individuals who have succumbed to other forms of the disease, owing to the presence of a foamy, usually blood-stained fluid resulting from the development of a terminal lung oedema. In the case of pneumonic plague it is also often possible to find patches of blood-stained sputum on the body or clothes, or in the environment of the corpses.

Examination of gram-stained smears and cutaneous inoculation of guinea-pigs are the most suitable methods for dealing with these materials which usually abound in plague bacilli.

Bubo puncture

Unless putrefaction is advanced, satisfactory results may be obtained by puncturing the affected lymph-nodes of victims of bubonic plague according to the technique recommended for bubo puncture of patients but with a thicker needle. The saline washings obtained in this way may be used for smear examination, cultivation on ordinary or selective media, and, in cases of special diagnostic importance, for direct inoculation of test animals, particularly of guinea-pigs by the percutaneous route.

Venous puncture

If the dead bodies are fresh, satisfactory material for smear examination, cultivation on the usual, or preferably on selective, media, and for animal inoculation, particularly percutaneous infection of guinea-pigs, may be obtained by venous puncture.

An alternative method of obtaining blood from the dead bodies for such tests, which is usually not objected to by the public, is to puncture the heart.

Digitotomy

The method of digitotomy, practised in some South American plague foci, consists of (i) the amputation of a finger, preferably a forefinger, from plague-suspect dead bodies after preliminary skin disinfection with alcohol, and (ii) opening of the second or third phalanx lengthwise with the aid of a small bone-saw so as to obtain bone marrow for examination. The wound thus created may be covered up with a cotton pad which has been dipped into formol or other antiseptic and which is fixed with the aid of a bandage or of adhesive plaster, or the hand is simply placed under the clothing of the dead body. For transportation to the laboratory the amputated finger should be placed stump down into a jar containing a layer of absorbent cotton.

According to the experiences made in South America, smear examination of the bone marrow extracted from the amputated fingers usually proved unsatisfactory. However, good results were obtained by cultivation and animal experiments.

While it is impossible to carry out digitotomy in countries the inhabitants of which abhor any mutilation of dead bodies, it might be feasible in these circumstances to obtain bone marrow for post-mortem examination from the sternal bone according to the method used in clinical work. It should be noted that this method has given some promising results in plague patients.

Autopsy

Complete autopsies

There can be no doubt that among the methods of examining plague-suspect dead bodies that of performing complete autopsies is of unsurpassed value, not only because it facilitates a fully adequate selection and procurement of materials for laboratory examination, but also because owing to the usual presence of marked and rather characteristic gross signs it is as a rule easy to arrive speedily at a *prima facie* diagnosis. In actual practice, however, it is often out of the question to perform complete autopsies of plague-suspect dead bodies, not so much for lack of facilities as on account of strong popular objections to this method which it would be most unwise to overrule. Moreover, the performance of complete autopsies on the highly infective dead bodies of plague victims is fraught with such great risks that this method should be used by trained pathologists only, who possess special experience in plague work and are therefore fully competent to take the necessary precautions.

It is for these reasons impossible to recommend the performance of complete autopsies as a standard method for the examination of plague-suspect dead bodies.

Partial dissection

A much simpler and safer as well as often less resented method is that of performing partial dissections for the sole purpose of procuring suitable pieces of organs from the plague-suspect dead bodies for laboratory examination. When dealing with victims of bubonic plague, it is sufficient to excise the buboes or portions of the buboes. If no buboes are found, an incision ought to be made in the epigastrium and small pieces of the liver, or spleen, or both, should be excised after these organs have been brought into view with the aid of long clamps. To open the thorax in order to obtain pieces of the lungs is more difficult and risky. Moreover, since a secondary bacteraemia is practically always present in pneumonic plague, excision of pieces of the liver or the spleen ought to suffice in this as well as in other forms of the disease. It is of interest to add in this connexion that some workers have recently recommended the use of the viscerotome in place of excision methods to obtain material from the liver of plague-suspect dead bodies.

After the excisions have been made, the incision wounds must be closed with the aid of long strips of adhesive plaster or by the application of clamps or sutures.

The excised pieces of organs should be put into sterile glass jars provided with screw-caps or with ground-in glass stoppers for transport to the laboratory. If, in the absence of local facilities for examination, the jars have to be dispatched over fairly short distances, requiring a transport of some hours only or half a day at the most, they should be kept under refrigeration. For transport of the materials over longer distances it is necessary to pour adequate amounts of Broquet's fluid or of normal or 2% saline into the jars, which must be then carefully packed into solid containers.

The choice of the methods for examining the excised pieces of organs (and also of the materials removed in the course of complete autopsies) depends upon their state of preservation. If they have undergone longer transport without having been kept in preserving fluids or if the dead bodies were not fresh, preference must be given to cultivation on selective media and guinea-pig infection by the cutaneous route.

Puncture

A useful and usually not resented procedure for obtaining materials from plague-suspect dead bodies for laboratory examination is to puncture one or several suitable organs with the aid of a 5-ml or 10-ml syringe on which is mounted an 18-gauge (1.25-mm) needle 10 cm long, after each puncture to wash the needle and syringe in 3 ml of sterile saline kept in a 10-ml flask or tube, and to use the resulting suspension, which contains the puncture material from one organ or pooled materials from several

organs, for laboratory tests. Unless a bubo is present, it is best to take material from the liver, several parts of both lungs and, as has been recommended by some workers, also from the heart. Desirable as it would be to include the spleen as well, it is considerably more difficult to reach this than the above-mentioned organs.

The most important methods of examining the materials obtained by puncture are smear examination and guinea-pig inoculation by the cutaneous route. The inspection of smears is desirable not only in order to arrive at a presumptive diagnosis but also because the demonstration of a considerable quantity of cells proves that the organs in question and not the tissues surrounding them (e.g., the oedematous tissue usually present round the buboes) had been punctured.

As shown by large-scale experiences in Madagascar in particular, organ punctures properly done are a reliable means for the post-mortem diagnosis of plague. However, whether this or one of the other procedures enumerated above is chosen for such work depends upon the local conditions in the various plague areas.

PRECAUTIONS TO BE ADOPTED

Precautions to be universally adopted

Instruction and supervision of staff

To prevent unfortunate accidents, it is of the utmost importance thoroughly to instruct the staff, particularly the assistant staff, in their duties, laying emphasis upon the proper procedures to be used and upon the dangers confronting the workers if they deviate from this technique or omit to use the precautions prescribed for the different types of work. Adequate supervision must be exerted to ensure that the members of the assistant staff in particular never fail to follow the instructions laid down in these respects. It should be absolutely forbidden for any staff member to perform work for which he has not been properly trained.

Plague vaccination

It is essential to keep all members of the staff protected against infection by plague vaccinations repeated at suitable intervals. Booster doses ought to be administered at least twice yearly and during the epidemic periods of the infection every three months.

Proper maintenance of laboratories

Every effort must be made to keep both permanent and emergency laboratories in an adequate condition, particularly protected against access

of rats or other free-living rodents and free from fleas and other vermin, including flies. All captured rodents and all rodent carcasses must be carefully freed from fleas, which must be killed forthwith. Research work with living fleas should not be performed in plague-diagnostic laboratories but in separate premises.

As an additional precaution the infected rodents should preferably be kept in flea-proof containers instead of in cages made with wide-meshed wire netting or having doors made of such wire netting. In emergencies tall tin buckets covered by close-meshed gauze or wire gauze may be used.

All instruments used for the infection of test animals and for the examination of rodent carcasses or human dead bodies must be sterilized by boiling as soon as possible after conclusion of the work. The metal trays used for animal dissection should be autoclaved or washed with alcohol or with antiseptic solutions.

The dissected rodent carcasses must be kept in solid metal containers, if desirable in strong antiseptic solutions, pending incineration or sterilization in the autoclave. The food remnants and litter in the cages or containers in which the infected animals have been kept must be incinerated; the cages or containers must be adequately disinfected.

The gowns or overalls used by the staff for animal infection, dissection of test animals, human autopsies, or other potentially dangerous work must be sterilized as soon as possible in the autoclave, and the rubber gloves worn must be boiled immediately after completion of such work.

The discarded slides on which plague smears have been made should be immersed in a jar with an antiseptic solution and should be boiled before they are cleansed. All other glassware used for work with plague bacilli, particularly the disused cultures, must be autoclaved before cleansing.

Action in case of accidents

Rules on how to deal with accidents occurring in the course of plague laboratory work must be prepared and the staff must be made familiar with these instructions. The staff members should be advised not to use antiseptics if they contract wounds during their work, but to let the wounds bleed freely and then to soak them well in 70% alcohol.

The staff members must be urged to report laboratory accidents immediately so that they may be taken under observation and so that prophylactic treatment may be instituted, if necessary. The workers must be also instructed to report immediately if they develop even slight fever or feel otherwise unwell so that, if necessary, treatment with antibiotics can be commenced without delay. It must be emphasized in this connexion that plague is practically always a curable disease provided that adequate treatment is given immediately. It must also be explained to the staff that oral administration of tetracyline in 4 daily doses of 250 mg for 4-6 days, or—if such

antibiotics are lacking—daily administration of 3 g of sulfadiazine or sulfamerazine for a period of 6 days is apt to prevent the development of the disease in persons who have been definitely exposed to the risk of infection, e.g., through contact with pneumonic plague patients or through laboratory accidents, but that the indiscriminate prolonged use of sulfa-drugs by the staff members themselves is bound to lead to a sulfa-poisoning, particularly to serious and sometimes irreparable kidney lesions.

Precautions to be adopted in Different Phases of Plague Laboratory Work

During the various phases of plague-diagnostic work the laboratory staff must be kept protected against (*a*) the risk of contracting infection through the bite of blood-sucking insects, especially rodent fleas; (*b*) the possibility of directly contracting bubonic plague when performing post-mortems or otherwise handling contaminated material; and (*c*) the possibility of contracting pneumonic infection through the splashing or spraying of materials laden with plague bacilli. The precautions necessary to avoid these dangers or a combination of these dangers may be outlined as follows.

Flea-borne infection

To protect themselves against the possibility of a flea-borne infection, e.g. when entering plague-infected houses, or when coming in contact with persons or dead bodies or with rodents possibly harbouring infected fleas, the members of the laboratory staff should preferably wear a special costume. This consists 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 and, where necessary, by rubber gloves. A mask must be added if there is a risk of contracting infection through the splashing or spraying of infective material, particularly when coming in contact with patients possibly suffering from primary or secondary plague pneumonia.

The masks used for this purpose or during other phases of plague laboratory work should measure 4×6 inches (10×15 cm) and should consist either of a single layer of the now available cotton-flannel filter fabric between single plies of gauze, or of a cotton pad $^1/_2$ inch (1.3 cm) thick between two layers of gauze. Pieces of tape must be affixed to the four corners. The upper pair of these tapes is passed round the head above the ears and tied behind, while the lower two tapes are tied behind after they have been passed below the ears. People with prominent noses should insert cotton plugs at both sides of the nose to close the gaps left when the mask has been adjusted.

Since under tropical conditions it may be rather trying to wear the overalls described above, long linen stockings covering the feet and legs and tied up above the knees may be substituted.

A further most advisable method of protecting plague workers against flea-borne infection is to impregnate the garments or linen stockings worn by them with a suitable insecticide exerting a residual action. However, the advice of specialists is indispensable for using this method in an efficient and safe manner.

Direct bubonic or pneumonic infection

No special precautions are needed when examining plague materials in the laboratory by bacteriological methods or when carrying out serological tests, provided that such work is performed with the cleanliness and care indispensable for laboratory work in general.

When performing human autopsies, a proper costume must be worn, including a rubber apron, high rubber boots, and solid, long, rubber gloves. To avoid danger from splashing of infective materials, masks and preferably goggles as well should be worn.

Less stringent precautions are necessary when dissecting plague-infected animals, the more so because plague workers should learn to perform the autopsies with the aid of suitably long instruments, without touching the carcasses. Nevertheless, the use of rubber gloves is indispensable and masks should be worn to guard against the accidental splashing of infective materials.

Experimental infection of test animals should be carried out with the precautions used for animal dissection. Special care must be taken to avoid the spraying of infectious material while charging the syringes.

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