**RECOMMENDED LABORATORY METHODS FOR THE DIAGNOSIS OF PLAGUE**

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F. M. PRINCE, S. F. QUAN, P. WAGLE

**CONTENTS**

<table>
<thead>
<tr>
<th>General Methods</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear examination</td>
<td>459</td>
</tr>
<tr>
<td>Fixation</td>
<td>459</td>
</tr>
<tr>
<td>Simple staining</td>
<td>460</td>
</tr>
<tr>
<td>Gram staining</td>
<td>461</td>
</tr>
<tr>
<td>Special stains</td>
<td>461</td>
</tr>
<tr>
<td>Cultivation</td>
<td>461</td>
</tr>
<tr>
<td>General indications</td>
<td>462</td>
</tr>
<tr>
<td>Solid media</td>
<td>464</td>
</tr>
<tr>
<td>Fluid media</td>
<td>464</td>
</tr>
</tbody>
</table>

**Biochemical methods**

- Reactions produced in carbohydrate-containing media | 464 |
- Reactions produced in urea-containing media | 467 |
- Tests with desoxycholate citrate agar | 468 |
- Tests for hydrogen sulfide and indole production, methylene-blue reduction test, and methyl-red test | 468 |
- Tests for reduction of nitrates and production of nitrous acid | 470 |
- Supplementary methods | 472 |

**Phage identification tests** | 472 |

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* The World Health Organization is greatly indebted to the authors of this work, who accepted the onerous task of outlining recommended methods for the diagnosis of plague, and especially to Professor K. F. Meyer and Dr. R. Pollitzer, who not only drew up and circulated a very detailed questionnaire to their colleagues, but also prepared the work in its final form.

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### LABORATORY DIAGNOSIS OF PLAGUE IN ANIMALS

<table>
<thead>
<tr>
<th>Methods of Examination of Plague-Suspect Dead Rodents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple methods</td>
</tr>
<tr>
<td>Materials collected from mouth or mouth</td>
</tr>
<tr>
<td>Body punctures</td>
</tr>
<tr>
<td>Venous puncture</td>
</tr>
<tr>
<td>Dihydroxy</td>
</tr>
<tr>
<td>Auspicy</td>
</tr>
<tr>
<td>Complete autopsy</td>
</tr>
<tr>
<td>Partial dissection</td>
</tr>
<tr>
<td>Presence</td>
</tr>
</tbody>
</table>

### LABORATORY DIAGNOSIS OF PLAGUE IN FLIES (OR OTHER INSECT VECTORS)

<table>
<thead>
<tr>
<th>Methods of Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>From flies found dead or killed in the laboratory</td>
</tr>
<tr>
<td>In the infected premises</td>
</tr>
<tr>
<td>From infected premises</td>
</tr>
<tr>
<td>Picking at flies or other insect vectors</td>
</tr>
</tbody>
</table>

### LABORATORY DIAGNOSIS OF PLAGUE IN MICE

<table>
<thead>
<tr>
<th>Methods of Examination of Plague-Suspect Dead Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple methods</td>
</tr>
<tr>
<td>Materials collected from mouth or mouth</td>
</tr>
<tr>
<td>Body punctures</td>
</tr>
<tr>
<td>Venous puncture</td>
</tr>
<tr>
<td>Dihydroxy</td>
</tr>
<tr>
<td>Auspicy</td>
</tr>
<tr>
<td>Complete autopsy</td>
</tr>
<tr>
<td>Partial dissection</td>
</tr>
<tr>
<td>Presence</td>
</tr>
</tbody>
</table>

### LABORATORY DIAGNOSIS OF PLAGUE IN OTHER VECTORS

<table>
<thead>
<tr>
<th>Methods of Examination of Plague-Suspect Dead Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple methods</td>
</tr>
<tr>
<td>Materials collected from mouth or mouth</td>
</tr>
<tr>
<td>Body punctures</td>
</tr>
<tr>
<td>Venous puncture</td>
</tr>
<tr>
<td>Dihydroxy</td>
</tr>
<tr>
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</tr>
<tr>
<td>Complete autopsy</td>
</tr>
<tr>
<td>Partial dissection</td>
</tr>
<tr>
<td>Presence</td>
</tr>
</tbody>
</table>
methylene blue may be used, pleomorphic stain as Wayson's stain is particularly suitable for the staining of plague smear.

Perch dial is prepared by adding 10 ml of a saturated solution of safranin or red 40 dye to 100 ml of a 25% solution of potassium dichromate in distilled water. Apply for 1 minute and wash off.

Wayson's stain is prepared by adding 0.25 ml of 0.2% solution of 2% methylene blue and 0.25 ml of 0.5% safranin in 10 ml of absolute ethanol. Stain the unstained smears to 200 ml of 3% formalin and 100 ml of distilled water. The stain is not necessary to decontaminate staining. After 1 minute, fix for 5 minutes. Reproduce the staining for 1 minute, followed by thorough washing with water, and then dehydrated.

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

Gram staining

Since the proper application of Gram's method of staining depends largely upon familiarity with the reagents and procedures used, lesions upon altered techniques might cause harm rather than proving beneficial.

Stain solution has to be drawn to the following modifications of Gram's original method, which are now used as some of the leading laboratories.

(a) Leçassier's modification: Stain for 15-20 minutes with a 0.5% aqueous solution of nile blue. (b) Acetate solution of Gram's iodine solution (prepared by adding 2 ml of sodium acetate and 3 ml of acetone to 1 ml of distilled water) 1 g of sodium iodate and 3 ml of distilled water. Add the dye, and make up to 20 ml with distilled water. (c) Final wash 10ml for 10 seconds. (d) Stain for 15-20 minutes with acute red solution (prepared from 2 g of crystal violet and 1 g of acetone with 5 ml of distilled water). (e) Final wash 10ml for 10 seconds. (f) Stain for 15-20 minutes with 0.5% solution of safranin.

(b) Gieson's modification: Stain 20 minutes with 3% aqueous solution of gentian violet. (c) Acetate solution of Gram's iodine solution (prepared by adding 2 ml of sodium acetate and 3 ml of acetone to 1 ml of distilled water). (d) Final wash 10ml for 10 seconds. (e) Stain for 15-20 minutes with acute red solution 1 g of crystal violet and 1 g of acetone with 5 ml of distilled water. (f) Final wash 10ml for 10 seconds. (g) Stain for 15-20 minutes with 0.5% solution of safranin.

(c) Modified Gram stain: The usual modification of Gram's original method is carried out as follows: (a) Add a 2% solution of crystal violet for 1 minute, (b) Hydrogen peroxide (3%) for 3 minutes, (c) Add a 2% solution of acetone with crystal violet for 1 minute, (d) Distilled water for 1 minute, (e) Add a 2% solution of acetone with crystal violet for 1 minute, (f) Hydrogen peroxide (3%) for 3 minutes, (g) Distilled water for 1 minute, (h) Add a 2% solution of acetone with crystal violet for 1 minute, (i) Hydrogen peroxide (3%) for 3 minutes, (j) Distilled water for 1 minute, (k) Add a 2% solution of acetone with crystal violet for 1 minute, (l) Hydrogen peroxide (3%) for 3 minutes, (m) Distilled water for 1 minute, (n) Add a 2% solution of acetone with crystal violet for 1 minute, (o) Hydrogen peroxide (3%) for 3 minutes, (p) Distilled water for 1 minute, (q) Add a 2% solution of acetone with crystal violet for 1 minute, (r) Hydrogen peroxide (3%) for 3 minutes, (s) Distilled water for 1 minute, (t) Add a 2% solution of acetone with crystal violet for 1 minute.

Recommended methods: Modifications (b) or (c) 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 plaque laboratory work. It is desirable, however, that:

(1) A smear similar to that of Wayson may be prepared extraneously by admixing 2-3 drops of a saturated solution (6%, 15%, 15%) solution of methylene blue and 6-9 drops of Ziehl's carbol fuchsin 15 ml of tap water. Stains need 3-2 minutes in enclosed are exposed to this stain for 15-20 seconds, then washed and air-dried.

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

Note: Ziehl's carbol fuchsin is prepared by mixing 10 ml of a saturated solution of basic fuchsin in 85% ethanol to 80 ml of a 5% aqueous solution of carbolic acid (phenol).

Cultivation

General indications

Reserve of the media

Although slightly solid as well as moderately liquid media may be used for the cultivation of Pasteur, plaque (pH range from 6.5 to 7.6), a pH of 7.2, 7.5 is 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 Porcine-water media used for biochemical tests.

Incubation temperature

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

Cultures needed with pilius-suspect material should be inspected after they have been incubated for 24 hours, but in view of the slow growth of Paste, pustis they should be reincubated for a further period of at least 24 hours, preferably for an additional period of 2 or 3 days. Observations for 60 days is advisable in water 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 pumice 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 various laboratories for general purposes are suitable for the cultivation of Paste, pustis, if adjusted to a suitable pH.

Most plague experts use a broth prepared from the locally available kind of meat or with meat extract as base for their agar media. However, in the works in the Institute Pasteur, Paris, maintaining that meat broth exists to some extent an inhibitory action on the growth of Paste, pustis, recommend for its cultivation a solid medium prepared by adding 2% pumice to 2.5% pumice water and adjusting the pH to about 7.0. It is essential for the preparation of such media to use a suitable brand of pumice rich in amino acids.¹

(b) Blood agar

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

1. Making 1 litre or smaller amounts of a suitable agar medium;
2. Cooling to a temperature of about 45°C;
3. Adding approximately 5% sterile defibrinated sheep's blood;
4. Placing carefully and gently 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 blood, can be substituted. Horse's blood in particular, often available in laboratories, can be used for the preparation of blood agar in a concentration of 10% to 20%.

Workers in the Institute Pasteur, Paris, 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. Making 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. Aspirating a few ml of the mixture on agar sheets, which are then allowed to gel in a slanting position.

(c) Special media

Hyper-nitrogen 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 resolution forms on this medium was considered to be characteristic of Paste, pustis. This claim has not been fully substantiated as for on Paste, pseudotuberculosis is concerned, though it has to be admitted that the invasion forms shown by the latter on salt agar develop more slowly and are morphologically distinct from those of Paste, pustis. Nevertheless, the use of hyper-nitrogen agar for plague-diagnostic work has been largely given up in favour of other tests more clearly distinguishing the plague bacillus from other pasteurelae, particularly the pseudotuberculosis bacillus.

Sections-solid/agar medium. Certain agar media, e.g., those prepared with Hasting's hormone broth or yeast infusion broth, may be rendered intrinsically suitable for the isolation of Paste, pustis by the addition of (a) 0.025% sodium salicylate, 0.25 ml of a freshly prepared 10% solution of sodium salicylate, and (b) 1/1000–1/4000 of gentian violet (0.1% to 2.5%). 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 1/4000 may inhibit all bacterial growth. Apart from this drawback, however, the medium is enormously suitable because it inhibits the most troublesome contaminants met with in plague-diagnostic work, and also because the characteristic large colonies of Paste, pustis developing in 26 to 48 hours may be easily picked out and used for slide agglutination tests.

Copper sulfate agar. Addition of 0.5% copper sulfate to agar is another means of checking the growth of contaminating organisms, particularly those of the protein group of organisms, encountered in diagnostic work with the organs of plague-suspect rodents or human victims.

¹ Church, G. 1896. Bull. Soc. Path. etc. 37, 220
Fluid media

Media

The peculiar assembly and "stabiizes" growth of *Fus. pestis* in nutrient broth can no longer be considered of differential diagnostic importance, because such features, while not invariably present if the organism is cultivated, may be produced by the rough form of the *Pasteurella*-bacillary bacilles. Both cultivation of at otherwise unidentified plague stenites in, 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 ml of sodium chloride to 1000 ml of distilled water.
2. Stir for 30 minutes to dissolve the solids.
3. Add 0.06 ml of citric acid for a further 30 minutes.
4. Filter through paper and dispense in 75 ml bottles in tubes.
5. Autoclave at a pressure of 15 pounds per square inch (1.05 kg per cm²) for 20 minutes.

The pH of peptone water recommended by different workers varies from 7.8 to 7.6. It would be most desirable to adopt the first mentioned value (7.8), found satisfactory in recent studies on the biochemical properties of *Fus. 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. Boiling 3.5 g of each of the carbohydrates in 300 ml of distilled water.
2. Adding formaldehyde solution (4.4 ml 40% free formaldehyde solution) with the color change to pink to 300 ml of peptone water (Universal indicator added) and mix well.
3. Adding the thoroughly mixed solution to 500 ml of peptone water and mix well.
4. Autoclaving the mixture at a pressure of 15 pounds per square inch (1.05 kg per cm²) for 20 minutes.

The pH of peptone water recommended by different workers varies from 7.6 to 7.5. It would be most desirable to adopt the first mentioned value (7.6), found satisfactory in recent studies on the biochemical properties of *Fus. pestis*, as standard.

As shown by the tabulation:

- **Glycogen**: -
- **Glucone**: a
- **Glucon**: -
- **Lactose**: a
- **Lactate**: -
- **Malate**: -
- **Mannose**: -
- **Maltose**: a
- **Melitose**: a
- **Rhamnose**: a
- **Saccharose**: -
- **Sulfur**: -

Examination of tests: + = Acidification without gas formation
- = No acidification
a = Acidification present or absent (variable results)

As shown by this tabulation:

- **Glycogen** is not acidified by *Fus. pestis*
- **Glucone** is acidified by *Fus. pestis*
- **Glucon** is acidified by *Fus. pestis*
- **Lactose** is not acidified by *Fus. pestis*
- **Lactate** is not acidified by *Fus. pestis*
- **Malate** is not acidified by *Fus. pestis*
- **Mannose** is not acidified by *Fus. pestis*
- **Maltose** is acidified by *Fus. pestis*
- **Melitose** is acidified by *Fus. pestis*
- **Rhamnose** is not acidified by *Fus. pestis*
- **Saccharose** is not acidified by *Fus. pestis*
- **Sulfur** is not acidified by *Fus. pestis*
on the one hand and the medical in the other sense (Pest. pestis) and allied species on the other hand. Tests with the results of the tests recommended for this purpose are unsatisfactory in that, in contrast to the invariably negative reactions given by Pest. pestis, pseudotuberculosis bacilli cause variable reactions, while pseudotuberculosis in the strict sense sometimes fail to satisfy the requirements of media.

(2) A distinction between plague and pseudotuberculosis bacilli may be made with quite rare exceptions through tests with glucose-containing media and, as far as indicated by clinical experience, even more exactly with the aid of negligenm. Giysager, which has already been recommended for this purpose, gave in the experience of one worker inconsequent results with Pest. pestis, but confirmation of these discordant findings is still lacking.

(3) The reactions produced by Pest. pestis in glucose-containing media are variable, but since the differences met with in this respect are of regional origin of an individual character, tests with glucose are important for epidemiological studies.

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

- Carbon hydrates and fermentable, metabolites
- Sugaallur, Of limited value for the differentiation of plague bacilli from the pseudotuberculous bacilli.
- Starches, Epidemiological classification of strains.
- Glucose, Of these media, which are usually kept in laboratory tests, we wish to give a general distinction of the pulitar plus, including plague and pseudotuberculosis bacilli from strains belonging to the L. and group which in contrast to the mannitol, produce in those media plus as well as reduction.

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

Such fermentation sets are prepared by dropping small solid tubes (16 mm. 25 mm. with their open ends through larger tubes) (18 mm. 125 mm.) filled with 10 parts of the media in question. Different-colored solid media should be used to drop the larger tubes so as to facilitate the recognition of the various media and immediately after saturation to be done preferably in a sterile direction, for the fluid completely fills the smaller tubes. If no form or reaction and fermentation of the media, it means that the test has not in the upper part of the small inserted tubes.

For the actual performance of the test, sets of the above-mentioned carbohydrate media are associated with uniform 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 of what temperature the inoculated carbohydrate media should be incubated. Several workers, who recently studied the biochemical reactions produced by Pest. pestis, recommended an incubation at 37°C in view of the fact that inoculums 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 an lower standard incubation temperature. Nevertheless, some workers insisted that a lower incubation temperature be adopted, because the optimum temperature for the growth of Pest. pestis was about 27-28°C. Since, however, the few-mentioned observers obtained consistently good results in tests with carbohydrate media kept in the usual incubation, it seems legitimate to overstep this objection and to recommend the usually applicable incubation temperature of 37°C as standard for these tests.

Some discussion also exists regarding the length of time during which the carbohydrate cultures under test should be observed. It is understandable that positive results (i.e., modification, in the case of pseudomembrane bacilli as a rule manifest or at least evident within a few days. Nevertheless, in order not to overlook late solidifications, it has been recommended by most workers that the growth be observed over longer periods, ranging from one week to three weeks. An observation period of two weeks might be adopted as standard.

Cautiously the test, it is essential to keep the growths pastured by shaking the tubes after their daily inspection, and (6) in order to ascertain that the procedure has kept the organism alive, to examine subcultures on blood agar plates only 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 pseudotuberculous bacilli and not to that of contaminating organisms.

Resistance produced in agar-containing media

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

- Dextrose, 20 ml.
- Monopotassium phosphate (K₂HPO₄) 1.0 g.
- Sodium chloride (NaCl) 3.0 g.
- Water, 100 ml.

Dissolve the solids in the distilled water with a little heat and possible and sterilize by boiling through bacterial and fungi filters. Dispense under aseptic conditions in 3 ml. amounts into sterile tubes, which must be labeled overnight to ensure their identity.
Tests with deoxycholate citrate agar

As recently established, plague bacilli, if cultivated on deoxycholate citrate agar for 48 hours at 37°C, grow rather scantily in the form of reddish, pinpoint colonies, the medium retaining its original pinkish color, whereas during the same period pseudomonads produce abundant growth in large opaque colonies, which, like the medium in general, show a yellow color.

The medium used for this new differential test consists of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>1.24 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>1 g</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>1 g</td>
</tr>
<tr>
<td>Thioglycollate</td>
<td>2.2 g</td>
</tr>
<tr>
<td>Sodium thiosulfate</td>
<td>1.8 g</td>
</tr>
<tr>
<td>1% potassium solution of neutral red</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Dissolve the peptone in the water and add sufficient sodium hydroxide to bring the pH to 7.4 to 7.5. Boil for a few minutes and filter through paper. Let the agar solid in the peptone water for at least 15 minutes, then add it in boiling or in the cold. Add about 6 ml of sodium citrate and then the deoxycholate potassium oxalate as rapidly as possible. When the ingredients are dissolved, bring to a pH of 7.2 to 7.3 and add the neutral red solution, 9 ml of potassium oxalate and enough for 11 ml of the neutral red solution.

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:

<table>
<thead>
<tr>
<th>Test</th>
<th>Paste. prot.</th>
<th>Paste pseudomonas</th>
<th>Methylene-blue reduction</th>
<th>Methyl red</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen sulfide</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methylen-blue reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*As tested confirmed by a study of 186 strains.

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 x 30 mm filter papers soaked in a saturated solution of lead acetate as follows:

1. Dip the soaked paper strips in a Petri dish placed in the hot air oven for 1 hour.
2. Immerse a peptone water tube with the paper 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 the color of the strip during incubation.

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

1. Record 1% peptone water containing 0.5% sodium thiosulfate is added to about 40 ml perimeters into small test tubes plugged with white absorbent cotton wool.
2. After inoculation with the organs, more liquid is used and the plug is removed with 1 or 2 drops of a 5% lead acetate solution.
3. The tube is then kept overnight in the incubator at 37°C. If hydrogen sulfide is produced, the endumisolate of the plug will be found brown or blackened, while its negative shows no such discoloration will be formed.

Now, as stated above, further histochemical tests may be made with the same cultures.

Indole production: A simple test may be performed by:

1. Setting up the reaction in all projections of the plug of the cultures used for demonstration hydrogen sulfide production (as above) and putting on these sterilized cotton plugs
2. Overlaid the plate so that in the sterilized surface faces the cultures and incubating the forms for a further 24 hours.

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

Note: Phank’s indole reagent is prepared for these tests by dissolving 1 ml of 0.6% diaminobenzenesulfonylhydrazide in 55 ml of absolute ethanol and adding 2 ml of concentrated hydrochloric acid, and mixing the whole with equal amounts of 8% sodium hydroxide solution. It is then kept for further 8 hours. When this solution is used a pink color is produced.
An alternative simple method, depending on the solubility of indigo at 37°C, consists of hanging strips of filter paper, which are sterilized with a saturated solution of sodium acetate 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 indigo 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:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium metabisulphite</td>
<td>5 g</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>72 ml</td>
</tr>
<tr>
<td>Concentrated hydrochloric acid</td>
<td>25 ml</td>
</tr>
</tbody>
</table>

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 swirled to and fro a few times but not shaken vigorously. The mixture was then left to stand for about 5 minutes. A positive result was indicated by the appearance of a pink colour within 6 minutes. In other cases a supernatant layer, consisting of a red-violet colour, was formed within a few minutes. A modified modification is (i) to add 1 ml of ether to the cultures and to shake the tubes; (ii) to allow the ether to separate 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; and (iv) to allow the supernatant fluid to separate into the ether layer, and read and report the results accordingly.

Methylene-blue reduction tests are carried out with 24 hours' old cultures of *Pasteurella pestis* which have been incubated at 37°C for 24 hours. A positive result is indicated by complete decolourization, while a green colouration indicates a weakly positive and absence of decolourization a negative reaction.

Methylated tests are performed with cultures grown for 2-3 days at 37°C in a glucose-phosphate medium (peptone, 0.5 g; K2HPO4, 0.5 g; glucose, 0.5 g; water, 100 ml; pH 7.2). Tests are made by adding 5 drops of 0.92% solution of methyl red in 95% ethyl alcohol and observing the appearance of a red colour indicating a positive and reaction. Tests for reduction of nitrites and production of nitric oxide

Tests to demonstrate the production of nitrites or nitric oxide in the presence of substrates are of great clinical significance, since the presence of nitrites may be indicative of the presence of certain strains of the organism which have been found to react positively or negatively. They are of interest in that the variability shown in such tests by *Pasteurella pestis* strain has been found to be not of an individual character. It has been stated in this connection that: (a) combining these tests with tests utilizing glucose-containing media, the plague strain may be placed into three groups, namely:

1. A "negative" strain, showing no reaction with either medium, and producing nitric acid in nitric oxide medium;
2. A "positive" strain, giving a positive reaction in all media and producing nitric oxide in nitric oxide medium;
3. A "variable" strain, giving a positive reaction in one medium and a negative reaction in the other.

To show the reduction of nitrites to nitric oxide by *Pasteurella pestis*, workers in the Hilton Institute, Bombay, have recommended a simple test, for which the cultures were mixed overnight in a test tube containing 1% of nitrate and 0.1% of glucose. After incubation at 37°C for 24 hours, the contents of the test tube were examined for the presence of nitrite reductase and the formation of nitric oxide, which is indicated by the appearance of a pink colour.

In general, for a demonstration of nitric oxide reduction, the use is advanced of methylene blue or peptone water medium at pH 7.2-7.5, in which 1 g of potassium nitrate per 100 ml or even per 100 ml has been added and which after sterilization have been filled in 10 ml amounts into tubes. The reagents most consist of:

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td>Sodium nitrite</td>
<td>4 g</td>
<td>Methylene blue</td>
</tr>
<tr>
<td>2 N HCl</td>
<td>500 ml</td>
<td>6 N HCl</td>
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One part of concentrated nitric acid added with 3.5 parts of distilled water.

To carry out the tests, 0.2 ml amounts of solution A are added to the cultures after an incubation for 3 hours and then solution B is added drop by drop. Addition of 0.2-0.2 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 nitric oxide by the organisms under test, provided that they are free from nitrites. For practical purposes the absence of nitrites from the medium may be ascertained by testing samples before inoculation with the above reagents. No red colour should appear even after a period of time subsequent to nitrite addition.

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

In addition to the above-mentioned methods for the differentiation of plague and pseudotuberculous bacilli among which, according to the present state of knowledge, tests with thymine, urea-containing media, and with desoxyribonuclease in agar are the most important and in the procedures recommended in the sections above, some stress has been laid upon the use of mortality tests for a distinction between the invariably leptomeningeal Paste. pestis and the typically mobile Paste. pseudotuberculosis.

However, important as this distinction appears at first glance, the usefulness of mortality tests in practical laboratory work is limited for various reasons. Mortality is shown by the pseudotuberculosis bacilli only so long as they are cultured at comparatively low temperatures, in general, below 20°-22° C. For more important still, there can be no doubt that pseudotuberculosis strains can which, temporarily at least, show no mortality. 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 mortality of the organism. 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 inert or mobile.

For specific distinction between active bacterial infection and true immunity, it has been recommended that the subcutaneous and the lung-needle preparations be replaced by a per cent. intracutaneous injection which, while not interfering with the Brownian movement, abolishes the active mortality of the organism.

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

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

With the aid of a 1 mm deep suspension of the organism under test are made in each containing 0.5 ml of normal saline. Blood agar plates are then inoculated with 1 mm loopsful of the suspension, and plates are placed on the blood agar plate, but not on plate agar, whereas pseudotuberculous bacilli develop equally well on both media.

Plague Identification Tests

As shown by recent studies, a close-cut distinction may be made between plague and pseudotuberculous bacilli by properly conducted tests with plaque 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 10°-20° C.
2. The growth is then spreaded on dry agar plates, each growth being spread over a circle 8 cm in diameter, so that one can accommodate 10 different plates.
3. After three hours, the plates are inoculated with a suspension of bacteriophages which has been adapted to an invariant plaque 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 plaque-phage exists only solely on Paste. pestis strains, and not on pseudotuberculosis bacilli. Tests made at 37° C or on the other hand, have given unreliable results.

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

Method B
1. 0.5 ml of broth in a 200 cm roller tube are seeded with an 8 hour broth culture of one coliform plaque strain and this culture is incubated with shaking for 6 hours at 39° C.
2. 0.5 ml of a plaque-phage filtrate with a titre of 10° determined according to the technique described above, is then added to the culture and this is allowed to stand at room temperature for 1 hour.
3. The organisms are then removed by Szent filtration and a determination is made of the plaque titre of the filtrate (which is usually about 10°). Samples of sterile half 0.5 cm x 2 cm, properly sterilized in the meantime, are then plated at 0.5 cm squares of the filtrate (reduced to only 3 plates) and allowed to stand for 4 hours at room temperature for 2 hours.
4. The plates are then incubated under aerobic conditions and other sheltered and incubated or dehydrated in vacuo.
5. After they have become dry, the plates are sealed under vacuum and stored at room temperature.
6. To cut out the tests, 8 hour broth cultures of the organism under test are inoculated over the dry surfaces of blood agar plates and, while the surface is still wet, bacteriophage-stained strips are applied with sterile forceps to the cultures and then incised into place, represented streaked across the agar strips serving as controls.
7. The plates are then incubated at 39° C.

If plaque colonies are dealt with in this manner, 1 cm wide zones of lytic activity surrounding the bacteriophage-stained strips, usually become visible at 18-24 hours and the lysis extends into the surrounding parts of the colonies on prolonged incubation. Control tests with pseudotuberculous
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 tests of Pasteurella growth 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 excellent second method, are of great differential diagnostic value. In fact, some experts are inclined to assign a much greater value to them than to the bacteriologic tests outlined earlier, which they consider rather outdated. It should be noted in this connection that recent plague bacillus bacteriophages are maintained in some of the leading laboratories, such as the George Williams Hopkins Foundation, San Francisco, the Harvard Institute, Brussels; the Institut Pasteur, Paris; and the San Francisco Field Station of the Communicable Disease Center, US Dep of Health, Welfare, and Education.

Serological Tests

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) giving the antitoxin in two doses of 5 ml. at 2-5°C for 48 hours, (ii) performing a test for agglutination of bacilli by giving a single 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 dose of 5 ml. is reached. Then further doses of plague bacilli are administered by the intravenous route, until, as shown by the examination of samples obtained from the superior vein of the animals, a satisfactory agglutination titre has been reached.

An alternative method is to grow plague bacilli for 3 days at 25°C on a suitable high-quality agar in Reuss broth which have been seeded with 2-3 ml. of an 18-24-hours old broth culture of Pasteurella pestis. The growth is then harvested with the aid of 20 ml of normal (0.25%) saline, and 10 ml of a 1.5% formal 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, sufficient use has been made of the formaldehyde-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 intramuscular injection of these formaldehyde-killed antigens. The doses used varied from 1000 to 5000 million organisms administered at weekly intervals or on alternate days for two or three weeks. Even at the starting titre remained almost invariably the same that it was found to be necessary to administer immunization with two additional or in vitro plague strains in order to attain a titre of 2000 million formal-killed organisms. It was essential to use batches of several rabbits for serum production because considerable individual variations in the agglutinin response of different animals were found to exist.

Immunization of rabbits with two avirulent plague bacilli, preferably strains which have lost their virulence spontaneously, but which have been passed on for many generations after they have produced a basic immunity in their animals with the aid of killed antigens. The technique adopted is similar to the Institut Pasteur, Paris, which, since it has been followed without considerable modification by other workers, might serve as standard: (i) to grow the avirulent E. coli strain (isolated in Madagascar) for 48 hours at a temperature of 29-35°C; (ii) to prepare from this growth suspensions in normal saline containing 100 million organisms per ml. (iii) to inject rabbits intravenously at intervals of three days with six doses of such suspensions, amounting to 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 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 antigens. 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 advantage 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, it is necessary to subject them to the following tests before their usefulness as a diagnostic tool is established.

Tests of tests

For diagnostic purposes rapid slide tests may be performed by placing 5 ml. drops of the bacterial suspensions to be tested, which should have a titre of 2000 million organisms per ml., on a slide and admixing
The method is also of outstanding importance in making a certain diagnosis in the case of individuals who for the first time have recovered from disease clinically resembling plague.

In order to perform such tests, 30-65 ml. of blood are obtained by means of a suitable vein and the serum, separated off preferably by centrifugation, is tested according to either the rapid slide method or the claret tube method.

Rapid slide tests with the serum of patients or convalescents are performed in a manner analogous to that described above with live or formalized Pasteur cultures which have been grown for 1-2 days at 37°C. In view of the low agglutination titers to be expected, it is sufficient to use serum dilutions of up to 1:20; in fact, the proponents of the method have advocated using serum dilutions of 1:3 or 1:4. Controls in normal serum are indispensable to guard against spontaneous agglutination of the test organisms. This has also been advocated that their agglutinability should be demonstrated by including controls made with suitable dilutions of a known plague-strain serum.

The agglutination test may also be carried out with live or formalized suspensions of plague bacilli grown at 37°C. In a series of recent tests, particularly good results were obtained with formalized suspensions of viable plague bacilli grown for two days at 37°C with the following procedure:

1. 10 ml of normal saline were added to centrifuged tubes to 5 ml of formalized suspensions of Pasteur culture prepared according to the method described above (see page 475) and the tubes were subjected to centrifugation at 3,000 revolutions per minute for 20 minutes.

2. The mixture was refrigerated in 5 ml. of normal saline and centrifuged at 3000 g (5000 rpm) for 30 minutes to remove any gross Turbidity.

3. The supernatant was added to normal saline in a 50 ml. flask and stored under refrigeration with 10 ml. of paraffin oil to a total of 2,000 milliliters per ml. for use in agglutination tests with unknown sera.

4. The test mixture was subjected to centrifugation tubes 0.5 ml. of the mixture with equal amounts of serum dilutions ranging from 1:10 to 1:1,000. Results were taken after an interval of 30 minutes. The tests were then 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 serum 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 titers 1:600 and 1:1,000 were obtained in tube tests with convalescent
was examined on the 20th and 44th day, respectively, after the individuals concerned had fallen ill with bubonic plague. There was no doubt, therefore, that such tests are valuable for a retrospective diagnosis of the disease.

Precipitin tests:

A recently devised modified precipitin test with soluble antigen-antibody from the livers of animals which had succumbed to plague was found to be valuable in laboratory trials, even in instances where the material had been stored at 37°C for periods of three months. This method, therefore, deserves 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 mummified carcases, preferably the heart, spleen, lymph nodes, and lungs, if necessary immersed with neutral saline solution, are ground in a mortar with sterile sand and suspensions are prepared by the addition of 0.15 volume of normal saline.

2. After the suspensions have been transferred to test-tubes of Erlenmeyer flask filled with col-stopper, approximately 2 volumes of distilled water are added and the contents are gently but thoroughly agitated.

3. After the mixture has been allowed to stand at room temperature for 5-30 hours or 0.5 ml of the clear supernatant fluid is removed and controlled at 3700-4000 r.p.m. for 30 minutes.

4. Precipitin tests can conveniently be made by drawing equal volumes (0.25-0.3 ml of the clear fluid and serial dilutions of a placebo-serum known to be negative) into test-bottles having an inner diameter of 1.5-2 cm. As a rule, at least one dilution of antigen-serum must be included.

5. After the tubes are incubated for 2 hours at room temperature and then cooled, the tubes are read after 48 hours at 37°C and at 0°C.

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 antigen of Paste. pestis, but also with those of pseudomonas, vibrio and even of Paste. mallei. However, specific reactions were obtained when plague serum with the heterologous antigen were used.

Haemagglutination tests:

Haemagglutination tests with protein fractions of Paste. pestis, because they are strictly specific for this organism in the evolution of even Paste. pseudobacilleriodes, and because they are highly sensitive, do double form one of the most valuable methods for plague laboratory diagnosis, provided that the difficulty of preparing or preserving the antigen necessary for this work can be overcome.
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 (Mus) rattus in South Africa or the Bandicoot rata (formerly known as Gymnura rata) in India.

In place of these or other wild rodents species large-scale use has occasionally been made of exsanguination rats (particularly Rattus rattus) for plague-diagnostic work. However, it is rather difficult to ascertain in areas where plague is endemic or not to occur that the animals trapped for this purpose are free from the infection or are not carriers of the infection. To avoid this, it has been necessary to house the animals in their own environment before they are transported to the laboratories or under natural conditions in which they are to be employed. This method, though expensive, has been found to be the most economical and practical method of breeding these rats in captivity. It is possible to control their breeding and rearing, and to maintain them in good health.

In the opinion of many workers, preference has been given to the domestic species, especially in experimental work, as they are easier to handle and are less subject to contamination from other sources.

The use of white rats in plague laboratory work is of considerable importance in that, in contrast to other available species, these animals are practically unsuitable for infection with Pasteurella pestis.

It would be quite legitimate to utilize white rats not only for the purpose of differential Pasteur and pseudotuberculosis blood cultures, but also for plague diagnostic work as well. However, the animals should be carefully selected, and should be free from any infection that may affect the results of the test. They should be kept in a clean, well-ventilated room, and should be fed a diet that is rich in vitamins and minerals.

Methods of inoculation

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 consistently with the small and rather fragile white rats and the white rabbit. However, although the method of cutaneous infection is of outstanding value because it is the only method of which it is possible to obtain positive results in white rats, the method of cutaneous infection is not always available, and it is not always possible to obtain positive results in white rats. Therefore, it is essential to employ other methods of inoculation, such as the subcutaneous route, which is the most reliable and least hazardous of the available methods.
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 decreases the chances of isolating pure cultures, particularly in a terminal stage, where decomposition of normally surviving animals is apt to be rapid.

In order to produce green buboes which are most easily manipulated, most workers are in favor of using either the inside of the thigh or the skin of the lower abdomen for anesthica inoculation. To carry out the test in a reliable manner, it is necessary to remove the fur of the animal at the chosen site over an area measuring 2.5 cm² or about one square inch. Various procedures are recommended for this purpose, some workers using a depilatory (e.g., barb Roose wax) which is afterwards washed off, others a shaving blade or a scalpel. In the case of long-haired rats, the hair may be simply plucked off. Some workers advocate lightly scratching the skin, but with a wooden stick before inoculation is made, but since plague bacilli are capable of entering even through the intact skin, the minor skin defects produced by shaving or plucking the hair fully permit an entry of the organisms.

Various methods have also been recommended for rubbing in the infective material, some workers utilizing the moist platinum or chromic steel wires used for cutting with for this purpose or depositing the material with the aid of a pipette or distributing it with the aid of the inoculating needle, or with other suitable instruments. Others preferring sterile, mounted, cotton plugs which have been prepared to suspensions of the test material. The organism of plague-infected animals to be tested may be applied directly with the aid of forceps or cotton pledgets. Applying suspensions of infected material, one might use the multiple-pronged method recommended for筧minal 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 quickly carried out in the laboratory, while the rats are kept individually in and after infection, by temporary reflex or drainage from the site of the rats by a wooden cooper with a hole in the same through which the tail of the animal is drawn out with the aid of a long screen or clamp for making the incision.

2. The method produces positive results when properly done and not with good subcutaneous tissue, but such results are often obtained with contaminated materials and when dealing with some resistant organisms which produce fatal infections when administered subcutaneously.

Subcutaneous inoculation

The method of subcutaneous inoculation of experimental animals with plague-suspect materials is the most universally applicable of the procedures previously, 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 at all organs of plague-suspect rodents or of pooled fluids, as well as of the fluid suspensions plague material. Further, direct use may be made of fluid cultures of the blood of plague-suspect patients or human subjects and of the inoculum of persons suspected of suffering from pneumonic plague. However, in view of their great sensitivity to injection with pathogenic, white mice should not be used for tests with sputum.

In the ease of pneumonic infection, most workers recommend either the lower part of the thigh or the lower part of the abdomen as the site for subcutaneous inoculation in guinea-pigs or rats and the lower part of the abdomen near the groin region in the ease of white mice. Not more than 0.2 ml or 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 guineapigs and white rats. The test materials must be prepared and the inoculations must be made under aseptic conditions.

Subcutaneously infected laboratory animals usually succumb to the 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.

Interperitoneal inoculation

The diagnostic importance of interperitoneal 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 24-48 hours after inoculation and is a rule not delayed beyond three days. Interperitoneal inoculation is therefore particularly indicated for the rapid establishment of the diagnosis in instances of previously unobserved recent plague—e.g., in the case of rats found dead or freshly killed on board or in ports—and in human patients, especially in the early stages of suspected pneumonic or septicemic plague, when any signs of a serious localized infection are lacking. 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 interperitoneally 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 does not plague-suspect materials, these rules must be followed:

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

2. The number of too large amount of virulent materials should be avoided, because otherwise the animals, particularly mice, may rarely recover to remain without showing macroscopic evidence of plague and yielding culturability as quite no growth of their points. This precaution is particularly necessary when intraperitoneal inoculation is used to confirm the plague nature of suspected cultures.

3. The inoculations must be carried out under aseptic conditions. It is best to have the site of the inoculation from hair in an area of about 1 cm and to distill the site with alcohol before the inoculation is made.

4. Rabbits must be taken not to purchase intraperitoneal organs. To avoid this, it is best to hold the animals before injection with their heads downward and to introduce a sterile syringe needle with the syringe held as much as possible parallel to the skin, in the opposite direction, i.e., upward. The lower half of the abdomen should be slowly pulled up and fixed 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 amount injected should not exceed 0.1 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, inoculation by other methods, e.g., by the conjunctival route, is 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 excretion, is fraught with greater risk than the procedures described above.

Methods of examination

All experimental animals remaining 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 repeat animal experimentation. While, as a rule, white mice may 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, through not fully pathognomonic, are usually quite characteristic of the presence of plague. The most important features

related with, in addition to subcutaneous ecchymosis, in guinea-pigs are:

(a) the presence of a marked distention, and often of ulceration at the site of injection, or a boil 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 petechiation or ecchymoses of the latter; (c) the presence of intense ecchymosis at the site of inoculation and, more frequently, of variable sized purpurae of the last and, in the case of white rats and more especially in guinea-pigs, of the eye; (d) the very rapid death of animals infected by this route, marked characteristic changes in the internal organs are likely to be absent.

Material for bacteriological examination should be taken under aseptic precautions from the site of injection or the nose, 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 lungs, moreover, which are indubitable when examining decomposed carcasses, are necessary in the case of experimentally infected animals, if it is sought to be the irrevocable rule, they are dissected within a few hours after death or are killed in the interval.

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 broth-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 streaked with a thin spatula and material taken with a platinum or experiment-white loop. To obtain fresh blood it is best to use a sterile glass spatula provided with a rubber bulb, which is plunged in, if necessary after the surface of the heart has been scored. Such small glass spatulas may also be used conveniently for the collection of peritoneal exudates.

After material for cultivation has been taken, smear or impression films are made from the same organs. For the sake of economy and speed one and the same slide may be used for several or all of the organs in question, but it is well to prepare two such sets so that in addition to communication with Wayson's or another suitable 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 liver, the spleen, and the lungs—on a sterile dish or tube so that, unless some 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 bacteriological findings.
logical findings. Direct passages by the intestinal route are apt to prove particularly useful in such cases.

To what extent confirmatory tests must be made with the culture 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 plague or bacillary tests is indispensable in handling recipient rodents 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 full laboratory results of microscopic and macroscopic examination and of cultural tests as conclusive. Seric 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

Contaminated (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 sets 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 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 obviously more rapid than the method of active trapping, especially when one uses the technique of reducing the food supply, which results in the mice being eaten by the rats. In addition to these, however, trapping, on the other hand, is apt to be used on the widest possible scale (1) in the localities round the epidemic focus, so as to delimit the latter and to detect whether or not the infection spreads; and (2) during the off-seasons as well in the plague-affected localities, because then the rodents are few and far between, but the presence of the infection may be found by an examination of trapped animals or their nests 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 agents of the plague staff so as to keep the public as much as possible away from the dead animals or their nests.

If such workers can be employed, they should make systematic rounds to detect and collect carcasses not only inside but also outside houses and compounds. Such round 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 large tongs and, after they have been tagged, should be placed in metal containers with well-fitting lids for transport to the laboratory.

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 house. During the outbreaks in China it was found suitable to establish collecting posts for the depositing of rat carcasses found by the public by affixing small containers with lids on the outside walls of houses and compounds, menaces poles or the like, or by placing large cisterns or earthenware jars with lidning flaps at strategic points in the thoroughfares. Staff members visited these pots at least twice daily in the morning.

In the opinion of most workers snap-type traps should be used in preference to drop traps to catch rats and mice for plague-diagnostic work, employing bait 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 other bags for transport to the laboratory so as to prevent escape of the animals. Peanut-traps are handled in this manner, satisfactory numbers of rats can almost invariably be recovered for examination.

Wild rodents

No generally valid indications can be given for the collection of plague-suspect wild rodents in view of the profound differences in the biology 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 different or distinct habits and species given to hibernation, seasonal changes, and the like.

Collections of the plague-suspect species 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 epidemics, if no carcasses are
found, satisfactory results may be obtained by the use of traps suited to the site of the problem involved. Shooting with rifles or shotguns by trained marksmen has been found frequently suitable in the USA. Good results have been obtained also by stringing 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 recommend this procedure, while others are against it. Since, in view of the unpredictable tendencies often shown by rodents kept in captivity, it is necessary to consider 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 with the pooled organs of such animals are bound to give positive results if the animals are inciting plague or are in the incubation stages of the disease.

Since, however, some workers have had favorable experiences with animals succumbing to manifested 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 three or four weeks unnecessarily. If not suffering within this time, the animals must be identified, 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 stone chisel, or by administering a blow to the head.
2. Injection of the animals.

Experimental data indicate that the above-named methods have been found satisfactory in the cases given. In any event, it is to be advised that the animals be killed as promptly as possible, and precautions taken to prevent escape or dissemination by fleas. The animal remains should be incinerated immediately after killing. In addition to the control of fleas, the locales should be thoroughly fumigated, especially in case of heavy infestation, and all materials from which fleas might have multiplied should be burned. If a strong odor is present in the vicinity, the fumigation should be continued for a period of 2 or 3 days. All exposed human-handled objects should be washed with hot water and soap. The above procedures should be followed in all cases of plague outbreak in any locality.
containing a layer of about 20 gm 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 detection are available, the usual procedure is to forward to the laboratory the lymph nodes or pieces of the intestinal organs, or both, of the dissected animals (especially the spleen and liver) in a preserving fluid. For this purpose Bouquet's fluid is often recommended.

According to a recently issued United States Bureau of Animal Industry Order for the transport of animals no liquid should be fed to animals subject to export until the time of their examination in the United States or Canada. To prevent obstrucion of the pipes it is found desirable to feed them with 1,004 lb of formalin and 0.025% of formalin solution at 100°C for 30 minutes, in two bottles, and then held by dry heat and afterwards cooled with sterile solution.

It is found that pieces of plague tissue preserved in Bouquet's fluid contained no viable yeast (see page 460). A known period of at least two weeks, provided that the yeast is kept at room temperature or in a refrigerator. Positive experimental results were also obtained with pieces kept in the fluid for periods up to three days.

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

According to the findings made in this respect in Madagascar, normal saline suspension of materials from the liver and lungs of experimentally infected animals, obtained by puncture, 3-10 hours after death, remained viable for periods up to six days at a temperature of 10°C, but were no longer virulent after having been kept for three days at 15°C. Suspensions made from carcasses which had been kept for 48 hours at 25°C remained virulent for 24 hours only, though the organs of the infected 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 spleen and liver, or both, of the carcasses in saline solution or in Bouquet's fluid; or, as has been recommended by some workers, pools may be prepared instead from the bone marrow of the carcasses, collected from the mammalian bones of the animals with the aid of a syringe provided with a catheter thin and short bevelled needle. Whether organs are chosen, it is essential to use exclusively materials collected from records belonging to the same animal or from the same species for the preparation of individual pools, because otherwise, in view of the great sensitivity of the animal tests performed with such collected materials, organs or species which are not actually suffering from plague might be inoculated.

Methods of Examination

Two fundamentally different methods are used for examination of the organs of plague-suspect rodents: one is simple, and the other more complex. The first method is used for the examination of swabbed sections, while the second is used for the examination of swabbed organs, usually for subcutaneous implantation of the organs.

The methods suitable for individual examination of the foci of the organ cultures have already been described (see page 466). It is necessary to add that when dealing with multiple specimens or with carcasses which have undergone partial destruction, satisfactory results may be obtained by opening the tissues 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 preserve material from this material for animal inoculation, but some advocate the utilization of more concentrated sodium chloride solutions, as much as 10% or 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 is to recommend by other workers consists of (i) using the pieces of organs into a thick-walled tube in 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 test pools, under the method of individually examining the foci of cultures seems satisfactory. 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, type of work in which the method of individual examination is not to give disappointing results. The pooling methods are likewise invaluable for ascertaining whether the infection persists in areas of 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 a few, or possibly even single, virulent
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 rodents 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 of their place of capture is as follows:

1. As noted already, the rodents captured dead or killed at the spot must be taken to the laboratory in tightly closed tin or boxes, or in well-sealed paper or cloth bags.

2. To kill the ectoparasites in the laboratory, a 12-gallon (45-litre) jar with paper or woven bark and a tightly fitting lid has been placed on three or four short 6-inch (15 cm) high wooden blocks.

3. After about 30 to 45 minutes the ectoparasites have been placed under the jar with the bags of the animals. The jars are then closed and left for four hours. After this time the contents of the jars may be removed, and the fleas and other parasites are killed by exposure to heat as described above.

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 soft brush or a scroop, or simply to strike the carcasses sharply with a thin-ended file or another suitable instrument. However, many workers prefer to fill the white enamel basin used for ectoparasite collection with water and to immerse the carcasses before they are depilated, 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 tongs, preferably with curled ends, and put into empty vials, or, better still, vials filled with formaldehyde, or if the ectoparasites cannot be picked up afterwards, into vials filled with 2% acetic acid 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 field conditions:

The three principal methods for collecting live fleas in infested premises are: (1) exposure of guinea-pig plaques 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 at most 6 inches (15 cm) above the floors in the premises in question; (2) the use of tanglefoot paper or, preferably, of more elaborate artificial tangle-foot traps; and (3) the collection of fleas from those sweeping, using a small dustpan and brush and a fine-sieve.

Details of these three methods are as follows:

1. The tangle-foot paper, which is inspired by the method used for catching sparrows, is left in the premises for a number of days. It is placed on the floors, in corners, and in other places where fleas are likely to be found. The paper is treated with a mixture of 2% formalin and 2% benzene, which is applied with a brush or a sponge. After the paper has been left for two or three days, the fleas are collected in the manner described above.

2. A definite time of exposure should be fixed, and the paper should be removed when the time is up. The fleas are collected and preserved in the same manner as before.

3. The tangle-foot paper should be left in the premises for a number of days. It is placed on the floors, in corners, and in other places where fleas are likely to be found. The paper is treated with a mixture of 2% formalin and 2% benzene, which is applied with a brush or a sponge. After the paper has been left for two or three days, the fleas are collected and preserved in the same manner as before.
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 corridor is scraped off at a time. These materials as well as the nests eventually reached are placed in the proof bags or other containers for transport to the laboratory, assisted facilities for flue collection are available on the spot. Working in manically plague-infected localities it is most desirable to treat the burrows with cyanide or chloropicrin before digging operations are started. Otherwise, the materials removed from the burrows must be treated with cyanide, cyanide or chloropicrin.

In order to collect the ectoparasites from these materials large improved 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 size (e.g., of ½ inch [0.37 mm] gauge and of a gauge corresponding to that of an average pine cone). Before the materials to be examined are sieved, the apparatus is placed in a large white enamel pan which may be used during the experimental and collection of the ectoparasites.

Another suitable apparatus for collecting fleas from grass has been described by Retallack. To collect fleas from the little found in the nests of rodents, workers in the U.S. recently took advantage of Retallack's funnel described by Baker & Whorton. 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 mound 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 white material fastened to a stick over the ground near the burrow entrances and by placing the cloth into the burrows to a depth of approximately 3 feet or 1 meter 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 mouths of the burrows.

Fleas from Plague Vector Species

Cranston, in his study of the flea, Plagiodromus hirsutus, described its habits and biology in the context of plague epidemiology. Fleas are known to be vectors for the plague bacillus, Yersinia pestis, and their control is crucial in plague control programs.

Methods of Examination

Since bacteriological examination of plague-positive fleas often gives disappointing results, inoculation of test animals with blood 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 blood, the collected fleas must be examined with the aid of a dissection microscope under low power of an ordinary microscope in order to determine the species to which they belong. Suitable instruments and sterile 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 reserve the maximum number of fleas to be used per pool to 30, but to reduce this number whenever possible to 25-20 when great bite is used in test animals and do not more than 20 when mice experiments are performed.
4. Before using the blood for the preparation of the test suspensions, they must be thoroughly washed preferably several times in sterile animal saline.
5. Suitable mouths and points or glass rods may be used to stimulate the tail for a few drops of sterile normal saline have been added. Another convenient method is to put the flea onto a larger, moderately-stretched tissue and to immobilize it for a few seconds. After a few seconds saline normal saline have been added, a long narrow swab which has previously been moistened inside the larger sucking for sucking the flea. Some sterile mouth or glass powder or a small amount of serum serum may be added to facilitate immobility.
6. After immobility has been established, sufficient sterile normal saline is added to prepare suspensions or extracts. The suspensions are then transferred to small tubes and incubated for a short time until the blood particles have become deposited.
7. Either saline suspensions or saline extracts may be used for subsequent inoculation with the suspensions. Each with white mice are preferable as this facilitates the identification of two test animals for each pool—a procedure which is desirable to guard against infection death of the inoculation animal.

Comparative Value of Flea-feeding and Tissue-feeding Tests

In the opinion of some workers, tissue-feeding tests, since they form an exact means of ascertaining whether plague is present in the rodent hosts concerned, can be used exclusively in preference to the less expedient and comparatively more dangerous method of testing the fleas of the
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 presence of bacteria at the site of the bubonic bubo, laboratory examination can easily be obtained by collection of pus filling the thin-walled vesicle, which often breaks spontaneously. The diagnostic value of cases made with this material is considerable, because in positive cases it is invariably possible to arrive 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 pus content. It is likewise easy to confirm the validity of these findings by examining feces and finding the ages or blood-stage cultures, which must be made with these materials. If a rapid confirmation of the diagnosis is essential, the material obtained at bubo puncture may be used for the intraperitoneal inoculation of test animals, particularly of guinea pigs.

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 the infection which had hitherto been localized in the bubo. Though such misgivings are unfounded, if bubo punctures are made cautiously in the manner described and without any unnecessary manipulation of the bubo, the method of examination is neither unduly liable to cause of generalization of the disease or to involve danger to the operator. However, during a previously confirmed outbreak, it is as a rule not at all difficult to decide by clinical examination whether specific plague infection should be suspected or not. If bubo puncture is used for this purpose, the material should be obtained by blood culture, which must be made before specific treatment is started.
Blood Examination

In order to appreciate the value of bacteriological blood examination in bacillary plague, it must be kept in mind that, though passing invasion 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 secondary bacteremia becomes established, while in slight and early cases the infection becomes localized in the buboes. It follows, therefore, that a bile bacteriological examination of the blood is of diagnostic value only in the case of patients who are severely affected and tested late in the disease. In such patients bacteremia may become so pronounced that plague bacilli may be found easily in serum made from the finger blood. However, except in the case of very much involved patients it is necessary to obtain blood for laboratory examination by puncture of a vein. An amount of 10-12 ml of blood should be withdrawn under strictly aseptic conditions and should be used for inoculation of: (1) two agar slopes with 0.5-2 ml quantities of the blood; and (2) one or preferably two flasks, each containing 50 ml or, better yet, 90 ml of a suitable brain medium or of peptic digest 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 examinations on age of or, preferably, one of the growths appearing on these plates and on the primarily inoculated flasks should 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 growths on the plates are fully typical.

In isolated cases from laboratories it may be impossible to utilize the methods described above for streaking in broth or peptic digest flasks. However, there ought to be no difficulty under any circumstances in inoculating agar slopes at the bedside of the patient with 0.5-2 ml quantities of blood withdrawn from a vein. Such cultures will prove valuable for examination even if they do not reach the laboratories for some days. Further, autolysed solutions of 10% sodium thiosulphate in normal saline, filled in 5 ml quantities into previously sterilised screw-toped jugs 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 culture inoculation.

Podridonic Plague

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

Comparatively the most rapid means of confirming the diagnosis of septicemic plague is intraperitoneal inoculation of test animals, preferably of young white mice, with blood withdrawn from a vein of the patient. At the same time agars slopes and flasks with both peptic digest water ought to be inoculated with acute quantities of the blood as recommended in the case of bacillary plague.

Since it would be desirable to wait for the results even of direct animal inoculation tests before instituting specific treatment with antibiotics, a decision whether to start this therapy has to be reached on clinical grounds in 24 hours after the patients are seen. In view of the non-specific symptoms, however, slight signs of primary septicemic plague this decision is rather difficult, especially in early or sporadic cases. However, the rapid determination of the condition of the patient, since it is rarely seen in diseases other than septicemic plague, ought to attract attention.
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 conspicuous, if visible at all. To make matters worse, some of the heterogeneous organisms, including gram-negative species, may more or less resemble \textit{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 \textit{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.
Diphtheria

The method of diphtheria, practised in some South American plague stations, 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 posterior phalanges with the aid of a small bone-saw or knife to obtain bone marrow for examination. The wound thus created may be covered with a cotton pad which has been dipped into formal 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 deep down in a jar containing a layer of absorbent cotton.

According to the experiences made in South America, a mere 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 diphtheria in countries the inhabited of which abound in non-dead bodies, it might be feasible to use these circumstances to obtain bone marrow for post-mortem examinations from the normal bone according to the method used in clinical work. It should be noted that this method has given promising results in plague patients.

Autopsy

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 and permanent collection of materials for laboratory examination, but also because, saving the usual presence of marked and rather characteristic gross signs, it is the only sure way to arrive at a specific 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 many popular objections to this method which it would be most unwise to override. Moreover, the performance of complete autopsies on the highly infectious dead bodies of plague victims is fraught with such great risks that this method should be used only by trained pathologists or others 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.
PRECAUTIONS TO BE ADOPTED

Pneumonic plague

It is essential to keep all members of the staff protected against infection by pneumonic plague cases. Both hands and face should be washed thoroughly before handling any infected material. Proper handwashing is also important.

Preventive treatment of laboratory personnel

It is important to ensure that all laboratory personnel are protected against pneumonic plague. This can be achieved by providing them with appropriate protective equipment and training them in the proper handling of infected material. Regular health checks should also be conducted to ensure that all personnel are in good health.

Preventive measures for laboratory work

Laboratory work on pneumonic plague cases must be conducted in a well-ventilated area with proper safety measures in place. All infected material should be handled with care, and the area should be disinfected thoroughly after each use.

Preventive measures for handling infected material

When handling infected material, it is important to wear appropriate protective clothing, including gloves, masks, and gowns. The area should be thoroughly disinfected after each use, and all waste material should be disposed of properly.

Preventive measures for laboratory personnel

Laboratory personnel must be educated on the proper handling of infected material and the importance of maintaining good hygiene. They should also be provided with appropriate protective equipment and regular health checks.
antibiotics are looking—daily administration of 3 g of sulfadiazine or sulfathiazole for a period of 3 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 persons having plague or through laboratory accidents, but that the imaginative prolonged use of sulfadiazine by the staff members themselves is bound to lead to a sulfadiazine-resistant strain and sometimes an incurable kidney lesion.

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 rodents; (b) the possibility of directly contracting plague when performing post-mortem 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:

\section*{Respiratory Infections}

To protect themselves against the possibility of a fly-borne infection, e.g., when entering plague-infected homes, or when coming in contact with persons or dead bodies or with rodents possibly harboring infected flies, the members of the laboratory staff should preferably wear a special costume. This consists principally of a gown similar to that used by doctors, which, made of one piece, covers the whole body except the head and hands. This is draped over the opening in the neck and is also tied firmly round neck and wrists. The costume is completed by rubber or black leather boots and, where necessary, by rubber gloves. A mask must be added if there is a risk of contaminating 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 4.5 cm in diameter (10–15 cm) and should consist either of a single layer of the now available cotton-impregnated filter fabric or a single piece of gauze, or of a cotton pad, 1 in. (0.8 cm) thick between two layers of gauze. Pieces of tape must be affixed to the four corners. The upper part of these tapes is passed round the head above the ears and tied behind, while the lower two 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 advanced.

Since under tropical conditions it may be better trying to wear the masks 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 fly-borne infection is to impregnate the garments or clean stockings worn by them with a suitable insecticide, creating a residual action. However, the advice of specialists is indispensable for using this method in an efficient and safe manner.

Direct inoculation or pneumonic infection

No special precautions are needed when examining plague materials in the laboratory by bacteriological methods, or when examining infected tissues, provided that such work is performed with the cleanliness and care advisable for laboratory work in general.

When performing human autopsies, a proper costume must be worn, including 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 have the advantage of the aid of suitable long instruments, without touching the carcass. 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 splashing of infective material while changing the syringe.

SELECTED BIBLIOGRAPHY
