

Use of the Antiserum-Agar Plate Technique for Specific Identification and Isolation of *Pasteurella pestis*

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Pasteurella pestis colonies were specifically identified on antiserum-agar plates used for primary culture of tissues from experimentally infected guinea pigs. Both selective and nonselective antiserum-agar plates were used to identify *P. pestis* from guinea pigs kept at 22 C for periods up to 4 days after death from plague. Colonies identified as *P. pestis* on selective and nonselective antiserum-agar plates, by the appearance of precipitin rings following brief chloroform vapor treatment, remained viable and were subsequently purified on nonselective antiserum-agar plates. Isolates obtained in this manner were uniformly lethal when injected into mice and guinea pigs, and conformed to standard laboratory criteria for *P. pestis*. *P. pestis* was identified on selective antiserum-agar plates from the spleens of all guinea pigs killed by the isolates, and from a large majority of the mice. The practical value and confirmative nature of the method were demonstrated.

Recently, we reported a serological technique (1) for specifically identifying fraction I-positive *Pasteurella pestis* colonies on nutrient agar plates containing specific fraction I antibody. That report dealt with the production of specific anti-plague serum, a description of the new antiserum-agar plate method, and the establishment of the specificity of the method by use of stock laboratory bacterial strains.

The present study demonstrates that our antiserum-agar plate method can be conveniently and successfully applied for the specific identification and isolation of viable *P. pestis* from tissues of experimentally infected guinea pigs. This report describes (i) the use and comparison of nonselective and selective antiserum-agar plates for specifically identifying viable *P. pestis* from guinea pig tissues collected up to 4 days after the guinea pigs died of plague, (ii) the use of nonselective antiserum-agar plates for purification of bacterial isolates, and (iii) the testing of such isolates for bacterial properties characteristic of *P. pestis* to ascertain the validity of the initial identifications made on both nonselective and selective antiserum-agar plates used for primary culture of infected guinea pig tissues.

MATERIALS AND METHODS

Animal inoculation. To provide a source of contaminated tissues, each of six Hartley guinea pigs weighing 400 g was injected intradermally with 50,000

virulent *P. pestis* (strain Alexander) cells contained in 0.1 ml of 2.5% Heart Infusion Broth (Difco). Organisms for injection were grown on a Blood Agar Base slant (Difco) for 24 hr at 26 C. Tissue specimens obtained from this group of six guinea pigs were plated on selective and nonselective antiserum-agar plates to obtain colonies for identification and subsequent isolation and purification on nonselective antiserum-agar plates. Seventeen isolates obtained through use of antiserum-agar plates, as well as the original inoculating strain of *P. pestis*, were each tested for virulence in pairs of Swiss-Webster mice (18 to 20 g, injected intraperitoneally), and in pairs of Hartley guinea pigs (400 g, injected intradermally). Injected doses ranged from 250 to 750 organisms contained in 0.1 ml of sterile 2.5% Heart Infusion Broth.

Collection of tissue specimens from the original group of six guinea pigs. To obtain increasingly contaminated tissue specimens for testing the comparative efficiencies of the different types of antiserum-agar plates, specimens were taken from throats, buboes, spleens, lungs, and hearts of pairs of guinea pigs that had been dead for different lengths of time. Guinea pigs 1 and 2 were sacrificed when they were visibly ill on the sixth day following inoculation. Tissues from these guinea pigs were tested immediately. Guinea pigs 3 and 4 were held at 22 C for 24 hr after death from plague on the seventh day following inoculation. Guinea pigs 5 and 6 were held at 22 C for 4 days after death from plague on the eighth day following inoculation. Throat specimens were obtained by use of sterile cotton swabs moistened with sterile 2.5% Heart Infusion Broth. Bubo aspirates were obtained with 22-gauge needles and 2.5-ml syringes. Spleen, lung, and heart samples

were obtained by excising approximately 1 cc of tissue from the respective organs. All specimens were placed in 1-ml quantities of sterile 2.5% Heart Infusion Broth and were macerated with a sterile pipette prior to plating.

Preparation of antiserum-agar plates. Basically, antiserum-agar plates were prepared according to the method reported earlier (1); however, the Difco agar media were altered in most cases (plate types B, C, D, and E) to inhibit contaminants. Specific antiplague serum (1) was used at a final dilution of 1:128. In addition to antiplague serum diluted in 0.85% NaCl, the various types of antiserum-agar plates contained the following ingredients at the following final concentrations: type A, nonselective, 4% Blood Agar Base; type B, selective, 4% Blood Agar Base, 0.025% CuSO_4 , and 0.005% erythromycin lactobionate; type C, selective, 2.25% Desoxycholate Agar and 0.025% CuSO_4 ; type D, selective, 4.5% Desoxycholate Agar and 0.025% CuSO_4 ; and type E, selective, enriched azide agar (6) without added calcium and glucose. Antiserum-agar plates containing CuSO_4 were prepared by adding filtered CuSO_4 from 5% stock in distilled water, to the diluted antiserum immediately before mixing with nutrient agar.

*Identification and isolation of *P. pestis* on antiserum-agar plates.* Throat specimens from each of the six guinea pigs were applied to separate antiserum-agar plates of each type by streaking with a 1-mm wire loop and by spreading 0.1-ml samples with a glass spreading rod. Bubo, spleen, lung, and heart samples from each of the six guinea pigs were streaked with a 1-mm wire loop onto sections covering one-fourth of the agar surface of each type of antiserum-agar plate. Antiserum-agar plates of each type were incubated at 37 C for both 40 and 66 hr to determine the length of incubation required for each type of plate. Antiserum-agar plates of each type were exposed to chloroform vapor for different lengths of time to retain viability of some colonies, thus permitting further purification and study of the colonies, while at the same time assessing the ability of colonies appearing on different type plates to withstand chloroform vapor. Plates containing specimens from guinea pigs 1, 3, and 5 were treated with chloroform vapor for 30 sec. Plates containing specimens from guinea pigs 2, 4, and 6 were exposed to chloroform vapor for 1 min. Chloroform-treated plates were aired in an inverted-tilted position for 10 min, returned to 37 C, and observed 6 and 18 hr later for the presence of precipitin rings. Five *P. pestis* colonies (identified by precipitin rings) were picked from each of the five types of antiserum-agar plates containing bubo or spleen specimens obtained from the original group of six guinea pigs. Four of the five colonies from each type of antiserum-agar plate were streaked onto Blood Agar Base plates and incubated at 26 C for 48 hr to ascertain viability of the colonies exposed to chloroform vapor for 30 sec and 1 min. The fifth colony from each type of antiserum-agar plate inoculated with bubo or spleen specimens was streaked onto type A plates for further purification. Type A plates (used here for secondary isolation) were incubated for 40 hr at 37 C, exposed to chloroform vapor for 1 min, aired in an inverted-tilted position

for 10 min, and incubated at 37 C for 18 to 24 hr. From among *P. pestis* colonies (identified by precipitin rings) on type A secondary-isolation plates, one colony from each plate was transferred by a small wire loop to Blood Agar Base slants and incubated at 26 C for 18 to 24 hr to provide stock cultures of the isolates for subsequent characterization.

Testing of isolates to confirm the validity of initial identifications based on precipitin rings around colonies. Seventeen isolates were selected for testing from the new supply of isolates. These were subcultured on Blood Agar Base slants for 24 hr at 26 C. Also subcultured, for control purposes, were stock laboratory strains of *P. pestis* (Alexander and M23), *P. pseudotuberculosis* (PB1⁺), and *Escherichia coli* (type B). Bacterial growth from each isolate and control strain was suspended in sterile 2.5% Heart Infusion Broth and tested for bacterial properties characteristic of *P. pestis*. Fraction I production at 37 C was tested by our antiserum-agar plate method. Pesticin production at 37 C was tested by the method of Brubaker and Surgalla (3). Phage sensitivity at 22 C was determined with specific *P. pestis* bacteriophage provided by John D. Marshall, Jr., and by the basic agar plate method described by Baltazard et al. (2). Pigmentation at 26 C was determined on hemin-agar plates prepared by the method of Jackson and Burrows (5). Calcium dependence at 37 C was determined on magnesium oxalate-agar plates prepared by the method of Higuchi and Smith (4). Urease production at 26 C was tested with Urea Broth (Difco). Fermentation of glucose, lactose, melibiose, and rhamnose at 26 C was tested using 1% carbohydrate in 5 ml of 1.2% Purple Broth Base (pH 6.8, BBL). Fermentation tests were conducted in 16 × 125 mm screw-cap tubes with loosened caps. Each of the 17 isolates and the original parent strain (Alexander) of *P. pestis* were tested for lethality in pairs of mice and in pairs of guinea pigs following the animal-inoculation methods described earlier.

Mice and guinea pigs killed by the isolates were placed at 4 C within 24 hr after death and tested within an additional 72 hr by streaking spleen specimens (suspended in 2.5% Heart Infusion Broth) on type D antiserum-agar plates. Antiserum-agar plates were incubated at 37 C for 66 hr, exposed to chloroform for 1 min, aired for 10 min, and reincubated at 37 C. Plates were examined after 6 and 24 hr for the presence of precipitin rings characteristic of fraction I-positive *P. pestis*.

RESULTS

With all types of antiserum-agar plates used, *P. pestis* colonies were specifically identified from one or more tissue specimens taken from each of the six original guinea pigs (Table 1). Type A plates, incubated at 37 C for 40 hr, whether treated for 30 sec or 1 min with chloroform vapor, yielded precipitin rings without killing the colonies (0.5 to 1 mm in diameter). Type A plates could be read within 48 hr after inoculation and were useful for testing pure or slightly contaminated specimens (Fig. 1). Type B plates were similar to type A, but

TABLE 1. Identification of *Pasteurella pestis* from guinea pig tissues on selective and nonselective antiserum-agar plates

Guinea pig	Type of plate ^a	<i>P. pestis</i> identified by precipitin rings				
		Throat	Bubo	Spleen	Lung	Heart
1	A	-	+	+	+	-
	B	-	+	+	+	-
	C	-	+	+	+	-
	D	-	+	+	+	-
	E	-	+	+	+	-
2	A	-	+	+	+	-
	B	-	+	+	+	-
	C	-	+	+	+	-
	D	-	+	+	+	-
	E	-	+	+	+	-
3	A	-	+	+	+	+
	B	-	+	+	+	+
	C	-	+	+	+	+
	D	+	+	+	+	+
	E	-	+	+	+	+
4	A	-	+	+	+	+
	B	+	+	+	+	+
	C	+	+	+	+	+
	D	+	+	+	+	+
	E	+	+	+	+	+
5	A	+	+	-	+	-
	B	+	+	-	-	-
	C	+	+	-	-	-
	D	+	+	+	+	+
	E	+	-	-	-	-
6	A	-	-	+	+	+
	B	-	-	+	+	+
	C	-	+	+	+	+
	D	+	+	+	+	+
	E	+	+	+	+	+

^a Description of plate types given in Materials and Methods; + = positive, - = negative.

yielded smaller colonies and less-defined precipitin rings. Type C plates were similar to type D plates, but differed in that type C plates yielded better-defined precipitin rings (Fig. 2), while type D plates were more inhibitory to contaminants (Fig. 3). The selective nature of types C and D plates permitted the identification of *P. pestis* from throat specimens (Fig. 4 and 5). Individual *P. pestis* colonies were identified when a small quantity (1-mm loopful) of throat specimen was used (Fig. 4). Use of a large quantity (0.1 ml) of throat specimen resulted in a continuous precipitin ring surrounding a confluent mass of *P. pestis* (Fig. 5). Continuous precipitin rings could not be detected on type A plates inoculated with 0.1 ml

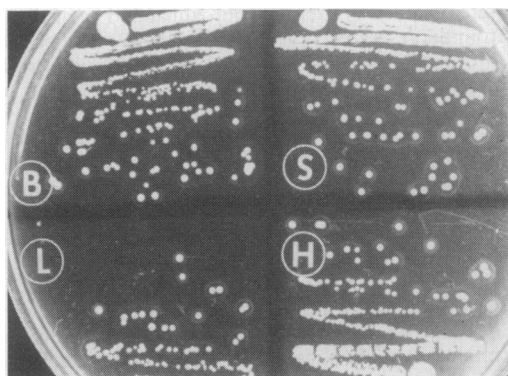


FIG. 1. Type A antiserum-agar plate streaked with bubo aspirate (B), spleen (S), lung (L), and heart (H) specimens from guinea pig no. 4. Plate was incubated for 40 hr prior to 1-min exposure to chloroform vapor. *Pasteurella pestis* colonies are surrounded by precipitin rings.

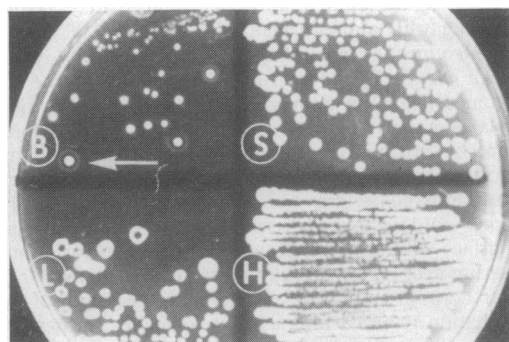


FIG. 2. Type C antiserum-agar plate streaked with bubo aspirate (B), spleen (S), lung (L), and heart (H) specimens from guinea pig no. 5. Plate was incubated for 66 hr prior to 30-sec exposure to chloroform vapor. Arrow points to *Pasteurella pestis* colony surrounded by precipitin ring.

of throat specimen (Fig. 6). Types C and D plates could be read within 48 hr after inoculation; however, better-defined precipitin rings were observed when these plates were incubated for 66 hr prior to treatment with chloroform vapor. Colonies 0.75 to 1 mm in diameter produced adequate precipitin rings, although the rings were less defined than those produced on type A plates. Faint precipitin rings appeared on types C and D plates within 6 hr after chloroform vapor treatment. Precipitin rings were well developed within 24 hr and remained visible for several days. Colonies incubated for 66 hr remained viable if exposure to chloroform vapor was limited to 30 sec. Colonies incubated for 40 hr did not survive this treatment. Type E plates could be read within 48 hr after inoculation; however, the precipitin

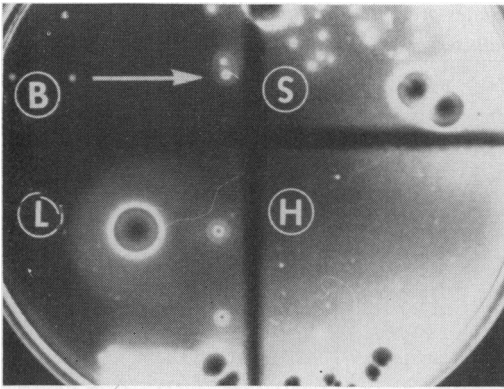


FIG. 3. Type D antiserum-agar plate streaked with bubo aspirate (B), spleen (S), lung (L), and heart (H) specimens from guinea pig no. 5. Plate was incubated for 66 hr prior to 30-sec exposure to chloroform vapor. Arrow points to *Pasteurella pestis* colonies surrounded by precipitin rings.

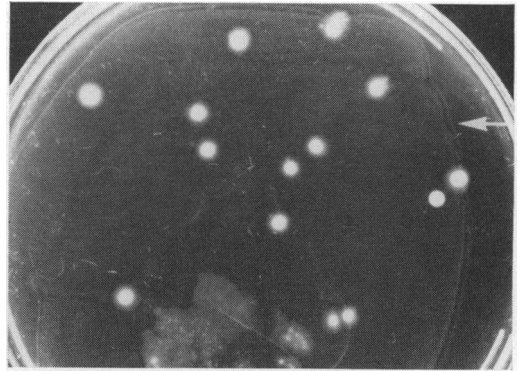


FIG. 5. Type D antiserum-agar plate containing 0.1 ml of throat specimen from guinea pig no. 4. Plate was incubated for 66 hr prior to 1-min exposure to chloroform vapor. Arrow points to precipitin ring surrounding confluent mass of *Pasteurella pestis*.

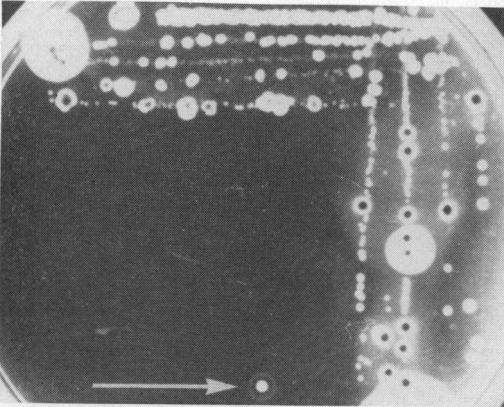


FIG. 4. Type C antiserum-agar plate streaked with throat specimen from guinea pig no. 4. Plate was incubated for 66 hr prior to 30-sec exposure to chloroform vapor. Arrow points to *Pasteurella pestis* colony surrounded by precipitin ring.

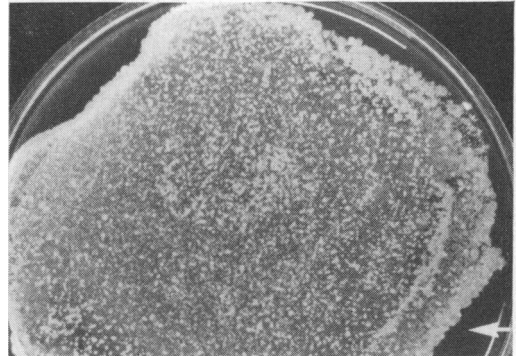


FIG. 6. Type A antiserum-agar plate containing 0.1 ml of throat specimen from guinea pig no. 4. Plate was incubated for 66 hr prior to 1-min exposure to chloroform vapor. Arrow directs attention to the absence of precipitin ring around mass of bacterial growth.

rings were less defined than those appearing on type A plates. Colonies on 40-hr type E plates withstood chloroform vapor for 30 sec. Precipitin rings were better defined when type E plates were incubated for 66 hr prior to chloroform vapor treatment. Colonies on the latter plates remained viable after exposure to chloroform vapor for 1 min.

P. pestis colonies identified on selective and nonselective antiserum-agar plates were readily purified on type A secondary-isolation plates. Isolates obtained from these plates were lethal for all mice and guinea pigs injected, and uniformly exhibited characteristics typical of *P.*

pestis (Table 2). *P. pestis* colonies were identified, with type D antiserum-agar plates, from the spleens of all guinea pigs dead as a result of inoculation with *P. pestis* isolates. Identification of *P. pestis* from mouse spleens proved more difficult because of interfering contaminants. Positive identifications were obtained, however, from 14 of the 17 isolates tested.

DISCUSSION

The antiserum-agar plate technique has inherent characteristics that should make it valuable for future use in the identification, isolation, and study of *P. pestis*. This method permits the identification, on primary-isolation plates, of *P. pestis* from contaminated tissues, thus reducing the amount of work and time required to identify

TABLE 2. Confirmative characterization of guinea pig isolates previously identified as *Pasteurella pestis* on selective and nonselective antiserum-agar plates

Isolate	Type of plate ^a	Guinea pig	Bacterial properties ^b											
			FI	PI	Ps	P	Ca	Le	Ure	Glu	Lac	Mel	Rha	
1	A	1	+	+	+	+	+	+	+	-	+	-	-	-
2	B		+	+	+	+	+	+	+	-	+	-	-	-
3	C		+	+	+	+	+	+	+	-	+	-	-	-
4	E		+	+	+	+	+	+	+	-	+	-	-	-
5	A	2	+	+	+	+	+	+	+	-	+	-	-	-
6	B		+	+	+	+	+	+	+	-	+	-	-	-
7	B	3	+	+	+	+	+	+	+	-	+	-	-	-
8	E		+	+	+	+	+	+	+	-	+	-	-	-
9	A	4	+	+	+	+	+	+	+	-	+	-	-	-
10	B		+	+	+	+	+	+	+	-	+	-	-	-
11	C		+	+	+	+	+	+	+	-	+	-	-	-
12	D		+	+	+	+	+	+	+	-	+	-	-	-
13	A	5	+	+	+	+	+	+	+	-	+	-	-	-
14	B		+	+	+	+	+	+	+	-	+	-	-	+
15	C		+	+	+	+	+	+	+	-	+	-	-	-
16	D		+	+	+	+	+	+	+	-	+	-	-	-
17	A	6	+	+	+	+	+	+	+	-	+	-	-	-
Alexander ^d			+	+	+	+	+	+	+	-	+	-	-	-
M23 ^e			-	+	+	+	+	+	+	-	+	-	-	-
PB1 ^f			-	-	-	+	+	+	+	+	+	-	+	+
<i>E. coli</i> ^g			-	-	-	-	-	-	-	-	+	+	+	+

^a Description of plate types given in Materials and Methods.

^b Bacterial properties: FI = fraction I production; PI = pesticin production; Ps = phage sensitivity; P = pigmentation; Ca = calcium requirement; Le = lethality for mice and guinea pigs; Ure = urease production; Glu = glucose fermentation; Lac = lactose fermentation; Mel = melibiose fermentation; Rha = rhamnose fermentation.

^c Acidification after 2 weeks.

^d *P. pestis* (strain Alexander, fraction I-positive).

^e *P. pestis* (strain M23, fraction I-negative).

^f *P. pseudotuberculosis* (strain PB1⁺).

^g *Escherichia coli* (type B).

P. pestis from tissues containing viable organisms. Although the method cannot replace the more rapid fluorescent staining test of Moody and Winter (8) in situations where immediate identification of *P. pestis* is mandatory, antiserum-agar plates containing specific fraction I antibody may be used to confirm the faster method often used to test specimens from human plague suspects or victims. Because the antiserum-agar plate test is specific for *P. pestis* when specific antiplague serum is incorporated into various nutrient agar media, it may become unnecessary to conduct all of the additional tests known for confirming *P. pestis*. This view is supported by results obtained both in this and in an earlier study (1). If, however, it is considered necessary to obtain isolates for performing complete characterizations, or for use in research, the present method readily permits it. This is possible because brief chloroform vapor treatment, used to release fraction I antigen from *P. pestis* colonies, promotes the formation

of precipitin rings without sterilizing the colonies. We have no evidence at this time that brief chloroform vapor treatment alters bacterial properties characteristic of *P. pestis*.

In addition to possible clinical use for confirming the presence of *P. pestis* in man, the antiserum-agar plate method may be useful in conducting epidemiological and ecological surveys in areas of the world where plague is suspected or known to exist. These possibilities are demonstrated by our results which show that *P. pestis* can be specifically identified and isolated from animal tissues on nonselective antiserum-agar plates, and that inhibitors and selective media can be used in combination with specific antiplague serum to increase the efficiency of the method. When combined with specific antiplague sera, selective media [such as enriched azide agar, developed by Knisely et al. (6) to isolate *P. pestis* from heavily contaminated materials such as soil and desoxycholate agar, and used by Marshall

et al. (7) to isolate *P. pestis* from human throats] become increasingly useful for testing contaminated specimens. Copper sulfate, recommended by Baltazard et al. (2) to inhibit *Proteus* species, also becomes more useful, especially when used in combination with antiplague serum and Desoxycholate Agar. Other inhibitors and selective media are available, but have not been tested in combination with specific antiplague serum in nutrient agar plates. This area should be explored because the ultimate potential of the antiserum-agar plate method for identifying *P. pestis* in heavily contaminated specimens may be directly related to the efficiency of the selective media employed in the antiserum-agar plates.

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