

# Pesticinogeny: a Characteristic Useful for Presumptive Identification and Isolation of *Pasteurella pestis*

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Current methods of identifying *Pasteurella pestis* rely heavily on tests specific for detecting fraction I, the envelope antigen. Pesticin I, a bacteriocin inhibitory for *P. pseudotuberculosis*, has been demonstrated in nearly all tested strains isolated from human infections. The results of using this characteristic as an identifying trait for *P. pestis* were compared with results reported for detecting fraction I by fluorescent-antibody and antiserum-agar techniques. Data indicate that, although certain atypical strains of *P. pestis* fail to react in one system or the other, a combination of these tests provides positive identification in all cases. Detection of *P. pestis* in contaminated materials is greatly facilitated, and the simplicity of this test makes it a valuable tool in the study of plague infections and an important adjunct to methods currently in use. The use of the pesticin I assay is not intended to replace other accepted techniques, but rather to supplement them and increase the effectiveness of plague investigation.

The identification of *Pasteurella pestis*, until recently, has been a time-consuming problem. Confirmation of the identity of the infecting organisms by accepted morphological, biochemical, antigenic, and animal infectivity studies requires approximately 2 weeks. With the development (16) of fluorescent antibody specific for fraction I, the envelope antigen of *P. pestis*, it has become possible to provide a presumptive diagnosis of plague within a matter of hours. Since many laboratories are not equipped to perform fluorescent-antibody examination of specimens for the presence of *P. pestis*, it seems important that alternative procedures should be available to the microbiologist for expediting plague detection. One highly specific method has been described (1) that uses a nutrient medium containing antiserum specific for fraction I. This report describes application of the pesticin assay (5) as a practical tool for presumptive identification and isolation of *P. pestis*.

## MATERIALS AND METHODS

Pesticin I is detected by its ability to inhibit growth of type I *P. pseudotuberculosis*, a species closely related to *P. pestis*. The major requirements for a successful assay are (i) a minimal level of  $Fe^{2+}$  and (ii) an excess of free  $Ca^{2+}$  in the medium. These conditions are satisfied by including calcium ethylenediaminetetra-

acetic acid (Ca-EDTA) in the medium. A proven formula for pesticin I assay agar, essentially the same as described by Brubaker and Surgalla (6), utilizes 4% Blood Agar Base (BBL) in distilled water. After sterilization at 121 C for 15 min, the agar is cooled to 45 to 50 C and sterile  $CaCl_2$ ,  $CaNa_2$ -EDTA (Geigy), and glucose are added to give final concentrations of 0.01 M, 0.1%, and 0.01 M, respectively. The base layer is poured into petri dishes, solidified, and allowed to dry at room temperature.

Specimens may then be streaked to insure that developing colonies are well separated. Isolated colonies appear after 24 hr at 26 C and reach a diameter of approximately 1 to 1.5 mm in 48 hr.

After the primary incubation at 26 C for 48 hr, each plate is inverted for 1 min over a 2 inch<sup>2</sup> (5.08 cm<sup>2</sup>) gauze pad saturated with chloroform. This sterilizes the surface of the colonies and prevents the cells from mixing with the indicator layer. After excess chloroform is allowed to dissipate, melted pesticin agar is cooled to 45 C and inoculated with type I *P. pseudotuberculosis* (ATCC 6902, American Type Culture Collection, Rockville, Md.) so that each milliliter contains  $10^5$  to  $10^6$  organisms. A suitable suspension can be prepared either by adding one drop of a 24-hr broth culture to 5 ml of melted agar or by suspending a 1-mm loopful of cells from an agar slope in 10 ml of broth and then adding 0.1 ml of the suspension to 5 ml of agar. The suspension is then pipetted evenly over the surface of the suspected colonies. Plates must then be incubated at 37 C and may be examined after 18 to 24 hr. Wide, clear zones

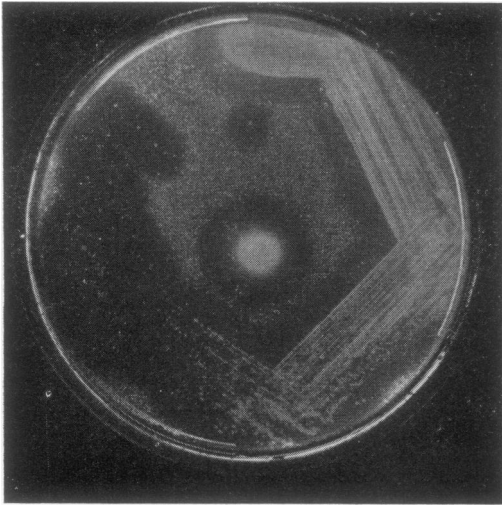


FIG. 1. *Pesticin I* inhibition of *Pasteurella pseudotuberculosis* demonstrated by clear zones around isolated colonies of *P. pestis*. Note positive control in center.

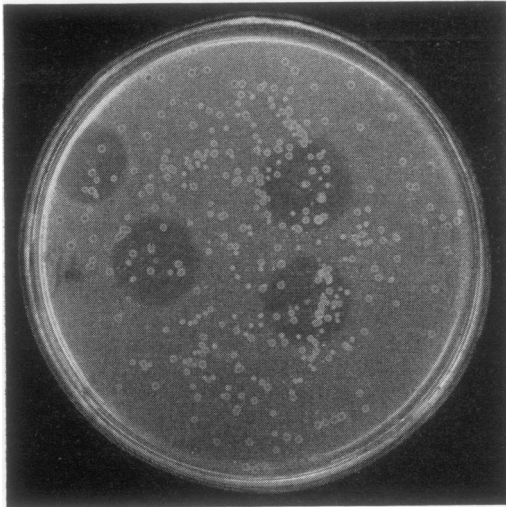


FIG. 2. Demonstration of *pesticin I* assay for detecting *Pasteurella pestis* in contaminated specimens.

of inhibition appear around colonies producing *pesticin I* (Fig. 1).

Moderately contaminated samples may be streaked directly on *pesticin I* assay medium. A more satisfactory method is to dilute the specimen and inoculate the agar with several levels to insure the best possible separation of colonies. The results of a typical test are shown in Fig. 2. The sample, bone marrow from an exhumed body (provided by Philip S.

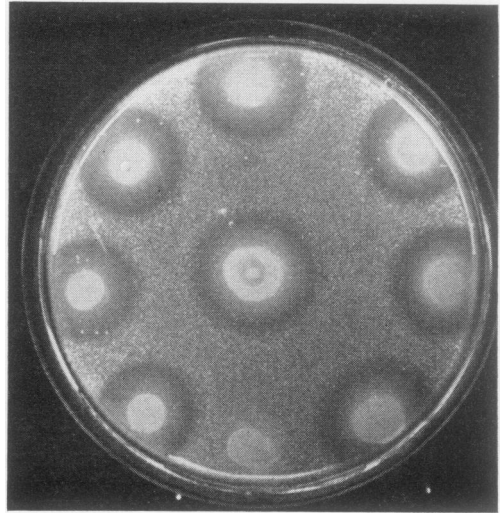


FIG. 3. *Pesticin I* assay used for testing several pure cultures at one time. Note positive control in center and negative control at top.

Brachman, U.S. Public Health Service), yielded approximately  $10^6$  *P. pestis* per g of tissue. This was roughly 1% of the total viable organisms recovered. When pure cultures are available, one plate may be used conveniently to test as many as six unknowns (Fig. 3). It should be strongly emphasized, however, that positive and negative controls must be included with each test to insure that the system is functioning properly.

If isolates of *P. pestis* are required, viable cells may be picked from a colony in the exact center of the *pesticin I* zone with an inoculating needle and purified by streaking to eliminate *P. pseudotuberculosis* carried over from the indicator layer. A convenient medium for differentiating between *P. pestis* and indicator organisms is Congo red agar (13). *P. pestis* from natural infections becomes dark red on this preparation and *P. pseudotuberculosis* appears pale pink.

Antiserum agar was prepared by the method of Albizo and Surgalla (1).

## RESULTS AND DISCUSSION

*Pesticin*, a bacteriocin-like substance produced by *P. pestis*, was first described by Ben-Gurion and Hertman (3). Studies by Brubaker and Surgalla (5) revealed the presence of two bacteriocins, *pesticin I* and *pesticin II*. Correlation of *pesticin I* production with the expression of fibrinolytic activity and coagulase was reported by Brubaker et al. (7). This relationship was further studied by Beesley et al. (2). The loss of *pesticinogeny* and the genetically linked fibrinolytic factor and coagulase were found to reduce the invasive powers of the organism (4). Subsequent studies suggested that, although

loss of pesticinogeny is associated with a reduction in virulence, infectivity may be unimpaired (M. J. Surgalla and E. D. Beesley, *Bacteriol. Proc.*, p. 75, 1969). This type of organism is apparently quite rare in natural plague infections. The literature covers two American strains isolated from nature that lack this property and yet retain other known virulence factors. One, strain G32, was isolated from ground squirrel fleas in Nevada in 1940; the other, Dodson, was isolated from a young boy in Arizona in 1967. Eleven hundred strains isolated from sylvatic plague and human infections in Viet Nam have been characterized, and the resulting data show that only three were nonpesticinogenic (J. D. Marshall, Jr., *personal communication*). Of 370

strains of *P. pestis* from Siberian and Mongolian foci examined by Timofeva (14), only three strains, isolated in 1923, 1945, and 1960, were nonpesticinogenic. Martinevskij (11) reported two interesting observations; strains from all natural foci examined, except from the "Petit Caucase," produce pesticin I and strains from the "Altai montagneux" focus not only produce pesticin I but are also sensitive to it. The apparent rarity of nonpesticinogenic organisms in human and sylvatic plague indicates the high stability of pesticinogeny and supports its value in identifying *P. pestis*.

The wide variation in the genetic make-up of *P. pestis* (9) points out the need for new methodology that can be applied at the level of the hospital diagnostic laboratory as well as in specialized facilities.

Table 1 shows some of the known varieties of the plague bacillus and indicates two properties, fraction I and pesticin I, that aid in the differentiation of *P. pestis* and *P. pseudotuberculosis*. A combination of testing procedures (Table 2), utilizing the detection of fraction I, pesticin I, phage sensitivity, and guinea pig lethality as indicators of the presence of *P. pestis*, can decrease the chance of obtaining false-negative or false-positive data.

Nonspecific inhibition of *P. pseudotuberculosis* by an unrelated organism has been reported by Brzin (8), who found that *Pseudomonas aeruginosa* strongly inhibits *P. pseudotuberculosis* types 1 through 5. We have found that *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacillus globigi*, and certain isolates of *B. subtilis* are also inhibitory. The mechanism of action of this

TABLE 1. Comparison of properties of typical and atypical strains of *Pasteurella pestis* and *P. pseudotuberculosis*

Strain	Genotypic properties	
	Fraction I	Pesticin I
<i>P. pestis</i>		
Alexander.....	+	+
Dodson.....	+	0
Bryans.....	+	+
M23.....	0	+
Nairobi.....	0	+
<i>P. pseudotuberculosis</i>		
PTB, type I A (ATCC 6902).....	0	0
Alaska type I B.....	0	0

TABLE 2. Reduction of false-positive and false-negative results in plague investigations utilizing a combination of two or more methods for detecting *Pasteurella pestis*

Strain	Fluorescent antibody	Antiserum agar	Pesticin I assay	Phage at 20 C	Interdermal guinea pig lethality
<i>P. pestis</i>					
Alexander.....	+	+	+	+	+
Dodson.....	+	+	0	+	± <sup>a</sup>
Bryans <sup>b</sup> .....	0	+	+	+	±
M23.....	0	0	+	+	±
Nairobi <sup>c</sup> .....	0	0	+	± <sup>d</sup>	0
<i>P. pseudotuberculosis</i>					
PTB type I A (ATCC 6902).....	0	0	0	0	±
Alaska type I B <sup>e</sup> .....	+	0	0	0	±

<sup>a</sup> Inconsistent dose response.  
<sup>b</sup> Winter, Cherry, and Moody (15).  
<sup>c</sup> Davis et al. (10).  
<sup>d</sup> High multiplicity is required.  
<sup>e</sup> Quan et al. (12).

inhibition is not clear; however, antibiotic production rather than bacteriocin or bacteriophage activity is likely. Other organisms tested and found to be negative include *Salmonella typhi*, *schottmuelleri*, *paratyphi A*, *enteritidis*, *gallinarum*, and *choleraesuis*; *Shigella dysenteriae*, *sonnei*, and *boydii*; *Proteus vulgaris*, *morganii*, *rettgeri*, and *mirabilis*; *Arizona arizoniae*; *Escherichia coli*; *Enterobacter aerogenes* and *cloacae*; *B. cereus*, *megaterium*, *thuringiensis*, *licheniformis*, and *brevis*. *Klebsiella pneumoniae*, *Neisseria catarrhalis*, *Spirillum serpens*, *Streptococcus faecalis*, *Staphylococcus aureus* and *epidermidis*, *Micrococcus luteus*, *Sarcina lutea*, *Brevibacterium linens*, and *Corynebacterium pseudodiphtheriticum* were also not inhibitory. Although false indication of pesticin activity may occur, careful observation of staining and colonial morphology should minimize this potential source of error.

The use of the pesticin I assay as presumptive evidence of the presence of *P. pestis* has a number of distinct advantages that make it a useful tool in plague investigation. The method is easy to use, and extensive training is unnecessary. There is no requirement for expensive and unusual equipment. The basic materials used in the test are common, inexpensive, stable, and easily obtained. Finally, application of the method provides the investigator with a viable isolate for further study.

We emphasize as we did in an earlier presentation (E. D. Beesley and M. J. Surgalla, *Bacteriol. Proc.*, p. 87, 1969) that the use of the pesticin I assay is not intended to replace other accepted techniques, but rather to supplement them and increase the effectiveness of plague investigation.

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