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Correlation of Autoagglutination and Virulence of Yersiniae

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Virulent strains of *Yersinia pestis*, *Y. pseudotuberculosis* and *Yersinia enterocolitica* invariably autoagglutinated in tissue culture media when grown at 36°C. Avirulent strains did not possess this property.

Yersinia pestis, *Y. pseudotuberculosis*, and *Y. enterocolitica* are animal pathogens which can cause a diversity of human disease. *Y. pestis*, the causative agent of bubonic plague in humans, is highly infectious and lethal to most rodents and many small mammals. *Y. pseudotuberculosis* and *Y. enterocolitica*, which produce less severe human infections, have been isolated from patients with gastrointestinal disease characterized by diarrhea or invasive involvement of the mesenteric lymphatics or both. Although *Y. enterocolitica* has been recovered from sporadic human disease for over 50 years, it is only recently that this organism has gained attention in the United States (3) due to its frequent isolation from a variety of animal, food, and water sources. The public health significance of such isolations has not yet been adequately assessed (3).

In the course of studies of virulence factors of *Yersinia* species, we have made the fortuitous discovery that strains which were virulent when fed to adult mice autoagglutinated when grown at 36°C in tissue culture media. Strains which failed to autoagglutinate were found to be avirulent. This observation has enabled us to develop a simple test for distinguishing virulent and avirulent strains of yersiniae. In addition to its value in preparing isogenic derivatives needed for studies of pathogenesis, we have found this test to be reliable for the rapid screening of large numbers of yersiniae from clinical and food sources. Thus, it is now possible to avoid the labor and expense of the animal and tissue culture models currently being used to screen such diverse isolates.

Many of the strains employed in this study are part of the stock culture collection of the Department of Hazardous Microorganisms, Walter Reed Army Institute of Research, Washington, D.C. Some strains were also provided by Wei Hwa Lee, USDA-FSQS, Beltsville, Md., Ira J. Mehlman, Food and Drug Administration, Washington, D.C., and Jean M. Alonso, Institut Pasteur, Paris, France. All strains were maintained at room temperature in screw-capped tubes containing 10.0 ml of semisolid medium (10.0 g of tryptose, 5.0 g of sodium chloride, and 5.0 g of agar per liter of water; Difco Laboratories). Organisms were routinely cultivated on Trypticase soy agar medium at 26°C.

The autoagglutination test was performed, using sterile disposable glass test tubes (13 by 100 mm) containing 2.0 ml of tissue culture medium. Although various tissue culture media gave satisfactory results, the data presented here were obtained by using RPMI-1640 medium with 10% calf serum and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Microbiological Associates, Walkersville, Md.). The incorporation of serum in the medium did not change the results but did stabilize the pH. Strains were streaked on Trypticase soy agar plates and grown for 2 to 3 days at 26°C. Cells from an isolated colony were inoculated into a pair of tubes. One tube was incubated for 18 h at 36°C, whereas the other was incubated for the same time period at 26°C. Routinely, ten colonies of each strain were tested to insure an adequate sample size. The growth in each tube was examined for evidence of bacterial agglutination. The growth of autoagglutination-positive (Ag^+) strains consisted of an irregularly edged layer of agglutinated bacteria which formed a flocculate covering the bottom of the tube. Usually, the medium in such tubes was clear. The growth of non-agglutinating (Ag^-) strains was distinctly different. Although some bacteria had settled out to form a smooth round pellet in the center of the bottom of the tube, the majority of the bacteria remained in suspension, therefore creating a turbid medium. When the various tubes were gently shaken, the agglutinated bacteria remained clumped whereas the non-agglutinated bacteria in the pellet formed a smooth turbid suspension.

This agglutination phenomenon was found to be dependent on the temperature of growth. Strains were scored as Ag^+ if the 36°C tube was positive and the 26°C tube was negative. Bac-

teria which agglutinated at both temperatures were considered to be false-positives. In most cases, these false reactions were produced by bacteria which formed colonies of a rough morphology. Although some strains exhibited better growth at one temperature or the other, this did not influence the autoagglutination phenomenon.

We selected colonies from representative wild-type virulent strains of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* and tested them for their ability to autoagglutinate. These cultures were found to contain both Ag^+ and Ag^- bacteria. Therefore, it was possible to select isogenic pairs of each of these strains differing in their ability to autoagglutinate. These isogenic pairs were then tested for virulence by oral feeding of adult mice. Groups of five Swiss albino mice (Walter Reed ICR) weighing 15 to 20 g were deprived of water for 18 h and then allowed to drink ad libitum from a 50-ml water suspension of each strain grown at 26°C containing about 1×10^9 bacteria per ml. Mice were examined twice a day for evidence of diarrhea and sepsis. Liquid feces as well as the spleens of dead mice were cultured by using standard bacteriological techniques to detect the presence of the challenge organism.

All Ag^+ strains proved to be virulent whereas their isogenic Ag^- derivatives were avirulent (Table 1). Virulence was defined as the ability of a strain to induce diarrhea or produce a fatal systemic infection after oral infection or both.

These results suggested that the autoagglutination test might be useful as a presumptive test of the pathogenic potential of yersiniae isolates. Over the last year we have tested 220 different strains for their ability to autoagglutinate. Four of 10 *Y. pestis*, 6 of 30 *Y. pseudotuberculosis* and 25 of 180 *Y. enterocolitica* strains were found to be Ag^+. All proved to be virulent to mice by the oral route. None of 185 Ag^- strains proved to be virulent. Nine strains of *Y. enterocolitica* autoagglutinated at both 36 and 26°C but were avirulent for mice. One strain of *Y. pseudotuberculosis* produced a positive reaction at both 36 and 26°C. This strain, however, did prove to be virulent. Therefore, of the 220 strains tested by our autoagglutination method, only one strain was presumptively falsely characterized as avirulent. Since the majority of the *Y. enterocolitica* strains were epidemiologically associated with human disease, we are presently testing a broad spectrum of environmental isolates to determine whether there is a general relationship between autoagglutination and mouse virulence.

The biochemical basis of this autoagglutination phenomenon is obscure. Nonetheless, there is without question a correlation between the autoagglutination and virulence properties of yersiniae. As a consequence, we are now examining the possible relationship of this phenomenon to known virulence determinants of yersiniae. At least five such determinants have been defined in *Y. pestis*: (i) V and W antigen (VW); (ii) purine synthesis (pur); (iii) fraction 1 (F1); (iv) pesticin-I-coagulase-fibrinolysin complex (PCF), and (v) the ability to absorb certain dyes (P) when grown on solid media (1). Determinants i, ii, and possibly v are also shared by *Y. pseudotuberculosis* (2). Recent evidence (unpublished data) suggests that some virulent strains of *Y. enterocolitica* also are VW positive. At least two biological roles have been associated with the V and W antigens: resistance to phagocytosis by neutrophils, and the survival and multiplication within free or fixed macrophages (4). We are currently investigating the possibility that the autoagglutination property and the VW antigen are indicators of the same virulence determinant. The fact that the production of the VW antigens and the ability to autoagglutinate are temperature dependent suggests that this is a reasonable possibility.

TABLE 1. *Comparison of virulence properties of autoagglutinating and non-agglutinating isogenic derivitives of Yersinia species*

Species	Strain	Autoagglutination	Virulence to mice	
			Diarrhea[a]	Death[b]
Y. pestis	195(Ag^+)	+	−	5/5
	195(Ag^-)	−	−	0/5
	MP6(Ag^+)	+	−	5/5
	MP6(Ag^-)	−	−	0/5
Y. pseudotuberculosis	13-13(Ag^+)	+	+	5/5
	13-13(Ag^-)	−	−	0/5
	13-14(Ag^+)	+	+	5/5
	13-14(Ag^-)	−	−	0/5
	13-10(Ag^+)	+	+	5/5
	13-10(Ag−)	−	−	0/5
Y. enterocolitica	Y7(Ag^+)	+	+	5/5
	Y7(Ag^-)	−	−	0/5
	4052(Ag^+)	+	+	0/5
	4052(Ag^-)	−	−	0/5

[a] Mice which were scored as diarrhea positive produced multiple soft to liquid stools over a 3- to 5-day period, usually starting on day 3 after bacterial challenge.

[b] Death occurred within 14 days.

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