COAGULATION OF HUMAN PLASMA BY *PASTEURELLA PESTIS*[1]

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Mushin and Kerr (1954) reviewed the literature describing the coagulation of various plasmas by intestinal gram-negative bacilli. There was agreement that these organisms coagulated citrated plasma because they metabolized citrate and released calcium ions. Other gram-negative bacilli have been found to coagulate both citrated and oxalated plasma. Billaudelle (1955) observed that fluid media containing citrated rabbit and sheep blood were coagulated by *Haemophilus pertussis* which also coagulated oxalated blood when tested by conventional techniques.

Jawetz and Meyer (1944) reported that a variety of strains of *Pasteurella pestis* coagulated citrated rabbit but not human plasma and that there was no correlation of the reaction with the virulence of the organism.

This report shows that some strains of *P. pestis* coagulate human plasma and that such coagulation depends on the strain, the anticoagulant and its concentration, the dilution of the plasma, the concentration of the test organisms, and its incubation period. The reaction could not be correlated with the lethality of a strain for mice.

MATERIALS AND METHODS

Preparation of bacterial suspensions. Thirty-two lyophilized strains of *P. pestis* were twice subcultured in brain heart infusion broth (Difco). The cultures were incubated for 24 hr at 28 C in a water bath with aeration by shaking at 230 strokes per min on a reciprocal shaker. The identity and purity of the cultures were checked with bacteriophage prepared from that originally isolated by Gunnison, Larson, and Lazarus (1951). *Staphylococcus aureus*, *Salmonella typhimurium*, and *Shigella flexneri* serotype 3 were also grown in brain heart infusion broth in the same manner. The number of viable bacteria was determined by conventional methods on blood agar base (Difco).

Plasma coagulation. The test procedure was that described by Fisk (1940), except that 0.5 ml plasma was inoculated with 0.1 ml of the bacterial suspensions and incubated at 37 C for 48 hr. Controls contained (i) 0.5 ml plasma plus 0.1 ml sterile brain heart infusion broth and (ii) 0.5 ml sterile brain heart infusion broth plus 0.1 ml of the culture. Human plasma was used unless stated otherwise.

Citrate determination. The utilization of citrate by the test organisms was determined chromatographically (Wood, 1959). Controls were (i) bacteria-free plasma plus brain heart infusion broth, (ii) bacteria-free plasma maintained at 4 to 8 C, and (iii) sodium citrate in water (2.0 mg per ml).

Virulence of P. pestis. The virulence of the organisms was determined in Namru mice (Garber and Hauth, 1950) which were inoculated subcutaneously with 0.1 ml of 10-fold serial dilutions (8 mice per group). Mice were observed for 2 weeks. The LD_{50} was estimated by a linearized grid method based on the dose-mortality studies of Goldberg et al. (1954).

RESULTS AND DISCUSSION

Several experiments were performed to determine optimal conditions for coagulation of plasma by *P. pestis*. Table 1 illustrates the effect of various anticoagulants, media, time, and dilution of plasma on the reaction. Undiluted citrated and oxalated plasmas were coagulated by strain A1122, whereas heparinized plasma was not. Coagulation, delayed in a 1:10 dilution of citrated plasma, did not occur in diluted oxalated plasma. Of the 4 media tested, brain heart infusion

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TABLE 1

Factors affecting human plasma coagulation by Pasteurella pestis strain A1122, grown at 28 C

Anticoagulant	Plasma, 0.5 ml	Plasma Inoculated with:											
		4.3 × 10^8 Cells grown in brain heart broth			3.1 × 10^8 Cells grown in heart broth			2.6 × 10^8 Cells grown in tryptose broth			2.5 × 10^8 Cells grown in nutrient broth		
		Coagulation at 37 C at hr:											
		4.5	21	48	4.5	21	48	4.5	21	48	4.5	21	48
Sodium citrate, 2 mg/ml	Undiluted	++	++	++	++	++	++	+	++	++	−	++	++
	1:10	−*	+	+	−	+	+	−	+	+	−	+	+
Potassium oxalate, 2 mg/ml	Undiluted	++	++	++	+	++	++	−	++	++	−	−	−
	1:10	−	−	−	−	−	−	−	−	−	−	−	−
Heparin, 0.01 mg/ml	Undiluted	−	−	−	−	−	−	−	−	−	−	−	−
	1:10	−	−	−	−	−	−	−	−	−	−	−	−

*No reaction.

broth gave the best results. Table 2 shows that 2 mg sodium citrate per ml plasma was the optimal concentration.

Table 3 shows the effect of different anticoagulants at the stated concentrations on the multiplication of *P. pestis* during static incubation in plasma. In the presence of citrate and oxalate, the organisms were markedly reduced in numbers. The reduction was less marked in the presence of heparin. Furthermore, coagulation of citrated or oxalated plasma was effected only by the largest inoculum.

It was of further interest to investigate the bactericidal effect of sodium citrate and to examine the possibility that plasma was either inhibitory to *P. pestis* or provided a poor growth medium. Table 4 shows not only that sodium citrate was bactericidal but that unheated human serum, although less so, was also bactericidal to *P. pestis* during static incubation. Small inocula of *P. pestis* grew well in unheated mouse and guinea pig sera under similar conditions. The high initial dose of organisms appeared to be necessary to overcome the factors preventing multiplication which are presumably present in plasma as well as in serum.

To determine whether the factors responsible for the coagulation of plasma were extracellular, Seitz filtrates of brain heart infusion broth cultures of strain A1122 were lyophilized and reconstituted to 10, 5, and 2 times their original concentration. None of these concentrates, used in 0.1-ml amounts, coagulated citrated plasma. It is, of course, possible that the coagulation factors were produced in such small amounts that much greater concentrations of culture filtrates would be necessary for their demonstration. This is suggested by the relatively large numbers of organisms required to produce coagulation under conditions of the test. We did not test for the possibility that Seitz filtration removed coagulation factors.

TABLE 2

Effect of sodium citrate concentration on plasma coagulation by Pasteurella pestis grown in brain heart infusion broth

Strain	Cells in Inoculum	Sodium Citrate, mg/ml	Plasma Coagulation at Hr:			
			6	21	26	48
51	3.4 × 10^8	2	−	+	+	+
		10	−	+	+	+
		20	−	−	−	+
68	4.6 × 10^8	2	−	++	++	++
		10	−	−	−	−
		20	−	−	−	−

The slow action of *P. pestis* due to the antibacterial action of sodium citrate and of plasma precludes the development of a rapid slide test with unconcentrated materials.

Citrate at 2 mg per ml of mouse blood was insufficient to prevent normal clotting. Although

TABLE 3

Test for plasma coagulation and multiplication by Pasteurella pestis incubated statically for 48 hr at 37 C in human plasma prepared with three anticoagulants*

Strain	Anticoagulant	No. of Cells		Coagulation	Strain	Anticoagulant	No. of Cells		Coagulation
		Inoculum	Recovered				Inoculum	Recovered	
A1122	Sodium citrate, 2 mg/ml	6.4×10^8	7.0×10^6	++	36†	Sodium citrate, 2 mg/ml	5.0×10^8	9.0×10^7	–
		6.4×10^5	3.1×10^1	–			5.0×10^5	1.1×10^1	
		6.4×10^3	0	–			5.0×10^3	0	
	Potassium oxalate, 2 mg/ml	6.4×10^8	2.4×10^5	++		Potassium oxalate, 2 mg/ml	5.0×10^8	1.7×10^7	–
		6.4×10^5	0	–			5.0×10^5	0	–
		6.4×10^3	0	–			5.0×10^3	0	–
	Heparin, 0.01 mg/ml	6.4×10^8	4.9×10^7	–		Heparin, 0.01 mg/ml	5.0×10^8	5.2×10^7	–
		6.4×10^5	3.9×10^3	–			5.0×10^5	7.9×10^6	–
		6.4×10^3	1.1×10^2	–			5.0×10^3	2.9×10^3	–

* Plasma 0.5 ml plus 0.1 ml brain heart infusion broth containing organisms.

† Broth control inoculated with 5×10^3 organisms and statically incubated at 37 C for 48 hr yielded 6.2×10^7 organisms.

TABLE 4

Bactericidal effect of sodium citrate on Pasteurella pestis incubated statically in fresh human serum at 37 C for 48 hr

	Pasteurella pestis			
	Strain A1122		Strain 36	
	No. of cells		No. of cells	
	Inoculum	Recovery	Inoculum	Recovery
Serum with sodium citrate, 2 mg/ml	6.4×10^8	1.7×10^6	5.0×10^8	2.9×10^7
	6.4×10^5	0	5.0×10^5	0
	6.4×10^3	0	5.0×10^3	0
Serum without sodium citrate	6.4×10^8	2.0×10^7	5.0×10^8	2.6×10^7
	6.4×10^5	3.8×10^2	5.0×10^5	2.0×10^6
	6.4×10^3	8.3×10^2	5.0×10^3	9.0×10^2

10 mg of citrate per ml prevented clotting, mouse plasma was not coagulated within 48 hr by strain A1122.

This strain coagulated undiluted citrated plasma of each of 15 human donors within 21 hr. Although 9 of the donors had received one or another kind of plague vaccine, all of the plasmas gave similar coagulation reactions.

Thirty-two strains of *P. pestis* were compared for their capacity to coagulate the pooled human plasmas of 10 of the 15 donors. Table 5 shows that plasma was coagulated by 17 of 20 strains of varying virulence and by 6 of the 12 avirulent strains. There was, thus, no correlation between coagulation and virulence for mice. None of the 32 strains utilized citrate.

In supplementary tests, as a control measure, *S. aureus* and *S. typhimurium* were found to coagulate human plasma, but the latter utilized citrate in the process. *S. flexneri* neither coagulated plasma nor metabolized citrate.

Two types of coagulum were noted in these experiments: the one (++ in the tables) was voluminous and gelatinous; the other (+) was

TABLE 5

Human plasma coagulation, mouse LD_{50}, and citrate utilization by 32 strains of Pasteurella pestis

Naval Biol. Lab. Strain No.	Cells/LD_{50}	Plasma Coagulation	Citrate Utilized	Naval Biol.Lab. Strain No.	Cells/LD_{50}	Plasma Coagulation	Citrate Utilized
Virulent:				127	7.0×10^0	+	−
16	1.5×10^0	++	−	131	5.0×10^1	+	−
32	4.2×10^5	−	−	137	2.0×10^1	++	−
37	1.0×10^1	−	−	138	1.8×10^1	+	−
39	9.0×10^0	+	−	*Avirulent*			
45	1.5×10^6	+	−	12	$>3.9 \times 10^7$	++	−
50	1.5×10^5	−	−	24	$>6.8 \times 10^6$	−	−
51	8.0×10^1	+	−	25	$>7.8 \times 10^7$	−	−
52	2.9×10^0	+	−	31	$>1.0 \times 10^7$	−	−
53	3.5×10^1	+	−	33	$>4.6 \times 10^7$	+	−
55	2.0×10^1	++	−	35	$>1.8 \times 10^7$	−	−
59	1.0×10^1	+	−	36	$>1.9 \times 10^7$	−	−
63	4.0×10^0	+	−	38	$>1.3 \times 10^7$	+	−
66	2.3×10^7	+	−	58	$>5.2 \times 10^7$	++	−
68	5.1×10^7	++	−	62	$>5.4 \times 10^7$	++	−
78	7.0×10^0	+	−	A1122	$>7.4 \times 10^7$	++	−
79	2.8×10^0	+	−	E.V.76	$>7.0 \times 10^7$	−	−

small in volume, strandlike, and with a clear supernatant. Microscopic examination of the latter type showed it to be composed of masses of bacteria and fibrin. This type of coagulum probably represented merely a quantitative difference either in available coagulation factor or fibrinogen.

Presumably there is no correlation between plasma coagulation and toxin production by *P. pestis*. Englesberg and Levy (1954) noted that strain A1122 elaborated less toxin than strain E.V. 76 under conditions favorable to toxin production. In this study only the former coagulated plasma.

The failure of Jawetz and Meyer (1944) to observe coagulation of human plasma may have been due to variation of any one or more of the many factors which affect the coagulation reaction. Our failure to show any correlation between virulence and coagulation of human plasma confirms their experience with rabbit plasma.

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SUMMARY

Human plasma was coagulated by some strains of *Pasteurella pestis*. Coagulation depended upon the growth medium, the anticoagulant and its concentration, the number of bacteria, and the length of the incubation period. The plasma coagulating factor was not demonstrable in culture filtrates obtained by Seitz filtration. The reaction was not referable to citrate utilization.

Mouse plasma containing sufficient sodium citrate to prevent spontaneous coagulation was not coagulated by the strain of *P. pestis* tested.

The virulence for mice of tested strains of *P. pestis* was not coincident with the capacity to coagulate human plasma.

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