DEMONSTRATION OF STAPHYLOCOCCAL CLUMPING FACTOR AND FREE COAGULASE IN SOFT AGAR MEDIA[1]

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Of the methods now available for the detection of coagulase positive staphylococci, none is designed to show both clumping factor and free coagulase activity in a single system. The observations reported here are by-products of a search for variant strains of staphylococci with particular reference to strain differences in the cell surface component known as clumping factor or bound coagulase. In this search, the soft agar technique, which has been applied to variation studies by a number of workers (McCarty *et al.*, 1946; Pike, 1946; Pittman and Davis, 1950; Lankford *et al.*, 1955; Finkelstein and Sulkin, 1958), has proved most rewarding. A comparison of the reactions of staphylococci in various soft agar media with the results of conventional coagulase and agglutination tests led to the method described here for simultaneous detection of free coagulase and clumping factor. The study also revealed that the formation of compact staphylococcal colonies in serum- and plasma-soft agar need not be due exclusively to antibody action.

MATERIALS AND METHODS

A series of 106 strains of staphylococci were examined for (a) free coagulase by the routine plasma clotting test, (b) clumping factor by both the slide and tube agglutination methods, and (c) appearance of colonies in fibrinogen-soft agar and in plasma-soft agar. Of this series, 92 strains were isolated from hospital patients and personnel, whereas 14 were laboratory stock cultures. Additional hospital strains were studied in soft agar containing varying concentrations of bovine fibrinogen (Armour fraction I), plasma or serum, and certain combinations of these substances with bovine albumin (Armour fraction V). Where strains were compared in tests employing fresh plasma, identical plasma was used throughout the experiment. Where the effects of fresh plasma and serum were compared, the source of the blood, whether human or rabbit, was the same donor.

Rehydrated compounded plasma (Difco) was used in the routine test for free coagulase; tubes were observed for clotting at the end of 1, 3, and 24 hr incubation at 37 C. Slide and tube agglutination tests for clumping factor (Duthie, 1954) were performed simultaneously. In each agglutination method an equal volume of the cell-saline suspension was mixed in (a) undiluted, fresh human plasma, (b) rehydrated plasma, and (c) 0.4 per cent bovine fibrinogen-saline, the average concentration of fibrinogen in normal plasma. Slide tests were considered positive if visible clumping occurred within 15 to 30 sec. In cases of delayed or no clumping, a loop of growth from a 16 to 18 hr trypticase soy agar (BBL) slant was suspended directly in undiluted plasma and in the fibrinogen-saline, and the slides were observed for the same intervals.

Basal soft agar was prepared by dissolving agar in trypticase soy broth (BBL) to make a 0.15 per cent agar medium and a 0.3 per cent agar medium. The former was dispensed in 10-ml amounts and the latter in 5-ml amounts to 1.5 by 12 cm screw capped tubes before sterilizing by autoclaving. To make 0.4 per cent fibrinogen-soft agar, 0.8 per cent solution of fibrinogen in trypticase soy broth was sterilized by positive pressure Seitz filtration and added in 5-ml amounts to tubes containing 5 ml melted, cooled, 0.3 per cent agar basal medium. In like manner, the proper amounts of sterile plasma, serum, or dilutions of the fibrinogen-broth were added to basal soft agar to obtain media of the desired composition. A 3 per cent albumin-trypticase soy broth, sterilized by positive pressure Seitz filtration, was similarly incorporated at 0.3 per cent final concentration into plasma-fibrinogen-soft agar.

An inoculum which resulted in 20 to 40 colonies

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per tube of soft agar medium was obtained as follows. For each strain to be tested, 5 ml trypticase soy broth was inoculated from an overnight trypticase soy agar slope culture. After 4 to 6 hr incubation at 37 C, the young broth culture was diluted serially using a 4-mm loop to introduce the organisms successively to (a) 10 ml sterile saline containing 0.05 per cent peptone, (b) 3 ml of the same sterile peptone-saline, and (c) 10 ml of the soft agar medium. All soft agar cultures were examined after 12 to 16 and 24 hr incubation at 37 C.

RESULTS

All strains of staphylococci tested produced an elongated, feathery type of colonial growth in basal soft agar medium as previously described by Finkelstein and Sulkin (1958). Of the 106 strains reported (table 1), 81 were positive and 25 were negative for free coagulase according to the routine plasma clotting test. Among the 81 free coagulase positive strains, 13 were auto-agglutinating and gave equivocal results in slide and tube agglutination tests. These 13 rough strains included 7 strains which proved to be mixtures of compact and diffuse colonies in plasma-soft agar and in fibrinogen-soft agar cultures. Results of the slide and tube agglutination tests with 0.4 per cent fibrinogen and rehydrated plasma agreed. In addition to the 65 strains which were positive for clumping factor in fibrinogen and rehydrated plasma, 2 other coagulase positive strains agglutinated only in fresh, undiluted, human plasma. Both these strains also produced spherical, compact colonies in 1 per cent plasma-soft agar, whereas in 0.4

TABLE 1

Clumping reactions and compact colony formation by coagulase positive and negative staphylococci

Total No.	Agglutination			Compact Colonies	
	0.4% Fibrinogen—saline	Rehydrated plasma[a]	Undiluted human plasma	0.4% Fibrinogen—soft agar	1% Plasma—soft agar
81 (Free coagulase positive strains)	65 +	65 +	67 +	71 +	73 +
	3 −	3 −	1 −	2 −	1 −
	13 ±[b]	13 ±	13 ±	1 ±	
				7 +/−[c]	7 +/−
25 (Free coagulase negative strains)	2 +	2 +	2 +	2 +	2 +
	19 −	19 −	19 −	23 −	23 −
	4 ±	4 ±	4 ±		

[a] Rehydrated plasma (Difco).

[b] ±, Doubtful results; rough strains in agglutinations; compact, slightly elongated colonies in soft agar.

[c] +/−, Mixtures of compact and diffuse colonies in soft agar.

TABLE 2

Effects of different concentrations of plasma, serum, and fibrinogen on the formation of compact colonies by typical strains of clumping factor positive and negative staphylococci

Strain No.	Clumping Factor	Compact Colonies in Soft Agar Medium Containing								
		Human plasma (%)			Human serum (%)			Fibrinogen (%)		
		1.0	0.1	0.01	1.0	0.1	0.01	0.4	0.04	0.004
5N-5, 5N-22, 96	+	+	+	−	+	±[a]	−	+	+	±
75, 79	+	+	+	−	+	−	−	+	+	±
2, 28	−	−	−	−	−	−	−	−	−	−

[a] ±, Intermediate colony type; compact, slightly elongated form.

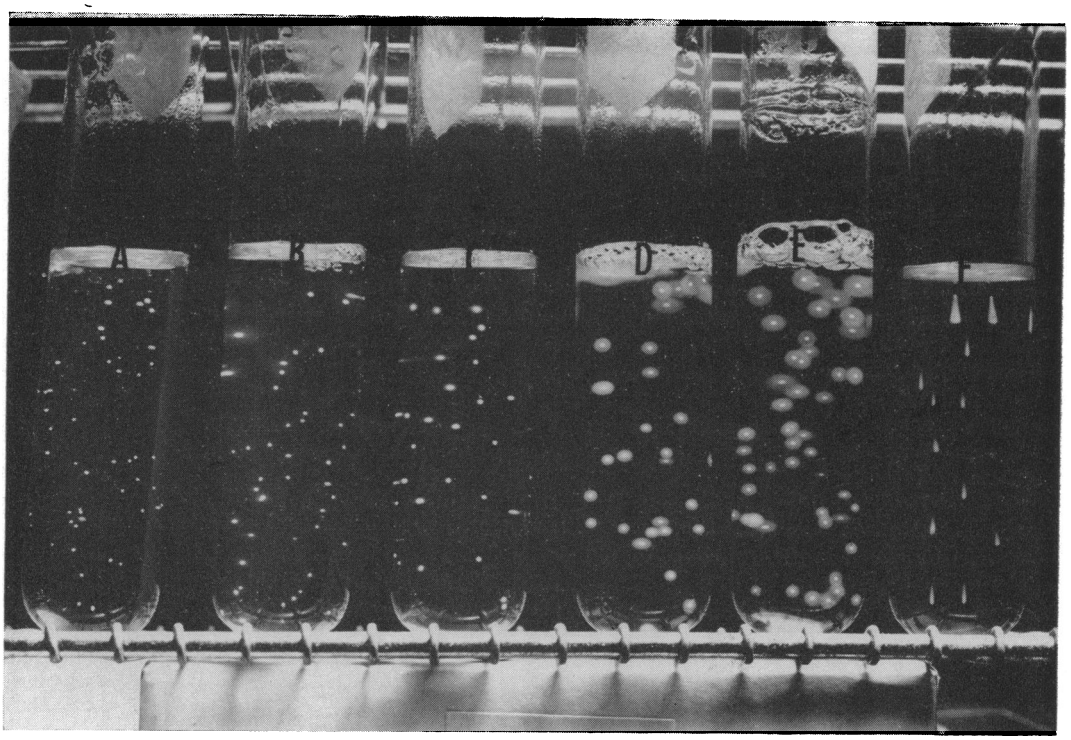

Figure 1. A staphylococcus (strain K-79) which is positive for free coagulase and clumping factor incubated 14 hr at 37 C in soft agar medium containing: (*A*) 1 per cent plasma; (*B*) 5 per cent plasma; (*C*) 0.4 per cent fibrinogen; (*D*) 1 per cent plasma and 0.4 per cent fibrinogen; (*E*) 1 per cent plasma, 0.4 per cent fibrinogen, and 0.3 per cent albumin; (*F*) basal medium only (control). Note compact colonies in all except control culture; coagulation zones in *B*, *D*, and *E*.

per cent fibrinogen-soft agar, one gave rise to diffuse colonies and the other to an intermediate colony type which was compact though slightly elongated, resembling a carpet tack in shape. With these 2 exceptions, all strains which formed compact colonies in 1 per cent plasma-soft agar also produced the same type colonies in 0.4 per cent fibrinogen-soft agar, and, conversely, strains which failed to develop compact colonies in fibrinogen-soft agar were negative for this feature in plasma-soft agar. The 23 free coagulase negative strains which were negative or doubtful for clumping factor by agglutination tests produced diffuse colonies in fibrinogen-soft agar and in plasma-soft agar.

When strains were compared in soft agar which contained varying concentrations of plasma, serum, and fibrinogen, those possessing the clumping factor formed compact colonies in 0.04 per cent fibrinogen-soft agar as well as in 0.1 per cent plasma- and 1 per cent serum-soft agar (table 2). A well defined coagulation zone was observed around colonies of free coagulase positive strains growing in 1 per cent plasma-0.4 per cent fibrinogen-soft agar (figure 1). Substitution of normal rabbit plasma or serum for human plasma or serum in these experiments yielded similar results. The addition of 0.3 per cent albumin to 1 per cent plasma-0.4 per cent fibrinogen-soft agar intensified the coagulation zone reaction and supplied a highly satisfactory medium for demonstrating both clumping factor and free coagulase activity, provided a 0.4 per cent fibrinogen-soft agar medium was also inoculated as a control. This method revealed strains which were positive for one of these properties exclusively and strains which were mixed populations with regard to clumping factor or free coagulase (figure 2). By transfer of individual colonies, it was possible to separate colonial types and to obtain pure substrains of variants from mixed populations. In table 3 are

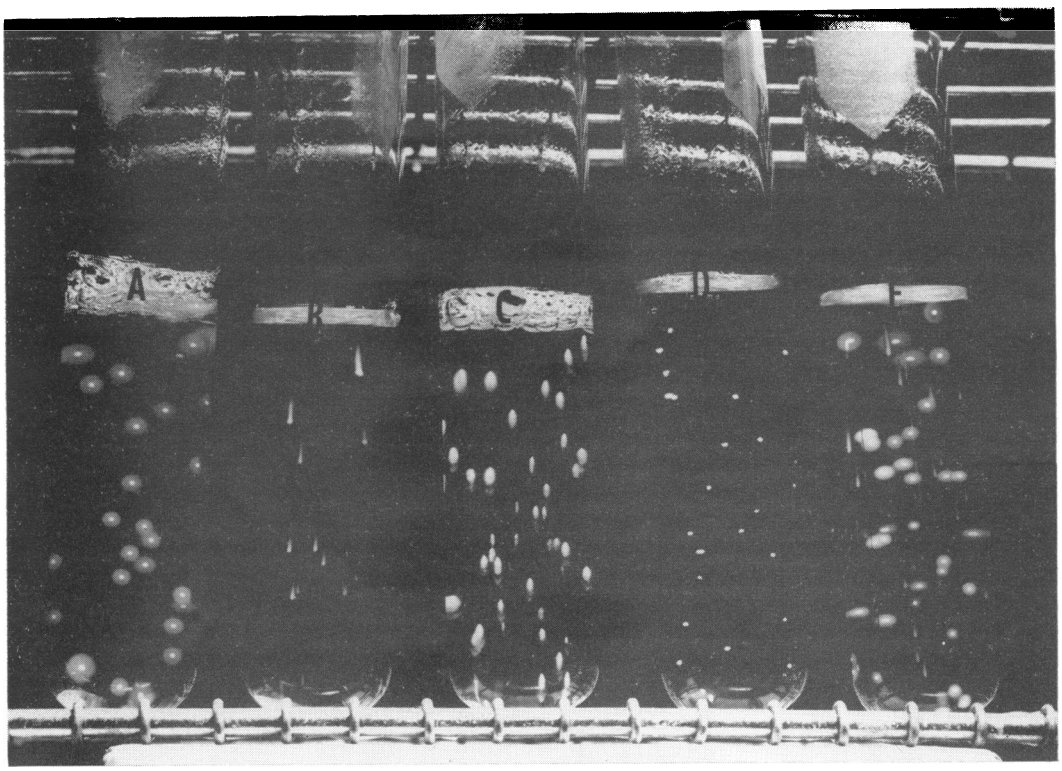

Figure 2. Growth of five different strains of staphylococci in albumin-plasma-fibrinogen-soft agar: (*A*) positive for both free coagulase and clumping factor; (*B*) negative for both free coagulase and clumping factor; (*C*) positive for free coagulase only; (*D*) positive for clumping factor only; (*E*) mixed population with respect to free coagulase and clumping factor.

TABLE 3

Demonstration of free coagulase (F)[a] and clumping factor (C)[b] activity in soft agar media

Strain No.	Free Coagulase (Routine Test)	Clumping Factor (Agglutination)[c]	Basal Soft Agar Medium Containing							
			1% Plasma		5% Plasma		0.4% Fibrinogen		1% Plasma plus 0.4% fibrinogen	
			F	C	F	C	F	C	F	C
5N-5, 79, 96	+	+	−	+	+	+	−	+	+	+
5N-22, 75	+	+	−	+	±[d]	+	−	+	+	+
2, 28, 64	−	−	−	−	−	−	−	−	−	−
6, 93	+	−	−	−	+	−	−	−	+	−
35, 65	−	+	−	+	−	+	−	+	−	+
18, 32, 95	+	+	Mixture							
			−	+	±	+	−	+	+	+
			−	−	−	−	−	−	−	−

[a] F, free coagulase as indicated by coagulation zone around colonies.
[b] C, clumping factor as indicated by compact colonies.
[c] Results of slide agglutination in 0.4 per cent fibrinogen and in rehydrated plasma.
[d] ±, Coagulation zones narrow or indistinct.

summarized the typical results which correlate clumping factor and free coagulase activity with the production, in soft agar media, of the compact colony and coagulation zone, respectively.

DISCUSSION

The high degree of correlation between nonspecific agglutination of staphylococci in plasma and their ability to coagulate citrated plasma lead to the assumption that a single property was responsible for both reactions (Much, 1908; Birch-Hirschfeld, 1934; Cadness-Graves *et al.*, 1943; Needham *et al.*, 1945). Substitution of fibrinogen for plasma in the slide test was recommended in order to avoid agglutinations due to specific antibodies (Berger, 1943). The observation that heat and merthiolate treated plasma retained its agglutinating property but no longer reacted in the tube coagulase test presented the first evidence that different factors were concerned in the two tests (Linsell and Gorill, 1951). Duthie (1954) distinguished between bound coagulase or clumping factor, which acts directly on fibrinogen to cause agglutination, and extracellular free coagulase, which is generally assumed to react with prothrombin or a closely related substance to cause the coagulation of citrated plasma.

To date approximately 300 strains of staphylococci have been compared in albumin-plasma-fibrinogen-soft agar and in fibrinogen-soft agar cultures as well as by agglutination and coagulase tests. These studies indicate that the soft agar method is effective for demonstrating both the clumping factor and free coagulase activity. Furthermore, it offers certain advantages over conventional methods, e.g., it overcomes the problem presented by rough strains in agglutination studies and it may reveal the heterogeneity of a strain with regard to coagulation zones and colonial compactness. Most coagulase positive strains produce spherical, compact colonies in 0.04 per cent fibrinogen-soft agar as well as in 0.1 per cent plasma- or 1 per cent serum-soft agar medium. Apparently plasma and serum are more effective than fibrinogen alone in reacting with staphylococci to form compact colonies. Jensen (1958) reported the regular occurrence, in the γ-globulin fraction of human serum, of an antibody which reacts with a cellular component of staphylococci belonging to serological type I of Cowan. Antibody action cannot be excluded as a contributing factor in the colony compacting mechanism in plasma- and serum-soft agar, but it is not necessarily the sole factor since all strains of staphylococci which are positive for the clumping factor also form compact colonies in bovine fibrinogen-soft agar. The presence of plasma or serum is essential for the coagulation zone phenomenon in soft agar as it is for the plasma clotting reaction in the routine coagulase test. Finkelstein and Sulkin (1958) noted that halos were visible occasionally around compact colonies in soft agar medium which contained high concentrations of serum. The combination of 0.3 per cent albumin, 1 per cent plasma, and 0.4 per cent fibrinogen in soft agar provides a medium in which even weak producers of free coagulase form remarkably clear coagulation zones. In basal soft agar any concentration of plasma or serum which is adequate for distinct coagulation zone formation is capable of compacting colonies of strains which have the clumping factor.

The formation of compact colonies by coagulase positive staphylococci in serum- and plasma-containing soft agar has been attributed to the action of specific antibody or to factor(s) resembling antibody in normal rabbit and human plasma and sera (Finkelstein and Sulkin, 1958; Hunt and Moses, 1958). If the findings which contribute to this interpretation are re-examined with respect to the effect of staphylococcal clumping factor on colonial form in fibrinogen-soft agar, it appears that antibody action in the usual sense is not the only possible explanation. Undoubtedly, specific antibody effect is operating at serum dilutions of 1:10,000 and more, but the compact "tailed" colonies which develop in soft agar containing 1:1000 rabbit antiserum appear to be the same as the "carpet tack" or intermediate colony type observed in the 0.1 per cent normal serum-soft agar and the 0.004 per cent fibrinogen-soft agar cultures herein described. Higher concentrations of serum, plasma, or fibrinogen in soft agar media result in the formation of spherical, compact colonies by strains of staphylococci which are positive for the clumping factor regardless of their free coagulase activity or, probably, serological type.

SUMMARY

A method which utilizes an albumin-plasma-fibrinogen-soft agar test medium and a fibrinogen-soft agar control medium for the simultaneous

detection of staphylococcal clumping factor and free coagulase activity has been described.

The formation of compact colonies in fibrinogen-soft agar appears to be an expression of staphylococcal clumping factor. Staphylococci which agglutinate in fibrinogen-saline also form compact colonies in fibrinogen-soft agar as well as in plasma- and serum-soft agar media. All but 2 of 106 strains of staphylococci produced the same type of colony (diffuse or compact) in bovine fibrinogen-soft agar and in human plasma-soft agar media.

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