APPLIED MICROBIOLOGY, June 1970, p. 911-914

Vol. 19, No. 6
Printed in U.S.A.

Relationship Between Staphylococcal Antiserum Titer and Zone Development on Immune Serum Plates[1]

K. F. WEISS AND RUTH N. ROBBINS
Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 24 February 1970

A workable relationship was established between the standard serum titers of staphylococcal immune antisera and the development of precipitin zones on serum agar around colonies of staphylococcal strains producing homologous antigens (enterotoxins). The standard titer of a serum is defined as the reciprocal of that serum dilution which, with 10 μg of pure enterotoxin per ml, will give a precipitin zone 10 mm in length in single gel-diffusion tubes after 7 days of incubation at 25 C. A numerical scale was set up for determining the intensity of precipitin zones on serum agar. A reading of 3 was considered optimum. This correlated well with a standard serum titer of 25, when 1 ml of such a serum was used per 20 ml of medium per serum plate. From this relationship, the minimum volume of serum required to give optimum precipitin zone development can be calculated.

The production in vitro of large quantities of staphylococcal enterotoxin, under a given set of conditions, depends in the last instance on the ability of the individual cells in a straphylococcal culture to produce large amounts of toxin.

High enterotoxin-yield isolates are obtained in the following manner: an 18- to 24-hr culture of a strain producing the desired enterotoxin is surface-inoculated onto a suitable solid medium containing an appropriate amount of sterile homologous antiserum. After a sufficient length of time of incubation, precipitin zones will form around the enterotoxin-producing colonies (2). The zone size, under constant conditions, depends on the amount of toxin produced and the concentration of the serum in the medium. There is a minimum amount of serum required to give precipitin zones having the necessary intensity for the zones to be seen. Holding all other factors constant, the amount of serum required, in turn, depends on the titer of the serum used.

The purpose of this study was to determine the standard titers (*see below*) of different lots of antisera and to correlate them to the minimum concentrations of antienterotoxins A, B, and C required, under standardized conditions, for isolating single enterotoxin-producing colonies on serum plates.

[1] Published with the approval of the Director of the Research Division, College of Agricultural and Life Sciences, The University of Wisconsin.

MATERIALS AND METHODS

Strains. The same strains and the same propagation methods were employed as described by Reiser and Weiss (1); however, only medium B consisting of 3% protein hydrolysate powder (PHP) and 3% NZ amine A (NAK) was used for the production of enterotoxin.

Antisera. Immune rabbit sera were prepared by the method of Robbins et al. (*in preparation*). The standard titer of an unknown serum was determined in the following manner. Single gel-diffusion tubes were set up from twofold serial dilutions of the serum. The dilutions ranged from 1:8 to 1:128, depending on the expected serum titer, the range of which was frequently known from the serum potency of previous bleedings of the same animal. A standardized antigen solution consisting of culture medium and containing 10 μg of enterotoxin per ml was placed above the agar containing the serum. The tubes were incubated for 7 days at 25 C, after which time the lengths of the precipitin zones were measured and plotted against the reciprocal of the serum dilutions. The standard titer of a serum, as defined here, is the reciprocal of that serum dilution which, under the stated conditions, gives a precipitin zone 10 mm in length.

Immune serum plates. The basal medium consisted of 3% PHP and 3% NAK, plus 1.8% agar-agar. Sufficient medium was added to petri plates containing the desired amount of sterile antiserum to give a total of 20 ml per plate. The plates were surface-dried and streaked with the appropriate test organisms. After 24 hr of incubation at 37 C, the plates were checked for zone development by viewing them against a 30-w fluorescent light at a distance of 10 cm. The zone

TABLE 1. *Precipitin zone development and standard serum titers*

Strain	Avg toxin yield (μg/ml)	Sera			Intensity scale				
		No.	Standard titer	Standard vol (ml)	1	2	3	4	5
					ml of serum used per 20 ml of medium / zone size (mm)				
100	9.3	A13	10	2.50	1.75	2.25 2.00	2.50	2.75	—[a]
					2.0	1.5 1.7	1.4	1.1	—
		A11	25	1.00	0.50	0.70	1.10 0.90	1.3	—
					2.5	2.3	1.8 2.0	1.4	—
		A14	45	0.55	0.30	0.50	0.70	—	—
					2.0	1.8	1.5	—	—
196	5.4	A13	10	2.50	2.00	2.25	2.75 2.50	—	—
					3.5	3.0	2.6 2.3	—	—
		A11	25	1.00	—	0.70	1.10 0.90	—	—
					—	2.2	1.3 1.8		
		A14	45	0.55	—	0.50	0.70	—	—
					—	1.7	1.2	—	—
S-6	450	B-10	15	1.57	1.10	1.30	1.70 1.50	—	—
					5.0	4.2	3.5 3.5		
		B-9	36	0.69	—	0.50	0.70	0.90	—
						4.2	3.5	2.7	—
		B-13	45	0.55	—	0.30	0.50	0.70	—
					—	4.0	3.8	3.2	—
C-243	82	B-10	15	1.57	0.90	1.10	1.50 1.30	1.70	—
					2.0	1.8	1.5 1.5	1.2	—
		B-9	36	0.69	—	0.50	0.70	0.90	—
					—	1.5	1.3	1.0	—
		B-13	45	0.55	—	0.30	0.50	0.70	—
					—	2.8	2.5	1.8	—
137	92	C-9	53	0.47	—	0.30	0.50	0.70	0.90
					—	2.3	2.0	1.5	1.2
		C-10	92	0.22	0.10	—	0.30	—	0.50
					2.1	—	1.5	—	0.8

TABLE 1—*Continued*

Strain	Avg toxin yield (μg/ml)	Sera			Intensity scale				
		No.	Standard titer	Standard vol (ml)	1	2	3	4	5
					ml of serum used per 20 ml of medium / zone size (mm)				
		C-8	170	0.15	—	0.10	—	0.30	0.50
					—	2.0	—	1.5	1.0
483	60	C-11	67	0.37	0.10	0.30	0.40	0.50	—
					2.4	1.8	1.3	1.0	—
		C-10	92	0.22	—	0.10	0.30 0.20	0.50	—
					—	2.0	1.2 1.5	1.0	—
		C-8	170	0.15	0.05	—	0.20 0.10	0.30	—
					2.3	—	1.2 1.5	1.0	—

[a] Not determined.

size was determined as the width of the annulus of the precipitin zone. Five intensity grades were used to denote the relative strength of the zone development. Grade 1 zones are barely visible. Grade 2 zones are not clearly defined but are discernible when the plate is viewed at right angle to the light source. Grade 3 zones are clearly visible from all angles. Grade 4 zones are very intense. The edges of grade 5 zones assume a rimlike appearance.

From preliminary work, it had been known that approximately 1 ml of antiserum B per petri plate, having a standard titer of approximately 25, was required to give easily discernible zones with strain S-6. Under the standardized conditions of the experiment, an intensity reading of 3 was obtained. Therefore, 1 ml of antiserum with a titer of 25 per 20 ml of medium was taken as the reference volume.

RESULTS AND DISCUSSION

The zone sizes and intensities for five dilutions of nine antisera obtained with nine staphylococcal strains are given in Table 1. There was very good correlation between the volumes of sera used and their corresponding zone intensities. This was true for all of the antisera regardless of toxin type or strain. In almost all cases, intensity readings of 3 (optimum) were obtained with amounts of sera per plate closest to the calculated standard serum volumes (SSV).

As could be expected, the precipitin zones became larger with decreasing serum concentration; however, there was no direct relationship between zone size observed on the serum plates and the amount of enterotoxin produced by a given strain as measured by single gel-diffusion tubes. Only a few colonies from a low enterotoxin-producing culture may form large zones on serum agar, rather than many or all colonies having small haloes. This is particularly true for cultures obtained through repeated single-colony isolation of large-haloed colonies. "Wild" strains of medium and low enterotoxin production usually present a considerable range of non-, low-, and large-haloed colonies. (At the Food Research Institute, after testing a considerable number of strains producing enterotoxins A, B, and C, the following ranges have been adopted: for enterotoxin A, low <2 μg/ml, medium 2 to 7 μg/ml, high >10 μg/ml; for enterotoxin B, low <5 μg/ml, medium 5 to 75 μg/ml, high >75 μg/ml; for enterotoxin C, low <5 μg/ml, medium 5 to 50 μg/ml, high >50 μg/ml.)

It should be pointed out here that occasionally antisera from a particular animal will be encountered which will give very faint precipitin zones in single gel-diffusion tubes although the length of the zones are in complete accord with the titer of the serum. For such a serum, an intensity reading of 3 may not be obtained on serum agar plates with the SSV. Pooling serum lots from several animals may alleviate this

TABLE 2. *Comparison between actual and standard serum volumes*

Antiserum	Strain	Calculated standard serum vol (ml)	Minimum vol (ml) used to give intensity reading of 3
A-13	100	2.50	2.50
	196		2.50
A-11	100	1.00	0.90
	196		0.90
A-14	100	0.55	0.70
	196		0.70
B-10	S-6	1.57	1.50
	C-243		1.30
B-9	S-6	0.69	0.70
	C-243		0.70
B-13	S-6	1.57	1.50
	C-243		1.30
B-9	S-6	0.69	0.70
	C-243		0.70
B-13	S-6	0.55	0.50
	C-243		0.50
C-9	137	0.47	0.50
C-11	483	0.37	0.40
C-10	137	0.22	0.30
	483		0.20
C-8	137	0.15	—[a]
	483		0.10

[a] Not determined.

problem. Adding larger amounts of serum to the plates may make the serum concentration too strong with regard to zone size; only a narrow band may form around the colonies, and if the enterotoxin production is very low it may take several days before any antigen-antibody reaction becomes visible.

On the basis of an intensity reading of 3 as optimum, any volume of antiserum used per 20 ml of medium, on which the precipitin zones reach an intensity reading of 3 after 24 hr of incubation at 37 C, multiplied by its standard titer ought to give 25. Alternately, dividing 25 by the standard titer will give the SSV required to produce an intensity reading of 3 under the conditions stated (Table 2). The agreement between the theoretical SSV and the volume actually used was very good. From this it may be concluded that once the standard titer of an antiserum has been determined, its minimum amount required in serum plates may easily be calculated.

Prolonged incubation of the serum plates may make it possible to use less serum than the SSV; however, the small amounts of serum saved may not warrant the extended incubation time.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI 07615 from the National Institute of Allergy and Infectious Diseases.

We thank M. S. Bergdoll for his encouragement in this study.

LITERATURE CITED

1. Reiser, R. F., and K. F. Weiss. 1969. Production of staphylococcal enterotoxins A, B, and C in various media. Appl. Microbiol. 18:1041–1043.
2. Sugiyama, H., M. S. Bergdoll, and G. M. Dack. 1960. *In vitro* studies on staphylococcal enterotoxin production. J. Bacteriol. 80:265–270.