

Assay of the Antibiotic Activity of Serum

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Received for publication 28 February 1969

One of the drawbacks of the "tube dilution" method for the assay of antibiotics in human serum has been illustrated by utilizing serum-sensitive and serum-resistant strains of *Escherichia coli*. In the case of serum-sensitive strains, it was found that fresh serum alone may account for the same degree of inhibition and thus yield minimal inhibitory concentrations identical to those obtained with serum combined with antibiotics, that is, "simulated" serum assay specimens. This fallacy of the method is discussed with regard to those instances in which laboratories were merely to utilize the patient's own coliform organism as the test organism, or with respect to the assay of, for example, polymyxins, in which inadvertently a R(ough) and therefore, serum-sensitive strain of *E. coli* were to be used as the indicator organism. It is recommended that serum-resistant laboratory strains of *Staphylococcus aureus* or *E. coli* of known antibiotic susceptibility be employed as the test organisms proper in order to circumvent the inherent bactericidal activity of serum.

Over the years, a variety of methods aimed at determining the antibacterial activity of patients' sera after chemotherapy have been developed. Of these, the serum dilution method probably has been the most widely used because of the ease with which this test can be executed. Ideally, the test is performed with a pre- and a posttreatment serum sample, both of which are assayed simultaneously. Many authors advocate the use of the patient's own organism (4-7, 11, 12) in these assays; thus, one determines the minimal inhibitory concentration (MIC) or, in addition, the minimal bactericidal concentration (MBC) of the patient's serum against the isolated pathogen. Other authors (3) recommend that the serum specimen from a given patient be assayed against a laboratory control strain of known antibiotic susceptibility. For control purposes, a test is set up in parallel utilizing the patient's pretreatment serum to which is added a known amount of the antibiotic being assayed. In any case, the results of these assays of the antibacterial activity of sera indicate the growth, inhibition, or death of the bacteria in a given dilution of serum.

It has been found that certain gram-negative organisms, such as various strains of *Escherichia coli*, *Klebsiella*, and *Enterobacter* that had been isolated from bacteremic or septicemic patients, are quite sensitive to the bactericidal activity of the patients' own sera and to sera from healthy human adult donors as well (9).

This study was conducted to determine to what extent fresh normal human serum alone is anti-

bacterially active as compared with the identical sera to which had been added a specified amount of a certain antibiotic. Specifically, an attempt was made to determine whether normal human serum is as active as serum combined with a given antibiotic against certain serum-sensitive strains of gram-negative organisms.

MATERIALS AND METHODS

Bacteria. *E. coli* strains 0:111, 2, 3, a control strain of *E. coli*, and a control strain of *Staphylococcus aureus* served as the test organisms. The two control strains are sensitive to all routinely employed antibiotics.

Media. Stock cultures were maintained on slanted nutrient-agar (Difco), from which they were transferred monthly. Nutrient broth (Difco) served as the diluent. The pour plate method was employed to monitor the number of organisms per milliliter of fluid medium: 1 ml of the appropriate dilution was transferred into each of two tubes, containing 10 ml of Trypticase Soy Agar (BBL) with added Yeast Extract (Difco), that had been held at 45 C. Plates were poured, allowed to solidify, incubated at 35 C for 16 to 18 hr, and examined.

Antibiotics. Ampicillin trihydrate (845 µg of active substance per mg) and kanamycin sulfate (785 µg of active substance per mg) were a gift from the Bristol Laboratories Division, New York, N.Y.; polymyxin B was obtained through the courtesy of Chas. Pfizer & Co., Brooklyn, N.Y. The antibiotic stock solutions were prepared in distilled water to 1,000 µg per ml or 1,000 units per ml of activity, respectively. They were stored at -15 C in small samples and were never reused after thawing.

Sera. Two normal adults, who had not received antibiotics for at least 1 month, were bled. The drawn blood was held at room temperature for 1 hr and subsequently was held at 4 C for 1 hr. The serum was separated from the clot and cells by centrifugation at 2,000 rev/min for 20 min. The serum from each adult (S- and T-serum) was dispensed in small samples into screw-cap vials which were frozen and kept stored at -65 C. As needed, vials were rapidly thawed in a water bath at 35 C.

Determination of serum sensitivity or resistance of test strains. The strains used were exposed to 10 and 50% fresh, as well as heat-inactivated (56 C, 60 min), S- and T-serum in nutrient broth. The bacterial inoculum was adjusted to yield roughly 10^8 organisms per ml at zero time. The final volume per assay and control tube was 2 ml. Immediately after inoculation with the test organisms, the tubes, each of which contained a $\frac{1}{8}$ by $\frac{3}{8}$ in Teflon-coated Alnico V magnet, were transferred to an MT-72 table containing the MS-7 water-immersible stirrer (Tri-R Instruments, Jamaica, N.Y.) in a water bath at 35 C. At 0, 1, and 3 hr, 0.35-ml samples were withdrawn, of which 0.25 ml was dispensed into 2.25-ml nutrient broth (1:10 dilution) and 0.1 ml was dispensed into 10 ml of nutrient broth (1:100 dilution). Amounts (1 ml) of each dilution were incorporated into duplicate pour plates. A strain was considered to be serum-sensitive if 90% or more of the organisms had been killed by 10% fresh serum within 1 hr, as serum-intermediate if between 50 and 89% had been so killed, and as serum-resistant if 49% or fewer of the organisms present at zero time had been rendered nonviable.

Broth dilution sensitivity tests. Assay tubes received 0.9 ml and control tubes received 1.9 ml of nutrient broth. The antibiotics were diluted twofold in nutrient broth. A 1-ml amount of the respective double-strength dilution of the antibiotic in nutrient broth was delivered into the corresponding tubes. All tubes were then inoculated with 0.1 ml of an appropriate dilution of each test organism to yield approximately 10^8 to 5×10^8 organisms per ml at zero time. The tubes were incubated at 35 C for 16 to 18 hr. The MIC in these tests was defined as the lowest antibiotic concentration giving complete inhibition of growth, as judged by visual inspection.

Determination of antibacterial activity of twofold diluted fresh human serum. Fresh serum was diluted twofold in nutrient broth. Assay tubes received 0.9 ml of nutrient broth, 1.0 ml of the double-strength dilution of serum, and 0.1 ml of the identical bacterial inoculum that was used in the broth dilution sensitivity tests. The final serum dilutions were 1:2 through 1:128. The MIC that was obtained with fresh, twofold diluted serum was interpreted as the highest dilution of serum yielding no macroscopically visible growth after 16 to 18 hr at 35 C.

Determination of antibacterial activity of human serum combined with antibiotics. Undiluted fresh serum was diluted twofold by the addition of an equal volume of antibiotic in nutrient broth (10 μ g of ampicillin per ml, 2 μ g of kanamycin per ml, or 20 units of polymyxin B per ml). Next, the serum was

further diluted twofold in nutrient broth. Each assay tube received 1.9 ml of the respective serum dilution (1:2 through 1:128) and 0.1 ml of the identical bacterial inoculum that was employed in the two previous assay methods. Thus, the first tube, representing a 1:2 dilution of serum, contained either 5 μ g of ampicillin per ml, 1 μ g of kanamycin per ml, or 10 units of polymyxin B per ml; the second tube contained twofold less serum and antibiotic, and so on. The MIC represented the highest dilution of serum, combined with antibiotic, that inhibited the growth of the test organism, as judged by visual inspection after incubation at 35 C for 16 to 18 hr.

RESULTS

Every test strain of organisms was screened with S- and T-serum in order to determine its sensitivity or resistance to the bactericidal activity of serum. Both sera yielded identical results. As shown in Table 1, *E. coli* strains 0:111 and 2 were found to be serum-sensitive, whereas *E. coli* 3, the control strain of *E. coli*, and the control strain of *S. aureus* proved to be serum-resistant. The latter control strain was noted to be agglutinated slightly in the presence of 50% fresh serum, a fact which was verified through phase-contrast microscopy. A smaller bacterial inoculum was deliberately chosen for the two control strains so that any minimal bactericidal effect on the part of the two fresh sera might be more readily demonstrable.

In the next series of experiments, the behavior of each test strain was examined simultaneously against fresh serum alone, antibiotic alone, and the combination of fresh serum and an antibiotic. Tables 2, 3, and 4 list the results obtained with ampicillin, kanamycin, and polymyxin B, respectively. Both sera yielded comparable results, as is evident from the "serum alone" control MIC values obtained in these experiments. Most striking were the data obtained with *E. coli* 2; a 1:16 dilution of serum alone was capable of inhibiting the growth of this serum-sensitive strain. In all experiments performed, fresh serum alone was found to inhibit this organism to approximately the same extent as did serum combined with any of the three antibiotics. This is also borne out by the finding that each antibiotic alone was much less effective than fresh serum alone, at least over the range of concentrations tested. At best, a minimal additive effect was observed in the case of serum combined with antibiotic against *E. coli* 2. However, this effect never was greater than twofold, thus suggesting a one-tube dilution variability due to experimental error. The results obtained with *E. coli* 0:111, the other serum-sensitive strain, indicated that 1:2 diluted fresh T-serum and 1:8 diluted S-serum could arrest the growth of this organism; the combination of

TABLE 1. Determination of serum sensitivity or serum resistance of the test organisms^a

Time after exposure to serum <i>hr</i>	<i>E. coli</i> 0:111 (10% T-serum)	<i>E. coli</i> 2 (10% T-serum)	<i>E. coli</i> 3 (10% T-serum)	Control <i>E. coli</i>		Control <i>S. aureus</i>	
				10% S-serum	50% S-serum	10% S-serum	50% S-serum
0	3.8×10^5	1.6×10^5	1.7×10^5	6.5×10^4	6.5×10^4	5.5×10^3	5.5×10^3
1	6.2×10^3	6.0×10^3	2.3×10^5	3.8×10^5	3.4×10^5	1.0×10^4	7.6×10^3
3	3.0×10^3	5.0×10^3	4.5×10^5	1.6×10^6	2.7×10^6	2.7×10^5	2.7×10^3

^a Values are expressed as the number of viable organisms per milliliter at specified times.

TABLE 2. Ampicillin-serum assays^a

Type of Assay	Organism	S-serum dilutions ^b						Control nutrient broth along	No. of organisms per milliliter at zero time	
		1:2 (5)	1:4 (2.5)	1:8 (1.25)	1:16 (0.6)	1:32 (0.3)	1:64 (0.15)			1:128 (0.08)
Serum plus Ampicillin	<i>Escherichia coli</i> 0111	—	—	+	+	+	+	+	+	2.2×10^5
	<i>E. coli</i> 2	—	—	—	—	+	+	+	+	1.6×10^5
	<i>E. coli</i> 3	+	+	+	+	+	+	+	+	2.2×10^5
	Control <i>E. coli</i>	—	—	—	+	+	+	+	+	9.1×10^4
	Control <i>Staphylococcus aureus</i>	—	—	—	—	—	—	+	+	5.8×10^4
Ampicillin alone	<i>E. coli</i> 0111	—	—	+	+	+	+	+		
	<i>E. coli</i> 2	+	+	+	+	+	+	+		
	<i>E. coli</i> 3	—	+	+	+	+	+	+		
	Control <i>E. coli</i>	—	—	+	+	+	+	+		
	Control <i>S. aureus</i>	—	—	—	—	—	—	+		
Serum alone	<i>E. coli</i> 0111	—	—	—	+	+	+	+		
	<i>E. coli</i> 2	—	—	—	—	+	+	+		
	<i>E. coli</i> 3	+	+	+	+	+	+	+		
	Control <i>E. coli</i>	+	+	+	+	+	+	+		
	Control <i>S. aureus</i>	+	+	+	+	+	+	+		

^a Symbols: +, visible growth; —, absence of macroscopic growth after 16 to 18 hr of incubation.

^b Values in parentheses indicate micrograms of ampicillin per milliliter of serum.

serum with any of the three antibiotics likewise resulted in a minimal additive effect. The serum-resistant strains, namely *E. coli* 3, the control *E. coli*, and the control *S. aureus*, were not inhibited at all by fresh human serum alone; furthermore, the MIC values obtained with the antibiotics alone coincided with those resulting from the combined activity of serum and antibiotic.

In a final series of experiments, it was found that the bactericidal activity of fresh human serum alone not only accounts for an MIC of from 1:8 to 1:16, but also results in an MBC of from 1:4 to 1:8, depending on the particular strain of serum-sensitive *E. coli* employed. The results are summarized in Table 5. In these experiments, all tubes without evidence of macroscopically visible growth were subcultured (one loopful per one-quarter sector of a chocolate-agar plate) and, furthermore, 1 ml was incorporated into one

Trypticase Soy Agar pour plate to determine the number of surviving organisms per milliliter. It is evident that the action of fresh serum alone may result in a "significant" MIC and MBC, depending on the respective strain of serum-sensitive organisms used.

DISCUSSION

The results obtained served to emphasize the following. A serum-sensitive or serum-resistant strain of *E. coli* or, for that matter, of other *Enterobacteriaceae* may be isolated from a bacteremic or septicemic patient's blood. Ordinarily, clinical microbiology laboratories do not determine the serum sensitivity or resistance of isolated coliform organisms with regard to the patient's own serum. If an antibiotic assay of the patient's serum is requested, some laboratories simply use the patient's coliform organism as the test strain,

TABLE 3. *Kanamycin-serum assays*^a

Type of assay	Organism	T-serum dilutions						Control nutrient broth alone	No. of organisms per milliliter at zero time	
		1:2 (1)	1:4 (0.5)	1:8 (0.25)	1:16 (0.12)	1:32 (0.06)	1:64 (0.03)			1:128 (0.015)
Serum plus kanamycin	<i>Escherichia coli</i> 0111	-	-	-	+	+	+	+	+	1.1×10^5
	<i>E. coli</i> 2	-	-	-	-	+	+	+	+	1.1×10^5
	<i>E. coli</i> 3	-	-	-	+	+	+	+	+	1.8×10^5
	Control <i>E. coli</i>	-	-	-	+	+	+	+	+	9.1×10^4
	Control <i>Staphylococcus aureus</i>	-	-	-	-	+	+	+	+	1.0×10^4
Kanamycin alone	<i>E. coli</i> 0111	-	-	-	+	+	+	+		
	<i>E. coli</i> 2	-	-	-	+	+	+	+		
	<i>E. coli</i> 3	-	-	-	-	+	+	+		
	Control <i>E. coli</i>	-	-	-	-	+	+	+		
	Control <i>S. aureus</i>	-	-	-	-	-	+	+		
Serum alone	<i>E. coli</i> 0111	-	+	+	+	+	+	+		
	<i>E. coli</i> 2	-	-	-	-	+	+	+		
	<i>E. coli</i> 3	+	+	+	+	+	+	+		
	Control <i>E. coli</i>	+	+	+	+	+	+	+		
	Control <i>S. aureus</i>	+	+	+	+	+	+	+		

^a Symbols are as explained in Table 2.^b Values in parentheses indicate micrograms of kanamycin per milliliter of serum.TABLE 4. *Polymyxin-serum assays*^a

Type of assay	Organism	T-serum dilutions						Control nutrient broth alone	Number of organisms per ml at 0 time	
		1:2 (10)	1:4 (5)	1:8 (2.5)	1:16 (1.25)	1:32 (0.6)	1:64 (0.3)			1:128 (0.15)
Polymyxin plus serum	<i>Escherichia coli</i> 0111	-	-	-	+	+	+	+	+	1.6×10^5
	<i>E. coli</i> 2	-	-	-	-	+	+	+	+	1.5×10^5
	<i>E. coli</i> 3	-	-	+	+	+	+	+	+	1.5×10^5
	Control <i>E. coli</i>	-	+	+	+	+	+	+	+	1.5×10^5
Polymyxin alone	<i>E. coli</i> 0111	-	-	+	+	+	+	+		
	<i>E. coli</i> 2	-	-	+	+	+	+	+		
	<i>E. coli</i> 3	-	-	+	+	+	+	+		
	Control <i>F. coli</i>	-	+	+	+	+	+	+		
Serum alone	<i>E. coli</i> 0111	-	+	+	+	+	+	+		
	<i>E. coli</i> 2	-	-	-	-	+	+	+		
	<i>E. coli</i> 3	+	+	+	+	+	+	+		
	Control <i>E. coli</i>	+	+	+	+	+	+	+		

^a Symbols are as explained in Table 2.^b Values in parentheses indicate units of polymyxin per milliliter of serum.

not realizing, of course, that the results obtained do not necessarily reflect the true antibiotic level of the serum specimen. Other laboratories may decide to divide the patient's serum in half and determine its MIC both against the patient's organism and against a laboratory control strain of either *S. aureus* or *E. coli*, the latter, for instance, when assaying polymyxin B. However, if not properly maintained, stock strains of *Entero-*

bacteriaceae may dissociate into R(ough) variants which are known to be exquisitely sensitive to the bactericidal activity of serum (10). Therefore, any laboratory that intends to employ the tube dilution method for the assay of, for example, polymyxin B or colistin sulfate, should ascertain the serum sensitivity or resistance of the isolated pathogen to the patient's own serum or, preferably, include a laboratory stock control strain of

TABLE 5. MIC and MBC values resulting from the bactericidal activity of two fresh human sera versus several strains of *Escherichia coli* (approximately 7.0×10^4 organisms/ml at zero time)*

Serum	Organism	Concn	Serum dilutions						Control nutrient broth alone	
			1:2	1:4	1:8	1:16	1:32	1:64		1:128
T-serum	<i>E. coli</i> 0111	MIC	—	—	—	+	+	+	+	+
		MBC	0	0	9.0×10^2					
	<i>E. coli</i> 2	MIC	—	—	—	—	+	+	+	+
		MBC	0	0	0	6.0×10^2				
	<i>E. coli</i> Ca	MIC	—	—	—	—	+	+	+	+
		MBC	0	0	0	3.0×10^3				
	Control <i>E. coli</i>	MIC	+	+	+	+	+	+	+	+
L-serum	<i>E. coli</i> 0111	MIC	—	—	—	+	+	+	+	+
		MBC	0	0	6.0×10^2					
	<i>E. coli</i> 2	MIC	—	—	—	+	+	+	+	+
		MBC	0	0	0					
	<i>E. coli</i> C	MIC	—	—	—	—	+	+	+	+
		MBC	0	0	7.0×10^0	8.0×10^1				
	Control <i>E. coli</i>	MIC	+	+	+	+	+	+	+	+

* *E. coli* C is a serum-sensitive strain used in these two experiments. Symbols: +, visible growth; —, absence of growth after 16 to 18 hr of incubation (MIC determination); 0, absence of any viable organisms; logarithmic numbers indicate numbers of organisms per milliliter after combined subculture and pour plating (MBC determination).

E. coli that has been shown to be resistant to a variety of samples of fresh human serum. Our laboratory divides the patient's serum specimen in half; one half is run against the patient's organism and the other half is run against a serum-resistant laboratory strain of either *S. aureus* or *E. coli*, both of known antibiotic sensitivity. These two control strains are maintained on Brain Heart Infusion Agar slants and are transferred biweekly; every 3 months, these strains are exposed to two samples of 50% fresh human serum to verify their serum resistance. Several vials of each strain suspended in 50% heat-inactivated horse serum in Brain Heart Infusion Broth are kept stored at -65°C (J. C. Sherris, personal communication).

An alternative procedure might be to inactivate the inherent bactericidal activity of the patient's serum prior to the performance of the dilution assay method. One possibility would be to heat-inactivate the fluid, since some antibiotics, including kanamycin and polymyxin B, are relatively heat-stable. However, other antibiotics, such as the various penicillins, are rather heat-labile (1), so that the laboratory would have to discriminate between heat-stable and heat-labile antibiotics.

Yet another possibility might be to add Liquoid (sodium polyanetholsulfonate) to the serum specimen at a final concentration of 0.05% (equivalent to $500\ \mu\text{g}/\text{ml}$), which is known to neutralize the bactericidal activity of serum (14). However,

Liquoid has been found to markedly inhibit the activity of kanamycin sulfate and polymyxin B in vitro, especially so in the presence of 10% fresh human serum (13); our latest results indicate that gentamicin sulfate also is inhibited by Liquoid, whereas cephalothin, chloramphenicol, lincomycin, the various penicillins, and tetracycline hydrochloride are not affected by this compound. Here, then, the laboratory would have to distinguish between antibiotics that are antagonized by Liquoid and those that are not. It should be added that 0.05% Liquoid in undiluted fresh serum, serum diluted 1:2 and 1:5, but not in higher dilutions of serum, gives rise to an almost instantaneous turbidity (13), which, of course, would obscure the macroscopic determination of the MIC over the range of these lower dilutions of serum.

Increasingly, various investigators use small holes punched into agar which are then completely filled with the appropriate serum dilution (2). Still other workers employ blotting-paper disks that have been soaked with the blood specimen directly and that are immediately placed on a thin layer of agar seeded with the proper test organism (15). It is known that serum is less protoplastcidal in agar than in fluid media (8). It would appear, then, that the use of agar-containing media might circumvent the bactericidal activity of fresh human serum. Preliminary experiments obtained in our laboratory indicate that diluted and undiluted fresh serum, instilled

into nutrient-agar wells or in the form of charged-paper disks, is not bactericidally active at all; no zones of inhibition of growth were noted. These results were obtained with pour-plated serum-sensitive strains of *E. coli*. However, when adding 10 or 20% serum to nutrient-agar deeps containing a total of 10^2 or 10^3 incorporated serum-sensitive organisms, the agar abolished the bactericidal activity of 10% serum, but not that of 20% serum. Clearly, further work is indicated to determine the suitability of solid media for the abolition of the bactericidal activity of biological fluids.

ACKNOWLEDGMENTS

This investigation was aided by a grant from the United Medical Research Foundation of North Carolina.

I thank Pat Reich for her skilled technical assistance.

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