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Influence of Different Media and Bloods on the Results of Diffusion Antibiotic Susceptibility Tests

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As part of an International Collaborative Study, the influence of medium and certain medium components on the results of disc susceptibility testing with nine antibiotics was examined and statistically evaluated. Four basic media, Trypticase Soy Agar, Grove and Randall's formula # 9 agar, and Mueller-Hinton agar with and without 1 mg of L-tryptophan per 100 ml, were used with six bacterial strains. Significant differences in zone diameters occurred with most antibiotics. The largest consistent differences were seen with tetracycline, and appeared to result from varying degrees of chelation of the antibiotic with free cations in the media. Reproducibility studies on different batches of Mueller-Hinton agar from two manufacturers showed some statistically significant differences, which were small except with tetracycline. Reproducibility between the products of a single manufacturer was excellent. The results of experiments to determine the effect of the addition of 5% blood of different species to agar medium showed that medium containing citrated or defibrinated horse, rabbit, sheep, or human blood yielded closely similar zone diameters, except that tetracycline zones were larger with citrated blood. A single type of medium should be selected for routine susceptibility tests whenever possible, and reproducibility of performance of the products of different manufacturers should be sought.

During the initial stages of an International Collaborative Study on antibiotic susceptibility testing techniques, experiments were carried out to determine the reproducibility of results from different laboratories when the same strains, reagents, media, and methods were used, and also to compare results on the same strain obtained by the routine methods used in the different laboratories.

These experiments indicated a number of actual and potential sources of variation that influence the results of susceptibility tests, and several participating laboratories undertook further investigations to define the significance and extent of these variations. The studies reported here were concerned with the reproducibility of results on different media and on different batches of medium. These variables appeared to be important contributors to differences in results between laboratories and bore directly on the selection of a medium for any proposed reference method. The study also included a statistical comparison of results obtained when blood-agar prepared with defibrinated and citrated bloods from several mammalian species was used. This information was pertinent since supplementation was required for satisfactory growth of some organisms with the media examined, and because there are geographic differences in the availability of different animal bloods.

The results of these studies have been described in abstract in the Report of the International Collaborative Study (Acta Pathol. Microbiol. Scand. Sect. B, Suppl. 217, 1971). They are presented here in detail with the technical and statistical methods employed.

MATERIALS AND METHODS

Microorganisms. The organisms used were clinical isolates obtained from patients in the University Hospital, University of Washington.

Antibiotic discs. Discs containing the antibiotics listed in the tables were used. All except those containing cephalothin were provided by Difco Laboratories. Cephalothin discs were obtained from Eli Lilly & Co. Discs were from Food and Drug Administration certified batches and were of the antibiotic contents specified by Bauer et al. (2).

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Media. The following basic media were used along with certain variations described in Results.

Trypticase Soy Agar (TSA) contained 15 g of a pancreatic digest of casein (Trypticase, BBL), 5 g of a papaic digest of soya meal USP (Phytone, BBL), 5 g of NaCl, and 15 g of agar in 1 liter of distilled water. The *p*H of the medium was adjusted to 7.3.

Grove and Randall's (6) formula #9 (GR) medium contained 17 g of a pancreatic digest of casein USP, 3 g of a papaic digest of soya meal USP, 5 g of NaCl, 2.5 g of dipotassium phosphate, and 15 g of agar in 1 liter of distilled water. The *p*H was adjusted to 7.3.

Mueller-Hinton (MH) agar contained dehydrated infusion from 300 g of beef, 17.5 g of an acid hydrolysate of casein, 1.5 g of starch, and 17 g of agar in a final volume of 1 liter of distilled water. The *p*H was adjusted to 7.4.

Unless otherwise specified, dehydrated commercial preparations of TSA and MH agar were used. MH agar was obtained from BBL and from Difco Laboratories. GR medium was prepared in the laboratory according to the above formula. The broths of each of these media were prepared in the laboratory and had the same constitution as their corresponding solid media except for the exclusion of agar. When blood-agar was used, it was prepared by adding 5% blood (final concentration) to TSA.

Bloods. Defibrinated and citrated horse, rabbit, sheep, and human bloods were obtained. The citrated bloods contained a final concentration of 1.25% sodium citrate, which was that concentration being used by the commercial enterprise supplying the animal bloods. Human blood was obtained from volunteers. All bloods were collected within 1 day of each other, refrigerated, and used within 1 week.

Disc diffusion susceptibility testing technique. The method used was based on that described by Ericsson et al. (4). Amounts of 60 ml of medium were used in 14-cm petri dishes, which gave a depth of approximately 4 mm. The inoculum was prepared by growing the test organism overnight in 5 ml of GR broth and diluting this in 0.9% sodium chloride solution. A 10^{-2} dilution was used for staphylococci and enterococci, and 10^{-3} for *Escherichia coli, Klebsiella* species, and *Proteus* species. Plates were seeded by flooding 2 ml of the inoculum onto the surface and drawing off the excess fluid with a pipette. The surfaces were then allowed to dry at room temperature, with the lids tipped, for 10 min before the application of discs. After this drying procedure, the surfaces of the agar had no visible film of fluid. A maximum of nine different antibiotic discs were placed on each plate and pressed onto the agar to insure contact. After placing the discs, a prediffusion period of 3 hr at room temperature was allowed before overnight incubation at 37 C. Inhibition zone diameters were measured with calipers. The end point was taken as an abrupt decline of growth. The plates were always coded and randomized before being read, and were read by two persons.

Broth dilution method. Twofold dilutions of the antibiotic were prepared in broth. The final volume in each tube was 2 ml. Each tube was inoculated with 2 drops of the inoculating suspension delivered with a pipette calibrated to deliver 30 drops per ml. The inoculating suspension was prepared by diluting an overnight broth culture to the MacFarland #1 barium sulfate standard (12). An uninoculated and inoculated control of the medium without antibiotic was also prepared. Tubes were incubated overnight at 37 C. The minimal inhibitory concentration (MIC) was taken as the lowest concentration of antibiotic which inhibited visible growth.

Statistical analyses. In the comparative studies to be described, four replicate plates of each medium were used for each organism unless stated otherwise. Single discs of each of the antibiotics under examination were represented on each plate. An analysis of variance was performed by use of the variance ratio, F, whenever more than two sample means needed to be compared. If a significant difference was found, the Student range test was applied to determine k, the minimum significant difference in zone diameters in millimeters. Statistically significant differences greater than 2 mm were considered to be of potential practical significance. Subsidiary investigations showed that differences in readings between the two readers rarely exceeded this figure.

RESULTS

Influence of different media on inhibition zone diameters. Comparisons were made of the results of diffusion tests performed with TSA, GR medium, MH agar, and MH agar + 1 mg of L-tryptophan per 100 ml (MH + T). The first three media were examined as possible reference media for continuation in the International Collaborative Study; MH with added tryptophan was included because acid hydrolysates of casein are known to be deficient in this amino acid and because a supplemented medium has been used in Japan for many years (10). Two strains of *Staphylococcus aureus* and one strain each of *Klebsiella* sp., *E. coli, P. mirabilis*, and an enterococcus were tested. Each strain was examined simultaneously on the four different media, with four replicate plates for each medium. Table 1 summarizes the results, and shows that there were significant differences between zone diameters on at least two of the media with most antibiotics and organisms. The major statistically significant differences are described below, and a detailed example of the type of results obtained is given in Table 2.

Methicillin and ampicillin zone diameters were larger on MH + T and MH agar than on GR medium and TSA with all organisms tested. Similarly, with penicillin, cephalothin, streptomycin, and kanamycin, zones on MH + T and MH agar were generally larger for all organisms, with the following exceptions. (i) The enterococcus produced consistently larger zones on GR medium and TSA for these antibiotics, and (ii) *S. aureus* 2 had largest zones on MH + T and smallest zones on MH agar with penicillin and

TABLE 1. *Magnitude of statistically significant differences (in millimeters) in mean zone diameters obtained on four different media*[a]

Antibiotic	*S. aureus* 1	*S. aureus* 2	*E. coli*	*Klebsiella*	*Proteus*	*Enterococcus*
Ampicillin	—[b]	—	NS[c]	NS	3.8 (MH + T > GR)[d]	—
Cephalothin	3.2 (MH + T > GR)	4.2 (MH + T > MH)	NS	1.5 (MH > TSA)	NS	1.8 (GR & TSA > MH + T & MH)
Chloramphenicol	1.4 (TSA > GR)	1.8 (TSA > MH)	NS	NS	4.6 (MH + T > GR)	1.7 (TSA > MH + T & MH)
Erythromycin	2.5 (MH + T > GR & MH)	1.6 (TSA > GR)	—	—	—	NS
Kanamycin	4.7 (MH + T > GR)	5.8 (MH + T > GR)	1.7 (MH & MH + T > GR)	3.2 (MH + T > GR)	3.6 M(H + T > GR)	3.4 (GR & TSA > MH + T)
Penicillin	4.5 (MH + T > GR)	4.5 (MH + T > MH)	—	—	—	2.2 (GR & TSA > MH + T)
Polymyxin B	—	—	3.5 (GR > MH + T)	2.5 (GR > MH + T & MH)	—	—
Streptomycin	7.4 (MH & MH + T > GR)	7.4 (MH + T > GR)	2.8 (MH & MH + T > GR)	5.0 (MH + T > GR)	6.0 (MH + T > GR)	—
Tetracycline	7.0 (GR > TSA)	—	7.8 (GR > TSA)	6.7 (GR > TSA)	—	7.2 (GR > TSA)

[a] Trypticase Soy Agar (TSA), Grove and Randall's formula #9 (GR), Mueller-Hinton agar (MH), and MH plus 1 mg of L-tryptophan/100 ml (MH + T).

[b] Not tested.

[c] No statistically significant differences in zone sizes on the four media.

[d] The average difference between the two media showing the largest difference in zone sizes. The differences noted are all statistically significant ($P = 0.05$).

TABLE 2. *Example of mean zone diameters in millimeters of S. aureus 1 on four media*

Antibiotic	Media				*k* value[a]
	TSA	GR	MH	MH+T	
Penicillin	42.2	40.5	43.2	45.0	1.2
Cephalothin	40.2	40.0	40.8	43.2	1.2
Tetracycline	21.2	28.2	25.5	25.8	1.1
Chloramphenicol	30.2	28.8	30.2	30.5	1.3
Erythromycin	34.2	33.0	33.0	35.5	1.2
Streptomycin	19.8	18.8	26.2	26.2	1.6
Kanamycin	28.0	25.8	30.2	30.5	0.9

[a] Minimum difference in millimeters between any two media required for statistical significance.

cephalothin. With chloramphenicol, zones were largest on TSA except for those with *P. mirabilis*, which averaged 4.6 mm larger on MH + T than on GR medium. All organisms tested behaved similarly with tetracycline in that zones were consistently 4 mm or more smaller on TSA than on the other three media. With polymyxin B, zones for both *E. coli* and *Klebsiella* were larger on GR and TSA than on the two MH media.

These experiments thus showed systematic differences in the effects of the different media with some of the antibiotics, but in other cases the results reflected strain differences. These differences could not be accounted for by the slight differences in *p*H between the media (TSA, *p*H 7.3; GR medium, *p*H 7.2 to 7.4; MH agar and MH + T, *p*H 7.4), or by differences in their growth-supporting activities. Except for the enterococcus, which gave equivalent growth on all media, growth was more luxuriant on MH + T and MH agar than on GR medium and TSA. With most antibiotics, zone edges were more diffuse and more difficult to measure on MH + T than on the other media.

Specific influence of medium on tetracycline zones. In the experiments described above, consistently and significantly larger tetracycline zones were obtained on MH agar and GR medium than on TSA. GR medium and TSA differed mainly in

that the formulation of GR medium includes dipotassium phosphate, whereas that of TSA does not. This reagent was therefore added to TSA in equivalent concentrations. Tetracycline zone diameters for *S. aureus* on the modified medium were comparable to those on GR and MH media. These observations suggested that TSA contained a substance which interfered with tetracycline activity and which was counteracted by phosphate. It is known that loss of tetracycline activity occurs by chelation of the drug with divalent and multivalent cations (15), and tests were made to determine whether this could account for the medium-associated differences (Table 3).

Broth dilution and agar diffusion tests were made with the use of TS and MH media supplemented with calcium, magnesium, and ferrous chlorides, sodium citrate, ammonium oxalate, and dipotassium phosphate. MIC values were increased and zone diameters were decreased by addition of the divalent cations, whereas the reverse was encountered when citrate, oxalate, or phosphate was added. Zone sizes for cephalothin and chloramphenicol were unaffected, thus excluding nonspecific effects on zone diameters. These results indicated that differences in free cation concentrations could cause differences in zone diameters and MIC values of the magnitude seen with different media.

Medium components were interchanged between TSA and MH agar to determine the source of the antagonizing substance in TSA. The results of interest are shown in Table 4. They indicate that the substance was present in Trypticase, a pancreatic digest of casein. Trypticase had no such effect in the presence of an acid digest of casein, indicating that the latter contained a neutralizing factor.

TABLE 3. *Effect of certain reagents on the activity of tetracycline against S. aureus in Trypticase Soy and Mueller-Hinton media*

Supplement	Trypticase Soy		Mueller-Hinton	
	MIC (μg/ml)	Zone (mm)	MIC (μg/ml)	Zone (mm)
None	5.0	23	1.25	30
$CaCl_2$, 5×10^{-3} M	5.0	23	2.5	22
$MgCl_2$, 5×10^{-3} M	10.0	20	2.5	25
$FeCl_2$, 5×10^{-3} M	20.0	11	2.5	16
K_2HPO_4, 5×10^{-3} M	2.5	24	1.25	31
K_2HPO_4, 1.6×10^{-2} M[a]	1.25	29	—	—
$Na_3C_6H_5O_7$, 5×10^{-3} M	0.63	29	0.63	33
$(NH_4)_2C_2O_4$, 5×10^{-3} M	2.5	29	1.25	28

[a] Amount of K_2HPO_4 in Grove and Randall's formula.

TABLE 4. *Effect of interchanging media components on tetracycline zone diameters of S. aureus*

Medium	Avg. zone diam (mm)
Mueller-Hinton agar (MH)	26
Trypticase Soy Agar (TSA)	20
MH with Trypticase substituted for Ca[a]	19
TSA with Ca substituted for Trypticase	25
MH with both Ca and Trypticase	26
TSA with both Ca and Trypticase	26

[a] Acid hydrolysate of casein.

Batch to batch reproducibility of MH agar. It is important for any well-standardized laboratory procedure to utilize media or reagents which have minimal variability in performance. For this reason it was necessary to determine the degree of batch to batch variability that a susceptibility testing medium would show. The reproducibility of 14 batches of GR was first tested. Two batches were of commercial dehydrated medium and 12 were prepared from combinations of four batches of pancreatic digest of casein USP and three of papaic digest of soy meal. Tests with a strain of *S. aureus* yielded largest mean zone diameter differences of between 2.5 and 5.3 mm with six of eight antibiotics tested. Our attention thus turned to the reproducibility of different batches of MH agar.

In an initial investigation, four batches of MH agar, two from each manufacturer, were tested. A fully sensitive strain of *S. aureus* was tested on five replicate plates from each batch with penicillin, methicillin, cephalothin, streptomycin, kanamycin, erythromycin, and tetracycline. The variance ratio, *F*, was used to detect significant differences between any of the four batches for each antibiotic. Significant differences were detected among the four batches with kanamycin, streptomycin, tetracycline, and cephalothin, but were of less than 2 mm except with tetracycline. With tetracycline, there was an average difference of 3.5 mm between the products of the two manufacturers. There was, however, good agreement between the two batches of each manufacturer.

In a second study, six additional batches of MH agar were tested, comprising three from each manufacturer. One strain of *S. aureus* and one of *E. coli* were tested. The findings with *S. aureus* were similar to those found in the initial study, but with *E. coli* the variability was greater. The results of this comparison are given in Table 5, where a number of significant differences can be

TABLE 5. *Differences in zone diameters on six batches of Mueller-Hinton agar*

Antibiotic	*S. aureus*		*E. coli*	
	k[a]	lmd[b]	*k*	lmd
Penicillin	1.5	1.9	Not done	Not done
Methicillin	0.9	1.7	Not done	Not done
Cephalothin	1.3	1.8	1.3	2.4
Streptomycin	0.7	1.6	1.2	1.6
Kanamycin	0.9	0.9	0.9	1.9
Erythromycin	1.0	0.9	Not done	Not done
Tetracycline	1.2	4.0	1.2	5.6
Ampicillin	Not done	Not done	2.0	2.5
Polymyxin B	Not done	Not done	0.7	2.4

[a] The *k* value represents the minimum difference in zone diameters (in millimeters) between any two batches required for statistical significance.

[b] The largest average difference that occurred among the six batches.

TABLE 6. *Differences in zone diameters between batches of Mueller-Hinton agar from manufacturers A and B*

Organism and drug	A (3 batches)	B (3 batches)	A and B (6 batches)
S. aureus			
Tetracycline	3.2[a]	1.2	4.0
E. coli			
Cephalothin	0.5	1.0	2.4
Tetracycline	2.8	1.0	5.6
Ampicillin	1.0	0.9	2.5
Polymyxin B	0.9	1.5	2.4

[a] The largest mean difference between zone diameters on different batches.

observed. The five differences which were greater than 2 mm were looked at further by comparing the reproducibility of the batches from each manufacturer separately. This information is shown in Table 6, and it can be seen that, with the exception of tetracycline, batches from a single manufacturer showed good reproducibility. With tetracycline, results were essentially identical among the batches from manufacturer B, but showed greater variation among those from manufacturer A.

Influence of four species of defibrinated and citrated blood on inhibition zone diameters. The extent to which the incorporation of blood into a test medium would affect zone sizes was a factor of importance, since it was recognized that the addition of blood is necessary for growth of certain organisms and since different species of blood are utilized in different locales.

TABLE 7. *Effect of blood on zone diameters*

Antibiotic and organism	Citrated vs. defibrinated[a]		Among bloods[b]		Blood vs. no blood[c]	
	Significance	lmd[d]	Significance	lmd	Significance	lmd
Ampicillin						
E. coli	NS[e]	—	NS	—	NS	—
Klebsiella	NS	—	**[f]	1.9	NS	—
S. aureus	NS	—	NS	—	NS	—
Cephalothin						
E. coli	NS	—	NS	—	NS	—
Klebsiella	NS	—	*[g]	0.7	**	1.2
S. aureus	NS	—	**	1.9	**	2.0
Cloramphenicol						
E. coli	NS	—	NS	—	NS	—
Klebsiella	NS	—	**	1.1	**	1.4
S. aureus	NS	—	NS	—	NS	—
Erythromycin						
S. aureus	NS	—	**	0.8	**	1.5
Kanamycin						
E. coli	NS	—	NS	—	NS	—
Klebsiella	NS	—	NS	—	NS	—
S. aureus	NS	—	**	0.7	NS	—
Penicillin						
S. aureus	NS	—	NS	—	NS	—
Polymyxin B						
E. coli	*	0.6	NS	—	NS	—
Klebsiella	**	0.7	*	0.6	**	0.8
Streptomycin						
E. coli	NS	—	**	1.0	NS	—
Klebsiella	NS	—	NS	—	NS	—
S. aureus	NS	—	**	1.5	**	1.9
Tetracycline						
E. coli	**	3.2	NS	—	**	4.7
Klebsiella	**	3.8	NS	—	**	5.1
S. aureus	**	4.7	NS	—	**	3.6

[a] Comparison of zones on citrated blood-agar and the respective zones on defibrinated blood-agar for all species of blood used.

[b] Comparison of zones on four types of blood-agar: horse, human, sheep, and rabbit.

[c] Comparison of zones on medium without blood to the respective zones with any of the four species of blood incorporated.

[d] The largest mean differences in millimeters.

[e] There were no statistically significant differences detected.

[f] Statistically significant differences were observed ($P = 0.01$).

[g] Statistically significant differences were observed ($P = 0.05$).

For these experiments, TSA plates were prepared with and without the addition of citrated or defibrinated human, horse, sheep, and rabbit blood. Single strains of *E. coli*, *Klebsiella* sp., and penicillin-susceptible *S. aureus* were tested on all media against the appropriate antibiotics.

Duplicate plates for each type of blood-agar were used for each organism. Statistical comparisons were made between results on citrated and defibrinated blood-agars, among results on the four species of blood, and also between TSA with and without blood. All comparisons yielding significant results, except those with tetracycline, were then retested with four replicates. The combined analyses are shown in Table 7. Data on significant results (except with tetracycline) are derived from the larger experiment.

In the comparison between citrated and defibrinated blood-agars, there was no significant difference in zone sizes on TSA containing citrated blood as opposed to defibrinated blood (regardless of species) except with polymyxin B and tetracycline. The differences in polymyxin zone sizes were small (0.6 to 0.7 mm) and cannot be considered of practical importance. For tetracycline, however, zones on citrated blood-agar were considerably larger than on defibrinated blood-agar for each of the species of blood. This difference can be explained by the effect of citrate in counteracting inhibition of tetracycline activity caused by chelation with certain cations.

TABLE 8. *Inhibition zone diameters of a strain of S. aureus tested on agar containing four different species of blood*

Antibiotic	k^a (mm)	Blood	Zone diam (mm)
Ampicillin			NS[b]
Cephalothin	0.7	Human	36.6
		Horse	36.5
		Sheep	35.7
		Rabbit	34.7
Chloramphenicol			NS
Erythromycin	0.6	Sheep	32.2
		Rabbit	32.1
		Human	31.5
		Horse	31.5
Kanamycin	0.7	Horse	24.8
		Rabbit	24.8
		Sheep	24.7
		Human	24.1
Penicillin			NS
Streptomycin	0.5	Rabbit	20.3
		Horse	19.2
		Sheep	19.2
		Human	18.7
Tetracycline			NS

[a] Minimum difference that is necessary in order for that difference to be statistically significant.

[b] No significant differences.

To compare the results among the four species of blood, both citrated and defibrinated blood agars of each species were included together in the analysis, except for tetracycline and polymyxin B which showed significant differences between citrated and defibrinated blood. The comparison showed a number of statistically significant differences in zone sizes on the different blood-agars, but all of these differences were 2 mm or less. Table 8 is an example of how the different bloods compared in actual zone diameters.

Most of the antibiotics tested showed little change in zone size (<2 mm) with the addition of either citrated or defibrinated blood as compared with TSA without blood. The exception to this was tetracycline, with which significant differences were again attributable to the influence of citrated blood.

DISCUSSION

The experiments described here showed that significant, and sometimes large, variations in antibiotic inhibition zone diameters may be encountered when different media are used for diffusion susceptibility tests. The relative order of zone diameters on the different media varied from antibiotic to antibiotic and in some cases with the bacterial species being tested. These results could not be explained by differences in growth rates, in agar content, or in initial *p*H. In the case of tetracycline, the activity of the antibiotic in diffusion tests was shown to be associated with variation in the concentration of free divalent cations in media, and thus with the extent of chelation of the antibiotic. This is believed to explain the differences encountered.

The majority of earlier studies by others led to similar conclusions, but for the most part they were not statistically evaluated. Bauer (1), Bulger and Nielson (3), Guthrie (7), Neter et al. (13), Trainer (14), and Kanazawa(11) all reported considerable influence of different media or medium components on the results of susceptibility tests. In contrast, however, Isenberg (9) concluded from a comparison of 11 media that results were essentially identical on all of them. More recently, Garrod and Waterworth (5) described a striking effect of differences in magnesium content of media and agars on the results of gentamicin susceptibility tests with some strains of *Pseudomonas aeruginosa*.

The consensus of these findings strongly supports the need for selection of a single medium formula for the majority of routine susceptibility tests so that reproducibility of results between

different laboratories can be facilitated. Even this step may not eliminate discrepancies because of batch to batch variation between the products of the same or different manufacturers. Our studies on different batches of MH agar from two manufacturers showed reasonably good reproducibility except with tetracycline, although statistically significant differences were detected with several other antibiotics. Comparison of different batches from a single manufacturer, however, showed a high degree of agreement. This indicates that it should be possible to obtain consistently reproducible media for susceptibility testing if criteria for their preparation and their performance are critically defined and disseminated. Recommendations to this effect have been made elsewhere (Acta Pathol. Microbiol. Scand. Sect. B, Suppl. 217, 1971), and Hoeprich et al. (8) have now described a fully synthetic medium which is satisfactory for susceptibility testing and opens up the possibility of the use of such a medium for reference purposes if not for day to day testing.

A modification of susceptibility test media which is frequently required involves the addition of blood to facilitate growth of more fastidious organisms. Since there are differences in the availability of particular species of blood in different parts of the world, we investigated the effect of citrated and defibrinated horse, rabbit, sheep, and human bloods on susceptibility test results. Neither the species of blood nor the methods of anticoagulation had any pronounced effect on results except with tetracycline. With tetracycline, larger zone sizes were obtained with citrated blood, presumably owing to the binding of free cations. The extent of this effect with media other than that tested would depend on the concentration of citrate used and the extent of tetracycline inactivation by the base medium. Although a number of other statistically significant differences in zone diameters were encountered, they did not exceed 2 mm. It thus appears that defibrinated blood from any of the mammalian species examined can be considered interchangeable for routine bacteriological purposes in susceptibility testing with the antibiotics examined.

It should be pointed out that the results presented here were obtained with a limited number of antibiotics and cannot be assumed to apply to all antimicrobial agents. Some comparisons with sulfamethizole, for example, have shown significant variability between the products of the two manufacturers of MH medium with a strain of *S. aureus* (up to 4 mm) but none with a strain of *E. coli*. Such observations reinforce the need for standardization of media, at least by performance tests. The diffusion method used in these experiments differed somewhat from the reference method proposed by the International Collaborative Study and from the method of Bauer et al. (2). They were, however, sufficiently similar that extrapolation of the conclusions appears justified.

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