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Effect of Mixed Cultures on Antibiotic Susceptibility Testing

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Careful studies of the antibiotic susceptibilities of mixtures of bacteria likely to be encountered in clinical cultures have shown that the results obtained are completely unreliable. Mixtures of resistant and sensitive species appeared either as "resistant" or "sensitive" depending upon the organisms and the drug. A number of sensitive species gave reactions interpreted as resistant when tested in combination. Since reactions of bacterial mixtures are completely unpredictable, the authors emphasize that antibiotic susceptibility testing be limited to pure cultures.

Despite the fact that most competent clinical microbiologists have stressed the importance of employing pure cultures of bacteria for antibiotic sensitivity tests, direct sensitivity testing of mixed cultures is still, unfortunately, a common procedure in many laboratories. Usually this means placing antibiotic disks on plates of media that have been inoculated with clinical material such as swab specimens from a wound or the pharynx and, after incubation, noting zones of inhibition of the mixed flora that are present. This practice is perpetuated by the erroneous concept that any antibiotic which can inhibit all organisms in the culture is the drug of choice for treating the patient. Although this fallacy is readily refuted on the grounds of rational therapeutics, there does not appear to be a documented microbiological basis for the objection to direct sensitivity testing. The present study was undertaken to investigate the effect of mixed bacterial cultures on antibiotic susceptibility testing.

MATERIALS AND METHODS

Organisms. The cultures employed in this study consisted of recent clinical isolates of *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus* Viridans Group, *Streptococcus faecalis, Corynebacterium species, Neisseria catarrhalis, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa.*

Susceptibility testing. Transfers were made from five well-isolated colonies of each organism to a tube containing 4 ml of Trypticase Soy Broth (BBL). A drop or two of sterile defibrinated sheep blood was added when streptococci, corynebacteria, or neisseria were being tested. The broth cultures were incubated at 35 C for 2 hr (6 hr for streptococci and corynebacteria) and were then used as inoculum for the test. Plates of Columbia Agar Base (BBL) were inoculated by uniformly streaking the entire surface of the agar with a cotton swab that had been dipped in the broth culture and drained of excess fluid by pressing against the inside wall of the tube. A second "purity check" plate of the same medium was inoculated with a loopful of the broth culture and streaked for isolation. Defibrinated sheep blood (5%) was added to the Columbia Agar base when streptococci, corynebacteria, or neisseria were tested. Fresh, high-potency discs were applied by means of a Difco dispenser, and each disc was pressed to the surface of the agar with sterile forceps. No more than eight discs were ever employed on a single plate. Five discs of each antibiotic were tested against each organism. Test plates were held at room temperature for 3 hr to allow diffusion of the antibiotics and were then incubated at 35 C for 14 hr. The sizes of inhibition zones were measured and the standard deviation and standard error were calculated. Organisms were interpreted as being "sensitive" if the zone diameter exceeded the minimum size previously determined by testing numerous isolates against that drug. These minimum diameters are listed in Table 1.

Combinations of organisms were tested by fishing three colonies of each organism into Trypticase Soy Broth and incubating as described above. Duplicate tubes were prepared for each combination of organisms, and each tube was used to inoculate five sets of plates, thus testing each combination against every drug 10 times. Inoculation and incubation were the same as previously described. The relative proportion of the two organisms in each inoculum was estimated by streaking the broth culture on an additional plate and noting their relative numbers. Table 2 lists the combinations of organisms employed and their relative numbers.

RESULTS

Zone sizes obtained with pure cultures of the test organisms and their interpretation are shown in Table 3.

TABLE 1. *Minimum zone diameter for interpretation as "sensitive"*

Drug	Disk potency	Minimum zone size
	μg	*mm*
Penicillin	10 units	25
Oxacillin	1	13
Ampicillin	10	11
Cephalothin	30	15
Cephaloridine	30	20
Polymyxin[a]	10	10
Streptomycin	10	12
Kanamycin	30	17
Erythromycin	15	16
Chloramphenicol	30	15
Tetracycline	30	15
Nalidixic acid	30	17

[a] Polymyxin B or colistin.

TABLE 2. *Combinations of organisms and their relative numbers*

Mixture	Ratio
Escherichia coli-Pseudomonas	1:1
E. coli-Klebsiella	2:1
Klebsiella-Pseudomonas	10:1
E. coli-Proteus	5:1
Klebsiella-Proteus	1:1, 2:1
Proteus-Pseudomonas	4:5, 1:5
Staphylococcus-E. coli	1:6, 1:2
Staphylococcus-Klebsiella	1:4, 1:2
Staphylococcus-Pseudomonas	1:6, 1:5
Staphylococcus-Proteus	*
Streptococcus faecalis-E. coli	1:3, 1:4
S. faecalis-Klebsiella	1:3, 1:6
S. faecalis-Proteus	*
S. pyogenes-E. coli	1:2, 1:4
S. pyogenes-Klebsiella	1:1
S. pyogenes-Proteus	*
S. pyogenes-Pseudomonas	1:2, 1:3
Staphylococcus-Streptococcus pyogenes	4:1, 3:1
Staphylococcus-Streptococcus faecalis	1:1
Staphylococcus-Corynebacterium	1:7, 1:5
Streptococcus Viridans-*S. faecalis*	1:4, 1:3
S. Viridans-*Corynebacterium*	1:8, 1:1
S. Viridans-*S. pyogenes*	1:3
Corynebacterium-S. faecalis	3:1
Corynebacterium-S. pyogenes	7:1, 5:1
Neisseria-S. pyogenes	1:1, 2:1
Neisseria-Staphylococcus	1:8, 1:6
Neisseria-Streptococcus Viridans	1:4, 1:20
Neisseria-Corynebacterium	1:1

* Unable to determine ratio due to spreading of *Proteus*.

TABLE 3. *Mean zone diameters and standard deviations of test organisms*[a]

Drug	*Staphylococcus aureus*				*Streptococcus faecalis*			*Streptococcus pyogenes*			*Streptococcus* Viridans group			*Corynebacterium* species			*Escherichia coli*			*Klebsiella pneumoniae*			*Proteus mirabilis*			*Pseudomonas aeruginosa*			*Neisseria catarrhalis*		
	MZD	SD		Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity	MZD	SD	Sensitivity
Penicillin	16	0.8	R		17	0.9	R	40	0	S	37	0.5	S	33	0.6	S	8	0.5	R	0	0	R	0	0	R	37	0.1	S
Oxacillin	18	0.6	S		0	0	R	27	0.5	S	21	1.0	S	12	1.0	R	6	0.4	R	0	0	R	0	0	R	15	0.6	R
Ampicillin	21	0.6	S		26	1.0	S	42	0	S	40	0.9	S	38	1.0	S	23	0.8	S	0	0	R	26	2.0	S	40	2.0	S
Cephalothin	33	0.9	S		19	0.7	S	42	0.9	S	38	0	S	40	0.9	S	22	0.4	S	20	0.5	S	26	1.0	S	36	3.0	S
Cephaloridine	29	0.7	S		17	0.8	R	46	0.9	S	39	0.8	S	45	0.8	S	25	0.7	S	22	1.0	S	22	1.0	S	34	2.0	S
Streptomycin	20	0.5	S		0	0	R	14	3.0	S	16	2.0	S	25	0.8	S	17	0.7	S	0	0	R	20	0	S	19	1.0	S
Kanamycin	23	0.6	S		0	0	R	0	0	R	13	0.6	R	25	1.0	S	21	1.0	S	23	0.9	S	24	2.0	S	23	2.0	S
Ethrythromycin	33	0.8	S		0	0	R	32	0.5	S	37	0.5	S	36	0.9	S	15	0.4	R	7	4.0	R	0	0	R	31	1.0	S
Tetracycline	26	0.8	S		0	0	R	27	0.8	S	26	1.0	S	24	2.0	S	19	1.0	S	0	0	R	0	0	R	25	1.0	S
Chloramphenicol	29	1.0	S		24	1.0	S	31	0.9	S	34	2.0	S	34	1.0	S	26	2.0	S	18	0.8	S	25	1.0	S	33	2.0	S
Polymyxin	3	4.0	R		0	0	R	0	0	R	0	0	R	10	1.0	S	13	0	S	13	0.8	S	0	0	R	11	1.0	S
Nalidixic acid	11	0.8	R		0	0	R	0	0	R	0	0	R	4	6.0	R	26	0.5	S	19	0.5	S	27	1.0	S	24	3.0	S

[a] Abbreviations: (MZD) mean zone diameters; (SD) standard deviation. (R) resistant and (S) sensitive, based on data in Table 1.

TABLE 4. *Combinations of sensitive and resistant organisms appearing "resistant"*

Sensitive	Resistant	Drug[a]
Streptococcus pyogenes	*E. coli*	pen, oxa, erythro (9, 0.5)
Streptococcus pyogenes	*Klebsiella*	pen, oxa, amp, erythro, strep (1, 4.0), tetra
Streptococcus pyogenes	*Proteus*	pen (11, 1.0), oxa, erythro, tetra
Streptococcus pyogenes	*Pseudomonas*	pen, oxa, amp, ceph, lor, erythro, strep (4, 6.0), tetra (12, 0.7)
Streptococcus pyogenes	*Staphylococcus*	pen (14, 0.7)
Corynebacterium	*Staphylococcus*	pen (19, 0.4), poly
Corynebacterium	*Streptococcus faecalis*	pen (23, 0.8), lor (18, 0.8), erythro, strep, kana, tetra, poly
Corynebacterium	*S.* Viridans	kana (9, 0.7), poly
Corynebacterium	*S. pyogenes*	kana (4, 5.0), poly (3, 3.0)
Streptococcus Viridans	*S. faecalis*	pen (22, 1.1), oxa, lor (16, 0.7), erythro, strep, tetra
Neisseria	*Staphylococcus*	pen (11, 0.9), poly, nal (11, 0.6)
Neisseria	*Streptococcus pyogenes*	kana (8, 6.0), poly (4, 5.0), nal (3, 5.0)
Neisseria	*Streptocossus* Viridans	kana (10, 0.8), poly, nal
Neisseria	*Corynebacterium*	nal
Staphylococcus	*E. coli*	oxa, erythro (12, 0.6)
Staphylococcus	*Klebsiella*	oxa, amp, erythro (8, 0.5), strep (4, 4.0), tetra
Staphylococcus	*Proteus*	oxa, erythro (2, 4.0), tetra
Staphylococcus	*Pseudomonas*	oxa, amp, ceph, lor, erythro, strep (11, 0.8), kana (9, 1.0), tetra (11, 0.5)
Staphylococcus	*Streptococcus faecalis*	oxa, lor (16, 0.7), erythro, strep, kana, tetra
Staphylococcus	*S. pyogenes*	kana (1, 3.0)
Escherichia coli	*Pseudomonas*	amp, ceph, lor, strep (12, 1.0)
Escherichia coli	*Klebsiella*	amp, strep, tetra
Escherichia coli	*Streptococcus faecalis*	strep, kana, tetra, poly
Escherichia coli	*S. pyogenes*	kana (6, 5.0), poly, nal
Escherichia coli	*Proteus*	tetra, poly
Escherichia coli	*Staphylococcus*	poly (7, 2.0), nal (12, 1.5)
Proteus	*Klebsiella*	amp, strep
Proteus	*Pseudomonas*	amp, ceph, lor, strep (11, 0.8), kana (8, 6.0)
Proteus	*Streptococcus faecalis*	lor (17, 0.8), strep, kana, nal
Proteus	*S. pyogenes*	kana (1, 3.0), nal (1, 3.0)
Proteus	*Staphylococcus*	nal (12, 1.5)
Streptococcus faecalis	*Klebsiella*	amp
Streptococcus faecalis	*Pseudomonas*	amp, ceph
Klebsiella	*Pseudomonas*	ceph, lor, kana (10, 0.7)
Klebsiella	*Streptococcus faecalis*	lor (16, 0.8), kana, poly, nal
Klebsiella	*S. pyogenes*	kana (3, 5.0), poly, nal (4, 4.0)
Klebsiella	*Proteus*	poly
Klebsiella	*Staphylococcus*	poly (8, 0.5), na (12, 2.0)
Pseudomonas	*Proteus*	poly
Pseudomonas	*Staphylococcus*	poly (5, 3.0), nal (12, 2.0)
Pseudomonas	*Streptocloccus faecalis*	poly, nal
Pseudomonas	*S. pyogenes*	poly, nal (1, 3.0)

[a] Abbreviations: pen, penicillin; oxa, oxacillin; erythro, erythromycin; strep, streptomycin; tetra, tetracycline; amp, ampicillin; ceph, cephalothin; lor, cephaloridine; poly, polymyxin; kana, kanamycin; nal, nalidixic acid. Numbers in parentheses represent the mean zone diameters in millimeters and the standard deviations. Absence of numbers indicates no zone.

TABLE 5. *Combinations of sensitive organisms appearing as "resistant"*

Mixture	Drugs[a]
Staphylococcus-Streptococcus pyogenes	Oxacillin (9, 1.0), streptomycin (6, 3.0)
Staphylococcus-Neisseria	Oxacillin (10, 0.8)
Staphylogoccus-Pseudomonas	Chloramphenicol (14, 0.7)
Streptococcus pyogenes-Escherichia coli	Streptomycin (10, 0.9)
S. pyogenes-Klebsiella	Cephaloridine (19, 0.6); Chloramphenicol (15, 0.5)
S. pyogenes-Proteus	Cephaloridine (18, 0.8); Streptomycin (8, 3.0)
S. pyogenes-Pseudomonas	Chloramphenicol
S. pyogenes-Neisseria	Streptomycin (7, 5.0)
S. pyogenes-Corynebacterium	Streptomycin (6, 4.0)
S. pyogenes-S. Viridans	Streptomycin (2, 4.0)
S. Viridans-*Corynebacterium*	Streptomycin (10, 1.0)
S. Viridans-*Neisseria*	Streptomycin (12, 0.6)
S. faecalis-Pseudomonas	Chloramphenicol
E. coli-Klebsiella	Cephaloridine (19, 0.5)
E. coli-Proteus	Cephaloridine (20, 1.0)
E. coli-Pseudomonas	Nalidixic acid (14, 2.0)
Klebsiella-Pseudomonas	Nalidixic acid (10, 0.6)
Proteus-Pseudomonas	Chloramphenicol (14, 1.0); Nalidixic acid (13, 0.9)

[a] Numbers in parentheses represent the mean zone diameters in millimeters and the standard deviations, respectively. Absence of numbers indicates no zone.

Combinations of organisms resulted in zone diameters ranging in size from the largest of the two zones obtained when the organisms were tested separately to smaller than either of the two "pure-culture" zones. Thus in many cases the combination of a sensitive organism with a resistant species resulted in a zone size interpreted as "resistant." The combinations of organisms giving such reactions are listed in Table 4.

Combination of the oxacillin-resistant diphtheroid with the oxacillin-sensitive *S. pyogenes* or *Neisseria* resulted in zone sizes interpreted as "sensitive" when tested against oxacillin.

A number of sensitive organisms gave zone sizes interpreted as "resistant" when tested in combination. These are listed in Table 5.

DISCUSSION

This study has clearly shown that the use of mixed cultures may give completely unreliable results regarding the antibiotic susceptibility of the component organisms. The appearance of a reaction interpreted as "resistant" from a combination of two resistant organisms could certainly have been predicted. The combination of a sensitive and a resistant organism may obviously appear as either "sensitive" or "resistant" depending upon the organisms and the drug. A surprising finding was that the combination of two sensitive organisms could give a "resistant" result.

It is not implied that the results obtained with the organisms tested apply to all strains. Since the mechanisms remain unclear, it might be expected that considerable strain variation occurs, and that it is not possible to predict the result of any combination.

The selection of organisms for this study was made only for investigational purposes and was believed to represent combinations of saprophytes and pathogens that are likely to occur in direct sensitivity testing. In actual clinical practice, susceptibility studies would not be done on *Corynebacterium* species or *N. catarrhalis*, rarely on *S. pyogenes*, and on members of the Viridans Group of streptococci only when they are isolated from areas of the body that are normally sterile. Likewise, in the routine diagnostic laboratory, penicillin, oxacillin, and erythromycin are only tested against gram-positive species (and *Bacteroides*), cephalothin against both gram-positive and gram-negative isolates, and the remainder of the drugs are only tested against gram-negative species. Nalidixic acid is only tested against gram-negative isolates from the urinary tract.

It is worth noting that the therapeutic goal in infectious diseases is elimination of the etiologic agent or agents rather than the entire microflora. Treatment of a streptococcal infection of a wound or throat should be directed solely toward the eradication of *S. pyogenes*, with the minimum disturbance of the normal commensal organisms. It has been recognized for some time that the commensal flora play an important role in the local resistance to infection, and that displacement or elimination of these organisms may result in serious superinfection (1).

Proponents of direct sensitivity testing often cite the time saved by circumventing isolation and identification of bacteria. In life-threatening infections, good clinical practice consists in obtaining the appropriate cultures and starting empirical treatment without delay. It would be folly for a physician to defer treatment of a critically ill patient only to be misled by completely unreliable laboratory results obtained by direct sensitivity testing.

There are certain instances when the determination of antibiotic susceptibility prior to the isolation and identification of the organism is justifiable. Such circumstances are limited to those situations where the infection is almost always monobacterial (such as positive blood or spinal fluid cultures), or when the specimen has been treated to inactivate all bacteria other than the specific pathogen (such as sputum containing many acid-fast bacilli).

It is obvious from the results obtained in this study that the dictum so often stated (and so often ignored) that antibiotic susceptibility tests should be performed only with pure cultures has a sound factual basis.

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LITERATURE CITED

1. Louria, D. B., and T. Kaminski, 1962. The effects of four antimicrobial drug regimens on sputum superinfection in hospitalized patients. Amer. Rev. Resp. Dis. 85:649–665.