THE SENSITIVITY OF HEMOPHILUS PERTUSSIS AND RELATED ORGANISMS TO VARIOUS ANTIBIOTIC AGENTS

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W. J. Stafseth
Major professor

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By

Chester A. Hornbeck

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SENSITIVITY OF HEMOPHILUS PERTUSSIS AND RELATED ORGANISMS TO VARIOUS ANTIBIOTIC AGENTS

INTRODUCTION

During recent years, new antibiotic agents have been discovered in rapid succession. Their importance can hardly be exaggerated, yet they are, collectively, not a panacea for all infectious diseases. Some of these antibiotic agents are spectacularly effective against a very few organisms, while others are active against a wide variety. On the other hand, some organisms are more or less resistant to all of these agents. Among the infections which have generally resisted treatment with antibiotics is pertussis. It should be noted, however, that most of the published studies are concerned with streptomycin, chloromycetin, or aureomycin.

In many reports, the primary study consisted of a determination of the sensitivity in vitro of the organism to the antibiotic in question. This is, of course, desirable since it may provide some basis for prediction of the success of the antibiotic in preventing or curing the disease. Care must be taken in evaluating any in vitro test since promising results are not always borne out in clinical trial.

Hegarty, Thiele, and Verwey (1945) presented evidence that streptomycin in a concentration of 15 mcg/ml was bactericidal to H. pertussis in vitro. When infected mice were treated with 0.5 mg doses, the survival time averaged approximately twice that of the controls, but there was still an ultimate mortality of 90 per cent.

Saiber and Homans (1951) demonstrated that 0.2 to 0.3 mcg/ml of chloromycetin inhibited growth of H. pertussis in vitro. In subsequent

in vivo experiments 100 per cent protection was achieved in some instances when the intranasal route of inoculation was used. In intracerebral infection chloromycetin was much less effective.

Bell, Pittman, and Olson (1949) showed that aureomycin hydrochloride given subcutaneously to mice in apparently non-toxic doses not only delayed the time of death but also prevented deaths due to prior intracerebral infection with H. pertussis. No in vitro experiments were reported. Preliminary clinical trials with children suggested that aureomycin given orally in apparently non-toxic doses shortened the clinical course of the disease.

A number of other investigators (Alexander and Redman, 1949; Day and Bradford, 1952; Shih Man, 1950) have published their findings on the sensitivity of H. pertussis and similar organisms to a number of antibiotics. In the majority of cases, however, only one method of testing was used. In the matter of investigating the possibilities of the synergistic effect of combinations of antibiotics against H. pertussis, very little has been done.

This study was planned with three major objectives, as follows:

I. Sensitivity of H. pertussis and related organisms to various antibiotics as determined by the use of antibiotic discs and a solid medium.

II. Sensitivity of H. pertussis and related organisms to various antibiotics using a liquid medium.

III. Synergistic or antagonistic action of various combinations of antibiotics.

The use of both solid and liquid media was intended to afford a more complete basis for evaluating the antibiotic agents under test. Also it was hoped that the results would be of some significance in comparing the two methods.

The choice of the related organisms, Brucella bronchiseptica and the parapertussis bacillus rested largely upon the work of Kendrick and Eldering (1938), and Anderson (1952). The former investigators showed by agglutination-absorption techniques and cross agglutination reactions that the three organisms are related but not identical. Anderson (1952) in a recent report, indicated that these organisms have a common "O" antigen, whereas variations of the antigenic structure of the three organisms were apparently due to slightly different "B" and/or "A" antigens, based on differentiation according to Kaufmann (1951). Both Br. bronchiseptica and the parapertussis bacillus have been reported as the cause of respiratory infections in human beings (Shih Man, 1950, and Eldering and Kendrick, 1951). Because of the similarity of these two organisms to H. pertussis it seemed reasonable to include them in the study. The parapertussis bacillus has never been given an official generic designation. Since its taxonomic position has not been clarified, it will be referred to here merely as the parapertussis bacillus.

Part I

SENSITIVITY OF H. PERTUSSIS AND RELATED ORGANISMS TO VARIOUS ANTIBIOTICS AS DETERMINED BY THE USE OF ANTIBIOTIC DISCS.

Materials

The medium used in this portion of the study was Bordet-Gengou medium which was made up as follows: The Bordet-Gengou agar base medium (Difco) was hydrated by suspending 4.5 gm in 100 ml of a one per cent solution of glycerol in distilled water and heating to boiling to dissolve the agar completely. It was then autoclaved for fifteen minutes at 15 lbs. pressure (121C). The medium was allowed to cool to approximately 45C and aseptically enriched with sterile sheep blood to 15 per cent by volume. After thorough mixing plates were poured, incubated at 37C for 24 hours to eliminate contaminated plates, and stored in a refrigerator.

The antibiotic discs used were the Bacto Sensitivity Discs, manufactured by the Difco Laboratories, Detroit 1, Michigan. The following antibiotics were tested: bacitracin, chloromycetin, dihydrostreptomycin, penicillin, aureomycin, and terramycin. Each of these agents was supplied in three concentrations which are indicated in table 1.

The lyophilized cultures of the three test organisms were supplied by the Western Michigan Section Laboratory of the Michigan Department of Health. The 6 H. pertussis and 11 parapertussis cultures were isolated from cough plates collected in Grand Rapids from children with whooping cough. Br. bronchiseptica 22067 was isolated at Thorndyke Memorial Hospital, Boston, from a child with symptoms of whooping cough. This culture was received in Grand Rapids from Dr. Martha Ward,

Communicable Disease Center, Chamblee, Georgia. The culture Br. bronchiseptica 2464 was isolated from a ferret by Dr. W. Ferguson, Michigan Department of Health, Lansing.

Methods

Prior to conducting the tests, the cultures were reconstituted by suspending the contents of the ampule in approximately 0.2 ml of sterile physiological saline, plating on the Bordet-Gengou medium, and incubating at 35 C, for 24 to 48 hours. After suitable growth had been established, enough growth was removed by loop and emulsified in 2 ml of sterile saline to give a suspension of approximately 1.2 billion organisms per ml. Previous tests had shown that 0.3 ml of such a suspension, when spread evenly over the surface of the plate, provided the necessary confluent growth and the suspending saline was quickly absorbed by the medium.

After the inoculum had been spread over the plate and had been absorbed by the medium, the sensitivity discs were aseptically placed upon the surface using flamed forceps. No more than three discs were placed on the surface of the medium to avoid crowding. Since the discs come in three concentrations of each antibiotic, one plate was sufficient for determining the effect of one antibiotic.

After the discs had been placed on the surface of the medium, the plates were incubated in an upright position for 24 hours at 35 C. In the case of H. pertussis, the incubation period was increased to 48 hours. The results were read by noting the diameter of the clear zone of no growth which surrounded the discs.

TABLE 1

Sensitivity of H. pertussis, parapertussis bacillus, and Br. bronchiseptica as indicated by antibiotic discs
Mean diameter in centimeters of inhibition zone with each concentration of antibiotic

Organism	Penicillin Units			Terramycin Mcg			Dihydrostreptomycin Mcg			Bacitracin Units			Chloromycetin Mcg			Aureomycin Mcg		
	0.5	1	10	10	30	60	1	10	100	2	10	20	10	30	60	10	30	60
H. pertussis																		
23919	0.6	0.8	1.4	1.5	2.0	2.6	0.8	1.1	1.3	0.7	1.5	2	3.4	3.7	3.9	0	1.2	1.6
18323	0.5	0.7	1	1.2	2.1	2.6	0.6	0.8	0.9	2.0	2.4	3.2	3.6	3.8	4	1.0	1.7	2.0
23360	0.5	0.6	1.1	1.6	2.7	2.9	0.5	0.6	0.9	1.8	2.9	3.9	3.3	3.9	3.9	2.0	2.2	2.6
19605	0.7	1.1	1.9	2.1	2.5	2.9	0.8	0.8	1	1	1.3	2.8	3.3	3.8	4	0	1.8	2.3
18560	0	0.9	1.5	3.2	3.6	4	1	0.8	2.2	1.5	2.8	3.1	3.7	3.9	4	0	1.6	3.0
10536	0.8	1	1.2	2.1	2.9	3.4	0.7	1	1.2	2.0	2.8	3.1	3.1	3.5	3.7	0.8	2.1	3.7
parapertussis bacillus																		
23788	0	0	0	2.7	3.1	3.3	0	0	0.9	0.7	1.8	2.2	2.5	3	3.5	0.8	1	1.3
17903	0	0	0	2.5	2.9	3.6	0	0	0.8	0.9	1.7	2.1	2.7	3.2	3.8	1.1	1.7	2.5
23807	0	0	0.7	2.8	3.1	3.5	0	0	1.1	0	1.5	1.8	3.0	3.3	4	0.8	1.2	1.9
22345	0	0	0	2.8	2.8	3.2	0	0	0.8	1	0.9	1.6	2.8	3.4	3.8	2.0	2.1	2.8
23456	0	0	0.5	2.7	3.0	3.5	0	0	1.1	0.6	0.9	1.6	2.9	3.5	4	1.2	1.8	2.8
23910	0	0	0	2.6	3.0	3.6	0	0	1.2	0.9	1.7	2.0	2.9	3.2	3.5	1	1.6	2.8
23054	0	0	0	2.7	3.1	3.4	0	0	0.7	0.9	1.3	1.7	3.8	4	4	1.3	2.6	2.8
21851	0	0	0	2.2	2.5	3.1	0	0	1.6	0.8	1.1	1.8	2.8	3.2	3.6	0.9	1.3	1.9
23310	0	0	1.1	3.0	3.8	4	0	0	1.2	1.1	1.6	1.8	2.5	3.7	4	1.4	2.8	2.9
21838	0	0	0	3.2	3.8	4	0	0.7	1.1	0.9	1.8	2.0	3.0	3.4	3.9	1.6	1.8	2
21551	0	0	0.7	3.0	3.7	3.9	0	0	1.6	0.6	1.1	1.4	3.2	3.6	4	2.8	3.2	3.3
Br. bronchisepticus																		
22067	0	0	0	1.5	1.7	1.8	0	0	0.6	0	1.0	1.2	1.5	2.1	2.3	0.6	1.7	1.9
2464	0	0	0	1.7	1.9	2.1	0	0	1.3	0	0	0.5	1.5	1.7	1.8	0	0	0.7

Results

Several duplications of the tests with antibiotic discs were made, and the results are given in table 1. The figures represent the mean of the recorded diameters of the clear zones, in centimeters. These results are shown in a condensed form in table 2, where the results are expressed as an average of the observations with cultures of each species.

Sensitivity of H. pertussis. Six cultures of H. pertussis were tested, and these were all inhibited to some degree by each of the antibiotics tested. According to the size of the zone of inhibition, the greatest effect was observed with chloromycetin and the least effect with penicillin and dihydrostreptomycin. Terramycin, bacitracin and aureomycin, all appeared to have the same general inhibitory effect.

Sensitivity of the parapertussis bacillus. Unlike H. pertussis, the parapertussis bacillus was almost completely resistant to penicillin and was inhibited by dihydrostreptomycin only in the 100 meg amount. Only one of the 11 cultures tested showed any inhibitive zone with the 10 meg disc. Chloromycetin and terramycin were the most effective. Bacitracin and aureomycin also were inhibitory in all three concentrations although the zones were not as large.

Sensitivity of Br. bronchiseptica. Only two cultures of this organism were tested and there was some difference in their sensitivity. However, both were resistant to penicillin and dihydrostreptomycin. They showed less resistance to bacitracin and aureomycin. Again terramycin and chloromycetin appeared to be the most effective of the antibiotics.

Discussion

The results with H. pertussis are qualitatively similar to those with the other two organisms. Penicillin had a considerably greater inhibiting action upon H. pertussis than upon either the parapertussis bacillus or Br. bronchiseptica. Dihydrostreptomycin showed results which were very similar to that of penicillin. Hegarty et al., (1945) showed that H. pertussis was inhibited by 3 mcg of streptomycin, but mice infected with H. pertussis and treated with streptomycin showed a 90 per cent mortality rate. The observations recorded in table 2 indicate that there is an inhibiting effect with 1 mcg, but that the inhibitory effect is not markedly increased by a much greater concentration. Since penicillin has been shown to be of little value in animal or clinical tests with H. pertussis as the infecting agent, and streptomycin but little better, it seems possible that zone size may not be a reliable criterion for choice of a suitable agent in animal tests. On the other hand, chloromycetin, which displayed a mean inhibition zone diameter of 3.4 cm in a concentration of 10 mcg has been shown to be active against experimental pertussis infections. Sarber and Hemans (1951), have shown that 0.3 mcg of chloromycetin per ml is inhibitory to H. pertussis and that in treatment of mice infected with H. pertussis, 100 per cent survived in some cases.

Terramycin and bacitracin showed an intermediate inhibitory effect as compared with chloromycetin and dihydrostreptomycin. Aureomycin gave results showing appreciably more inhibition than dihydrostreptomycin or penicillin, though much less inhibition than chloromycetin. This is an interesting finding since Bell et al., (1949) have reported promising

results in animals with aureomycin and, to a lesser extent, in clinical treatment of children. However, in tests with mice, the dosages were as high as 10 mcg, which would produce a relatively high blood level. The increased inhibiting effect of higher concentrations of aureomycin as compared with streptomycin or penicillin as shown in table 2 would give an indication of possible inhibition at high concentrations of the antibiotic in the blood.

It is of note that Brande and Dockrill, (1952) have reported that the inhibition zone diameter when using aureomycin may be considerably less than some other antibiotic, yet in a liquid medium it is much more effective. Successful treatment of clinical infections due to strains sensitive to aureomycin in the test tube indicated that the tube method was more reliable in their study.

From the results given in table 1, it is evident that penicillin and dihydrostreptomycin have little effect upon the parapertussis bacillus in any concentration. This result is not surprising since Alexander and Redman (1949) found in a recent study that when using Bordet-Gengou plates containing a concentration of 1,000 mcg of streptomycin, a considerable number of colonies of parapertussis bacilli grew from the original inoculum. The authors concluded from this result that there were some extremely resistant organisms initially present in every population examined. One might, therefore, expect to find isolated colonies in the clear zone of inhibition surrounding the antibiotic disc; however, none was found. A possible explanation is that the occasional clear zone found around the disc containing the highest concentration of dihydrostreptomycin did not include a large enough number of organisms to demon-

TABLE 2

Summary of Results with Antibiotic Discs Showing Mean Diameter Observed with Several Cultures.

	H. pertussis	Parapertussis bacillus	Br. bronchi-septica
Penicillin (Units)			
0.5	0.5	0	0
1	0.8	<0.1	0
10	1.3	0.2	0
Terramycin (Mcg)			
10	1.9	2.7	1.6
30	2.6	3.2	1.8
60	3.0	3.6	2.0
Dihydrostreptomycin (Mcg)			
1	0.8	0	0
10	0.9	<0.1	0
100	1.2	1.1	0.9
Bacitracin (Units)			
2	1.5	0.8	0
10	2.3	1.4	0.5
20	3.0	2.1	0.9
Chloromycetin (Mcg)			
10	3.4	2.9	1.5
30	3.7	3.4	1.9
60	3.9	3.8	2.1
Aureomycin (Mcg)			
10	0.6	1.4	0.3
30	1.7	2.1	0.9
60	2.5	2.3	1.3

strate extremely resistant colonies.

In the case of parapertussis, the most effective antibiotic is chloromycetin, followed very closely by terramycin. Of noticeably less effectiveness are aureomycin and bacitracin. Br. bronchiseptica followed the same pattern, i.e., penicillin and dihydrostreptomycin had little or no effect, chloromycetin and terramycin proved to be the most effective and very nearly identical in their effect, and aureomycin was similar to bacitracin.

The results given in table 2 indicate that as far as relative sensitivity to antibiotics is concerned, parapertussis is more sensitive to all antibiotics used than is Br. bronchiseptica.

Conclusions

1. Of the organisms studied, Br. bronchiseptica was the most resistant, the parapertussis bacillus was of intermediate resistance, and H. pertussis the least resistant to the antibiotics penicillin, terramycin, dihydrostreptomycin, bacitracin, chloromycetin, and aureomycin.
2. H. pertussis was sensitive in some degree to each of the six antibiotics.
3. There was very little variation among the sensitivity of the six strains of H. pertussis to the antibiotics studied.

Part II

SENSITIVITY OF H. PERTUSSIS AND RELATED ORGANISMS TO VARIOUS ANTIBIOTICS USING A LIQUID MEDIUM.

According to plan, when data had been accumulated on the effect of various antibiotics upon the test organisms using the disc method, the next step was to study the tube dilution method with the same agents and cultures. The purpose was to determine more exact endpoints of inhibition, and also to obtain data for comparison of the two methods. This comparison seemed especially important with aureomycin, since Braude (1952) has pointed out discrepant results between test tube and disc with this antibiotic. He showed that aureomycin was much more effective in broth cultures than was suggested by the use of aureomycin discs. However, his observations did not include H. pertussis.

Medium

Since we had had considrable experience with the semi-synthetic medium described by Cohen and Wheeler (1946), this medium was chosen for the sensitivity studies. It contains casein hydrolysate, various salts, starch, and yeast dialysate, and is a clear faintly yellowish fluid. The formula as published by Cohen and Wheeler (1946) follows.

Fluid Medium for H. pertussis

(Casein hydrolyzate medium with yeast dialyzate)

Casamino acid (Bacto)	10 grams
Sodium chloride (A.C.S.)	2.5 grams
Monopotassium phosphate, KH_2PO_4(A.C.S.)	0.5 gram
Magnesium chloride, $MgCl_2.6H_2O$ (A.C.S.)	0.4 gram

Starch, soluble, powdered	1.5 gram
Calcium chloride, $CaCl_2$ (A.C.S.), 1 per cent solution	1 ml
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$ (A.C.S.), 0.5 per cent solution	2.5 ml
Copper sulfate, $CuSO_4 \cdot 5H_2O$ (A.C.S.), 0.05 per cent solution	1.5 ml
Cysteine hydrochloride, 1 per cent solution	3.0 ml
Yeast dialysate	50 ml
Distilled water to make	1 kg

The casamino acid, salt, phosphate, and magnesium chloride were dissolved in part of the water. The starch, dissolved separately by heat in part of the water, was added and then the remaining ingredients. After making up to 1 kg, the pH was adjusted to 7.2-7.3. The medium was dispensed in 6 ml amounts in 175-x 22-mm tubes and autoclaved at 10 lbs. pressure for fifteen minutes.

The yeast dialysate was prepared from Fleischmann pure dry yeast, type 2019, dialyzed against distilled water at 78-80 C for 7 hours.

The antibiotics were generously furnished by Dr. P. C. Fink of the Chas. Pfizer Co., Rahway, New Jersey.

Methods

Two strains of H. pertussis, 10536 and 18560, one strain of the parapertussis bacillus, 21838, and one strain of Br. bronchiseptica, 22067, were chosen after preliminary testing as typical and representative of the three species. Approximately two weeks before conducting the experiments these organisms were removed from the dried state by suspending the pellets from the lyophile tube in sterile physiological saline. This suspension was spread over the surface of

Bordet-Gengou plates and the plates incubated until growth was established, usually 48 hours. Incubator temperature was maintained at 35 C throughout, unless otherwise stated. Several successive transfers were made, and the cultures studied for typical morphology and hemolysis. When the criteria for smooth strains had been satisfied, isolated colonies were streaked on Bordet-Gengou agar slants and incubated for 48 hours. At the end of this period another transfer was made, incubated for 24 hours, harvested by pipetting 2 ml of the liquid medium into the test tube and gently rubbing off the surface growth. This suspension was transferred to 125 ml Erlenmeyer flasks containing approximately 10 ml of liquid medium. Gram stains were made before transferring the suspension to the flasks to disclose any gross contamination. After 24 hours incubation, portions were removed and Gram stained for purity. After purity was established, other portions were inoculated into the flasks as described above. This procedure was carried out for three successive days. At this time, samples of each strain were both stained and plated on Bordet-Gengou medium to rule out morphological changes and loss of hemolytic ability. If no change was observed, the culture was considered to be satisfactory for the remainder of the test.

Upon the day of the test, the antibiotic under study was dissolved in a measured amount of the liquid medium to provide a known concentration of antibiotic. Dilutions were made, in which the concentration was twice that to be tested, to allow for addition of an equal quantity of the culture.

One ml of each concentration was placed in a 15 x 90 mm test

tube. To each tube was added 1 ml of a one billion per ml Cohen-Wheeler suspension of H. pertussis. Previous tests showed that a final density of 0.5 billion per ml of H. pertussis was necessary to insure growth. The parapertussis bacillus suspension was also adjusted to one billion per ml. All density determinations were made with a Genco photelometer using the pyrex glass suspension from the National Institutes of Health, representing an opacity of 10 billion organisms per ml. The Br. bronchiseptica suspension was diluted to 2 million organisms per ml since this organism is much less fastidious than the other two, and grows more rapidly. Controls consisting of 1 ml of Cohen-Wheeler medium plus 1 ml of the seed suspension were included. All tubes were then incubated for 48 hours. At the end of this period, suitable dilutions were made, and portions plated on Bordet-Gengou plates. The plates were incubated for 96 hours in the case of H. pertussis, and 48 hours for the two other organisms. After the incubation period colonies were counted, morphology and staining properties observed, and results recorded as percentage of surviving organisms, arrived at by comparing number of colonies on test and control plates.

Results

The results of the sensitivity tests are shown as an average of several tests in tables 3, 4 and 5. From the data on H. pertussis in table 3, it may be seen that penicillin, dihydrostreptomycin, chloromycetin and neomycin, in this order, are considerably more effective against this organism than are aureomycin, bacitracin and terramycin, with the method used. The latter three antibiotics are

very similar to each other in their effectiveness. The data in table 4 indicate that the parapertussis bacillus is generally more resistant to all the antibiotics except polymixin and neomycin. Neomycin and polymixin, respectively, were the most effective agents against parapertussis. Chloromycetin, terramycin and aureomycin were respectively somewhat less effective, and bacitracin, penicillin and dihydrostreptomycin much less effective.

The data in table 5 show that chloromycetin, aureomycin, polymixin and neomycin were, respectively, the most effective against Br. bronchiseptica, showing quite comparable results. Terramycin, dihydrostreptomycin, bacitracin and penicillin were all much less effective in their action.

Additional experiments, using solid medium. In order to obtain further data on the sensitivity of the test organisms, Bordet-Gengou agar plates were prepared containing known amounts of the various antibiotics. The choice of the concentrations was governed by results with the discs since comparison of disc and liquid medium methods was desirable. The preparation of the medium has already been described (Methods, Section I). Before pouring the plates, the proper concentration of antibiotic in 1 ml was added to 49 ml of the blood agar medium, thoroughly mixed and poured. After the plates had cooled, and had been incubated to disclose any contamination, they were seeded with 0.35 ml of a saline suspension containing approximately 80 organisms per .03 ml. The plates were incubated for 48 to 96 hours, colonies counted, and results expressed in per cent of survival, as compared to control plates inoculated in the same manner at the same time.

Since polymixin discs in three concentrations, and neomycin in one concentration, had become available, plates were prepared as in Part I, and these data are included with results of the plate method in tables 6 and 7.

Results

It is evident from these tables that in the majority of cases the results shown by the disc method were in agreement with results given by the poured plate method. The results shown by the tube dilution method were not in as good agreement with either the tube dilution or poured plate method if H. pertussis is considered. The results shown by the tube dilution were more comparable to results of the other two methods in the case of the parapertussis bacillus and Br. bronchiseptica. With neomycin discs of 10 mcg concentration, the inhibition zones indicated considerable effectiveness against the three organisms. Polymixin B discs of 5 mcg concentration produced inhibition zones considerably smaller than neomycin with the organisms tested.

Discussion

The results obtained in using a liquid medium should be considered in the light of results with antibiotic discs since it is especially important to establish the degree of correlation between the two methods.

In table 2 penicillin and streptomycin discs at respective levels of 10 units and 100 mgs produced inhibition zone diameters of no more than half the size of the inhibition zones of the other four antibiotics tested against H. pertussis. However, in the test tube, using liquid medium, table 3, these two antibiotics produced 100

TABLE 3

Per cent Survival of *H. pertussis* After Incubation for 48 hours in a Medium Containing Various Quantities of Antibiotics

Culture Number	Units or Micrograms									
	20	15	12.5	10	7.5	5	2.5	1	0.5	Control
				Aureomycin						
10536	0	12	15	17	42	63	62	76	91	100
18560	0	3	4	6	9	10	21	49	73	100
				Chloromycetin						
10536	0	0	0	0	0	0	0	41	46	100
18560	0	0	0	0	0	0	1	22	30	100
				Terramycin						
10536	8	11	16	21	24	24	24	53	73	100
18560	19	20	20	21	29	38	50	69	77	100
				Polymixin						
10536	0	0	0	0	0	0	17	25	95	100
18560	0	0	0	0	0	1	22	36	90	100
				Dihydrostreptomycin						
10536	0	0	0	0	0	0	0	0	0	100
18560	0	0	0	0	0	0	0	13	61	100
				Bacitracin						
10536	0	18	19	20	35	39	47	93	100	100
18560	0	19	24	29	37	43	52	84	96	100
				Penicillin						
10536	0	0	0	0	0	0	0	0	10	100
18560	0	0	0	0	0	0	0	0	19	100
				Neomycin						
10536	0	0	0	0	0	0	0	17	95	100
18560	0	0	0	0	0	0	1	35	39	100

TABLE 4

Per cent Survival of parapertussis No. 21838 After Incubation for 48 Hours in a Medium Containing Various Quantities of Antibiotics*

Antibiotic	20	15	12.5	10	7.5	5	2.5	1	0.5	Control
Aureomycin	1	1	3	4	4	4	40	56	73	100
Chloromycetin	0	0	0	1	2	6	10	36	40	100
Terramycin	1	1	1	2	2	2	33	38	53	100
Polymixin	0	0	0	0	0	0	0	19	74	100
Dihydro-streptomycin	63	71	79	Not Calculated						100
Bacitracin	12	18	20	27	32	37	62	79	86	100
Penicillin	42	47	50	62	75	80	88	98	100	100
Neomycin	0	0	0	0	0	0	0	0	2	100

*In units or micrograms.

TABLE 5

Per cent Survival of Br. bronchiseptica No. 22067 After Incubation for 48 Hours in a Medium Containing Various Quantities of Antibiotics*

Antibiotic	20	15	12.5	10	7.5	5	2.5	1	0.5	Control
Aureomycin	0	0	0	0	1	1	5	90	95	100
Chloromycetin	0	0	0	0	0	1	7	29	57	100
Terramycin	27	25	52	94	Not Calculated					100
Polymixin	0	0	0	1	10	69	72	91	96	100
Dihydrostrepto-mycin	32	56	62	89	Not Calculated					100
Bacitracin	84	91			Not Calculated					100
Penicillin	87	98			Not Calculated					100
Neomycin	0	0	0	1	1	2	4	36	53	100

per cent inhibition at the 2.5 unit level (penicillin) and the 2.5 mcg level (dihydrostreptomycin). Indeed, penicillin produced complete inhibition at the one unit level. No other antibiotic gave complete inhibition of both strains at the 2.5 unit or mcg level. These results suggest that both penicillin and dihydrostreptomycin in the form of discs used on Bordet-Gengou plates gave inhibition zones much smaller than their effectiveness in liquid medium would indicate. This discrepancy could be similar to that observed by Braude (1952) when studying aureomycin; however, there was good correlation between the effectiveness of aureomycin in the test tube and the success in clinical cases in his study. No similar correlation has been noted with either dihydrostreptomycin or penicillin in animal tests or clinical cases of infection with H. pertussis. In contrast to the foregoing, aureomycin at a comparable level of concentration of 10 mcg displayed a somewhat smaller zone of inhibition than did either penicillin or dihydrostreptomycin, yet at the 2.5 mcg level, there were survival rates of 62 and 21 per cent with strains 10536 and 18560, respectively, when tested with aureomycin. It must also be noted that aureomycin is distinctly more effective in treating experimental and clinical infections caused by H. pertussis (Hazen et al, 1951).

Inhibition zones produced by terramycin and bacitracin were similar and the results shown in table 3 indicate that there is reasonably close agreement between the effects of the two antibiotics in the liquid medium. Comparison of the zone diameter with the per cent of survival suggests that there might be a smaller percentage of H. pertussis surviving than shown in table 3. Polymixin produced an

inhibition zone at the 5 mcg level almost identical with penicillin at the 10 unit level; however, penicillin allowed no survivors at the 1 unit level while polymixin allowed survivors at the 2.5 mcg level. Of the antibiotics tested, chloromycetin and neomycin (table 7) respectively gave the best correlation in that the diameters of the inhibition zones were the largest produced by any of the antibiotics at the 10 mcg or unit level and inhibition in liquid media at the identical level was complete. Lack of better correlation between the solid medium disc method and the liquid medium method when testing the H. pertussis might be attributed to the slowness of growth of the organisms. After 48 hours in liquid medium some of the more sensitive antibiotics, aureomycin (Braude and Dockrill, 1952) for example, may have partially lost their effectiveness. Another possible explanation is the large initial inoculum, approximately 0.5 billion per ml final density, necessary to establish dependable growth in a stationary liquid medium. It is certainly a possibility that this large inoculum might be of an overwhelming nature and produce erratic results.

The parapertussis bacillus, table 4, gave results showing in general a better correlation with the disc method. Penicillin and dihydrostreptomycin discs gave very small inhibition zones, and when tested in the liquid medium the number of survivors was greater than 50 per cent at the 10 unit or mcg level. Again the best correlation between the results with the two methods was with chloromycetin.

Using the disc method, bacitracin showed inhibition zones much smaller than terramycin against parapertussis, and in the test tube, at any level of concentration, the per cent of survivors was greater

TABLE 6

Sensitivity of *H. pertussis* and Related Organisms to Various Antibiotics Incorporated in Bordet-Gengou Medium.

Antibiotic	Concentration per ml	Per cent Survival		
		H. pertussis	Parapertussis	*Br. bronchi-septica*
Chloro-mycetin	10 mcg	0	0	0
Neomycin	10 mcg	0	0	0
Disc Method*		2.2	2	1.8
Terra-mycin	10 mcg	0	0	0
Poly-mixin B	5 mcg	0	0	23
Disc Method*		1.4	1.2	0.9
Baci-tracin	2 units	22	100	100
Peni-cillin	10 units	0	100	100
Aureo-mycin	10 mcg	0	0	0
Dihydro-streptomycin	1 mcg	0	100	100

*Zone diameter (cm) of inhibition.

TABLE 7

Sensitivity of H. pertussis and Related Organisms to Various Antibiotics. A Comparison of Three Methods.

Antibiotic Concentration and Method	H. pertussis	Parapertussis	Br. bronchisentica
Chloromycetin 10 mcg			
Disc(cm inhibition zone)	3.4	2.9	1.5
Test Tube(Per cent Survivals)	0	0	0
Poured Plate(Per cent Survivals)	0	0	0
Neomycin 10 mcg			
Disc	2.2	2	1.8
Test Tube	0	0	0
Poured Plate	0	0	0
Terramycin 10 mcg			
Disc	1.9	2.7	1.6
Test Tube	6	1	1
Poured Plate	0	0	0
Polymixin B 5 mcg			
Disc	1.4	1.2	0.9
Test Tube	0	0	69
Poured Plate	0	0	23
Bacitracin 2 units			
Disc	1.5	0.8	0
Test Tube	24	28	91
Poured Plate	22	100	100
Penicillin 10 units			
Disc	1.3	0.2	0
Test Tube	0	62	100
Poured Plate	0	100	100
Aureomycin 10 mcg			
Disc	0.6	1.4	0.3
Test Tube	12	4	0
Poured Plate	0	0	0
Dihydro-streptomycin 1 mcg			
Disc	0.8	0	0
Test Tube	0	100	100
Poured Plate	0	100	100

with bacitracin than with terramycin. Aureomycin gave results quite similar to those obtained when using the H. pertussis, i.e. a reasonably large zone of inhibition using discs but survivors in the highest concentrations in the test tube. The correlation between the disc and test tube method was thought to be due primarily to the somewhat better growth of the parapertussis bacillus compared to H. pertussis.

Br. bronchiseptica, tables 2 and 5, gave results similar to those observed with the parapertussis bacillus. Penicillin and dihydrostreptomycin discs produced very small inhibition zones, and these antibiotics in the test tube showed but little inhibition. Chloromycetin and terramycin discs gave almost identical inhibition zones, 1.5 and 1.6 cm respectively at the 10 mcg level. In the test tube there was complete inhibition of the organism at this level with both antibiotics. There was evidence of the small inhibition zone of the aureomycin disc being misleading, as Braude (1952)reported. At the 10 mcg level the inhibition zone diameter was 0.3 centimeters as compared with a diameter of 1.5 centimeters at the same level of chloromycetin, yet there was complete inhibition with both agents in the test tube. Bacitracin gave results similar to those of penicillin and dihydrostreptomycin in that the discs produced small inhibition zones and the survival rate was more than 75 per cent with the highest concentration of antibiotic.

The general correlation of the two methods when using Br. bronchiseptica as the test organism was of the same order as with parapertussis. The agreement was much closer with these organisms than with H. pertussis.

TABLE 8

Decreasing Activity of a Number of Antibiotics Against *H. pertussis* and Related Organisms. A Comparison of Two Methods.

	Test Tube Method Using a Liquid Medium*	Disc Method Using a Solid Medium*
H. pertussis	Penicillin Dihydrostreptomycin Neomycin Chloromycetin Polymixin B Aureomycin Terramycin Bacitracin	Chloromycetin Neomycin Terramycin Bacitracin Polymixin B Aureomycin Dihydrostreptomycin Penicillin
Parapertussis	Neomycin Polymixin B Chloromycetin Terramycin Aureomycin Bacitracin Penicillin Dihydrostreptomycin	Chloromycetin Terramycin Neomycin Aureomycin Polymixin B Bacitracin Dihydrostreptomycin Penicillin
Br. bronchiseptica	Chloromycetin Aureomycin Neomycin Polymixin B Terramycin Dihydrostreptomycin Bacitracin Penicillin	Neomycin Chloromycetin Terramycin Polymixin B Aureomycin Dihydrostreptomycin Bacitracin Penicillin

*Denotes decreasing inhibition zone size in disc method and decreasing survival percentage in the test tube method.

Summary

Table 8 presents a summarized comparison of the overall results of the disc and dilution methods. The results with the test tube method were based on per cent of survival at comparable levels when determining the order of activity of the antibiotic in question. It is evident from table 8 that there is no correlation between the results of the two methods in the case of the H. pertussis. However, if antibiotics shown to be the most efficacious in the treatment of experimental pertussis infections in animals or clinical human infections are considered, Hazen et al (1951), the disc method is much more reliable. With either of the other two organisms used in this study, the results of the two methods are similar. There was a marked similarity of results between the poured plates and the disc method. Comparative results between the poured plate method and the test tube method were not as striking. It is interesting that with bacitracin the results with the two methods were nearly identical. As previously stated, bacitracin using the disc method gave an inhibition zone which might indicate a greater inhibiting action than is actually present.

Conclusions

Using the tube dilution method, the various antibiotic agents were tested for inhibitory action against H. pertussis, the parapertussis bacillus, and Br. bronchiseptica. Based upon the data, the conclusions were as follows:

1. H. pertussis was most susceptible to penicillin, dihydrostreptomycin, neomycin and chloromycetin,

and least to terramycin, bacitracin and aureomycin. This was in disagreement with the results with discs.

2. The parapertussis bacillus was most susceptible to neomycin and polymixin, least susceptible to dihydrostreptomycin, penicillin and bacitracin, and of intermediate susceptibility to chloromycetin, terramycin, and aureomycin. This was in agreement with the results shown by discs in a majority of the antibiotics tested.

3. Br. bronchiseptica was most susceptible to chloromycetin, aureomycin, neomycin and polymixin, and least susceptible to penicillin, bacitracin, dihydrostreptomycin and terramycin. This was in general agreement with the results with the discs in a majority of the antibiotics tested.

4. The results of both the antibiotic disc method and antibiotic containing Bordet-Gengou medium method were in close agreement; further both of these methods were more reliable than the tube dilution method using Cohen-Wheeler medium in predicting the most successful agents for treatment of infections as shown by clinical studies by other investigators.

Part III

STUDY OF SYNERGISTIC OR ANTAGONISTIC ACTION OF VARIOUS COMBINATIONS OF ANTIBIOTICS.

In recent studies Jawetz and coworkers (1952), studied the effect of a number of antibiotic combinations against both gram positive and gram negative organisms. No reports on synergistic action upon H. pertussis have been found in the literature. It was decided to determine if there were combinations of antibiotics which would display synergism or antagonism in their action on the three organisms under study.

Method

The concentrations of the antibiotics to be used in combination were based upon the results of each used alone in liquid medium, shown in tables 3, 4 and 5. The concentration of each antibiotic chosen was such that the surviving population was approximately 25 per cent of the control without antibiotic. Cohen-Wheeler medium was made up to contain 8 times the desired concentrations, to allow for dilution in the tests.

For each combination tested the set-up consisted of four 15 x 90 mm test tubes containing the following:

Tube 1. One half ml of each antibiotic in concentrations 8 times the final concentration required per ml.

Tube 2. One ml of one of the antibiotics appearing in the combination.

Tube 3. One ml of the other antibiotic present in the combination.

Tube 4. One ml of plain Cohen-Wheeler medium. This tube served as a control.

H. pertussis culture suspension prepared as in Part II was added, 1 ml to each tube. The procedure was repeated for the parapertussis bacillus and Br. bronchiseptica. After a suitable incubation period, 48 hours for H. pertussis and the parapertussis bacillus and 24 hours for Br. bronchiseptica, suitable dilutions were plated on Bordet-Gengou plates, the plates incubated and colonies counted. The results were expressed according to the following scheme:

Antagonism (Antag.)	Indicates the surviving population equals the control within normal dilution error.
Indifference (Ind.)	Indicates the surviving population is from 50 to 90 per cent of the control.
Slight synergism (+)	Indicates a surviving population of from 25 to 49 per cent as compared to the control.
Moderate synergism (++)	Indicates a surviving population of from 10 to 24 per cent.
Marked synergism (+++)	Indicates a surviving population of less 10 per cent.
Complete (Comp.)	Indicates no surviving population.

The above description of the synergistic activity is emperical particularly when defining "Indifference". It would probably be

fallacious to assume that one antibiotic in the combination would inhibit one portion of the microbial population and the other antibiotic of the combination an entirely different portion even though these agents are chemically different. It is more reasonable to presume that both agents, initially, exert to some degree an overlapping inhibitory effect upon the same organisms but may then act somewhat more selectively and separately upon different, more resistant survivors. The results from the various combinations are given in table 9.

Results

H. pertussis. Of the combinations in table 9, those containing bacitracin exhibit the most synergistic activity, either marked or moderate. Combinations containing dihydrostreptomycin also displayed synergistic activity though not marked. Penicillin combinations were of less activity. Neomycin combinations showed variable activity, i.e., indifference, complete inhibition, and moderate activity.

Parapertussis bacillus. All bacitracin combinations, and neomycin combinations, table 9, showed very similar and slightly synergistic activity in the majority of combinations. One bacitracin combination and one neomycin combination exhibited moderate activity. Dihydrostreptomycin combinations showed slight activity except that of dihydrostreptomycin and aureomycin which completely inhibited the parapertussis bacillus.

Br. bronchiseptica. Bacitracin combinations exhibited indifferent synergistic activity with the exception of bacitracin and terramycin, which was completely inhibitory. Neomycin combinations showed slight activity except with terramycin with which it was completely inhibitory.

TABLE 9

Synergistic Effects of Antibiotic Combinations upon H. pertussis and Related Organisms

Antibiotic Combinations	H. pertussis	parapertussis bacillus	Br. bronchiseptica
Penicillin plus Neomycin	+	+	+
Penicillin plus Dihydrostreptomycin	Ind.	Ind.	Ind.
Penicillin plus Bacitracin	+	+	+
Dihydrostreptomycin plus Bacitracin	++	+	Ind.
Dihydrostreptomycin plus Neomycin	++	+	+
Dihydrostreptomycin plus Chloromycetin	+	+	Antag.
Dihydrostreptomycin plus Aureomycin	++	Comp.	+
Dihydrostreptomycin plus Terramycin	++	+	++
Bacitracin plus Neomycin	+++	+	Ind.
Bacitracin plus Chloromycetin	++	+	Ind.
Bacitracin plus Aureomycin	+++	++	Ind.
Bacitracin plus Terramycin	++	+	Comp.
Neomycin plus Chloromycetin	Ind.	+	+
Neomycin plus Aureomycin	Comp.	+	+
Neomycin plus Terramycin	++	++	Comp.

Dihydrostreptomycin combinations ranged from antagonism to moderate activity. Penicillin combinations were of slight or indifferent synergistic activity.

Discussion

The results shown in table 9 are what might be expected i.e., H. pertussis, generally the most sensitive to the antibiotics tested, being the most susceptible appearing to be synergistic action, and the parapertussis bacillus, of intermediate sensitivity, displaying the same general sensitivity to the combinations but of a noticeably lower order. Br. bronchiseptica, which is the most resistant of the three organisms to single antibiotics also appeared to be the most resistant to the combinations. Of interest too is the fact that both H. pertussis and the parapertussis bacillus showed a fairly uniform degree of sensitivity, the former being of eather moderate or marked sensitivity, and the latter of generally slight sensitivity to the majority of combinations. On the other hand, the activity of the combinations against Br. bronchiseptica was quite variable, ranging from antagonism to moderate effectiveness in the combinations which included dihydrostreptomycin. This variance was not as marked in the other combinations.

Because of the unexpected results obtained when using single antibiotics in liquid medium, there seemed to be adequate justification for testing several of the combinations in Bordet-Gengou plate for confirmation of the effect observed in liquid medium.

The combination of penicillin and dihydrostreptomycin was selected since with all three organisms the synergistic activity

appeared to be indifferent. Three combinations which gave complete inhibition, one for each organism, were also tested.

The plates were prepared as described previously except that the two antibiotics were mixed together immediately before being added to the measured amount of Bordet-Gengou medium. The inoculum used was the same as described in Part II. The plates were incubated for either 48 or 96 hours depending on the organism, and survivors expressed as a per cent of the colonies appearing on the control plates. These results are given in table 10.

Results

With both combinations tested, H. pertussis exhibited indifferent sensitivity. The parapertussis bacillus exhibited the same general type of indifferent sensitivity although there appeared to be evidence that the combination of dihydrostreptomycin and aureomycin was slightly more active. Br. bronchiseptica showed indifferent sensitivity in the case of the penicillin and dihydrostreptomycin combination, and complete sensitivity to the combination of bacitracin and terramycin. Of the combinations tested, the only definite evidence of synergism noted was in the case of terramycin and neomycin tested with Br. bronchiseptica.

Discussion

The results in table 10 indicate again the lack of agreement between the two methods, particularly with H. pertussis and to a lesser extent with the parapertussis bacillus. In contrast, the results confirm the agreement of the two methods with Br. bronchiseptica as the test organism.

TABLE 10

Synergistic Activity of Antibiotic Combinations Using a Solid Medium

Antibiotic Combinations	Per cent of Survival		
	H. pertussis	parapertussis bacillus	*Br. bronchiseptica*
Penicillin	88	57	85
Penicillin plus Dihydrostreptomycin	88	85	92
Dihydrostreptomycin	82	77	68
Control	100	100	100
Aureomycin	70		
Aureomycin plus Neomycin	78		
Neomycin	75		
Control	100		
	This combination with *H. pertussis* only.		
Aureomycin		78	
Aureomycin plus Dihydrostreptomycin		55	
Dihydrostreptomycin		70	
Control		100	
	This combination with the parapertussis bacillus only.		
Terramycin			52
Terramycin plus Neomycin			0
Neomycin			83
Control			100
	This combination with *Br. bronchiseptica* only.		

The combination of dihydrostreptomycin and penicillin which exhibited "indifferent" synergistic activity in the liquid medium was indifferent in all cases when the Bordet-Gengou medium was used. If only this combination were used as a basis of comparison between the two methods, it would suggest good agreement. However, when the results of the other combinations previously shown to give complete inhibition are observed, distinct disagreement is seen with both H. pertussis and the parapertussis bacillus. Br. bronchiseptica, on the other hand, is completely inhibited regardless of the method. On the basis of these and some of the results shown in Part II, it appeared that synergistic activity of combinations of antibiotics against both H. pertussis and the parapertussis bacillus in the liquid medium method as described and the Bordet-Gengou medium method would have to be supported by further in vitro testing as well as animal tests before reliability of either method could be established.

While the results in table 10 disagree insofar as the two methods are concerned, the discrepancy between percentages of survival is not great with the single antibiotics. In all cases, the unit level, chosen as a result of the testing in liquid medium, was found to allow more than fifty per cent survival as was planned. In most cases, more than seventy per cent survival was noted. This being the case, the results of testing single antibiotics in a liquid medium may be considered reliable in terms of survivors, i.e., the concentration of an antibiotic necessary to inhibit a certain percentage range of less than fifty per cent of the three organisms may be relied on to inhibit, within limits, a like percentage on Bordet-Gengou medium. The

Activity of antibiotic combinations in liquid medium is not wholly predictable with respect to the activity found using Bordet-Gengou medium if H. pertussis and the parapertussis bacillus are to be considered. If Br. bronchiseptica is employed as the test organism, the activity of antibiotic combinations is more likely to be comparable with both methods.

Conclusions

1. Synergistic activity of various combinations of antibiotics against the three organisms in this study was demonstrated by use of Cohen-Wheeler liquid medium containing the various combinations.
2. The synergistic activity of certain antibiotic combinations against the pertussis and parapertussis organisms shown in the liquid medium were not duplicated by use of Bordet-Gengou medium.
3. The synergistic activity of certain other combinations of antibiotics in the liquid medium was duplicated when using Bordet-Gengou medium.
4. The percentage of surviving organisms resulting from the activity of a single antibiotic in the liquid medium remained comparable with Bordet-Gengou medium at the same antibiotic concentration with the three organisms tested.

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