In Vitro Susceptibility of El Tor and Classical *Vibrio cholerae* Strains to Trimethoprim and Sulfamethoxazole

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Trimethoprim and sulfamethoxazole were tested individually and in combination against El Tor and classical biotype strains of *Vibrio cholerae*. Determinations of the minimal inhibitory concentration in liquid media and by an agar-dilution method showed that classical strains were uniformly more resistant to sulfamethoxazole than were El Tor strains. In agar-dilution studies, trimethoprim was equally active against both biotypes. Combination of the agents produced a synergistic action against all strains tested. Testing for susceptibility to sulfonamide is suggested as a possible method for differentiating between El Tor and classical biotypes of *V. cholerae*.

Clinical studies in Iran (4) and East Pakistan (unpublished data) suggest that a trimethoprim-sulfamethoxazole combination may be effective in the treatment of clinical cholera. Although both studies were incomplete, the drug combination appeared to be effective in cholera caused both by the classical and by the El Tor biotypes of *Vibrio cholerae*. Since neither study employed the components of the combination individually, however, it is not possible to determine from such data the role of each component in achieving a desired therapeutic success. Sulfaguanidine has been reported to be of no value in the treatment of cholera caused by the El Tor biotype (10). Such information suggested the possibility that trimethoprim was the major, if not the only, active ingredient of the trimethoprim-sulfamethoxazole combination, and that the sulfa component played a minor role.

The in vitro studies reported here indicate that trimethoprim is equally active against both biotypes, whereas sulfamethoxazole is much less active against the classical biotype than against El Tor strains. A combination of the two components showed increased potency against both biotypes, and was particularly synergistic against the more resistant classical strains.

MATERIALS AND METHODS

The organisms used in these studies were from a variety of geographic areas, and included both classical and El Tor biotypes (Table 1). Prior to these studies, they had been stored lyophilized. At the time of the

experiments, they were passed on Trypticase Soy Agar (BBL) two to four times before being subcultured in broth for the studies. Susceptibility studies in liquid media were carried out with two classical and two El Tor strains, according to the method of MacLowry et al. (7). Briefly, serial twofold microdilutions of the antibiotics were prepared in Trypticase Soy Broth in plates. An equal volume of a 4- to 6-hr culture of the organism to be tested in Trypticase Soy Broth was then added to each well, the plates were covered, and the cultures were incubated at 37 C overnight. The next day, the plates were examined for visible growth. That dilution in which no growth was visible was considered to contain the minimal inhibitory concentration (MIC) of the antibiotic for that strain of organism. For combination studies, one antibiotic was diluted vertically in the microdilution plates and the other horizontally, thus producing a wide range of concentration combinations.

Because of the difficulty of ascertaining a precise end point in liquid media, an agar-dilution method was subsequently adopted. Culture plates were prepared from serial twofold dilutions of antibiotics in Trypticase Soy Agar. Four- to six-hour cultures of test organisms were diluted optometrically to 10^5 organisms/ml, and 0.01 ml was placed on a 1-cm diameter spot on the antibiotic-containing plates. Plates were incubated at 37 C for 48 hr, and spots were examined for visible growth at 24 and 48 hr. Four end points were noted: complete inhibition of growth at 24 hr, greater than 90% inhibition of growth at 24 hr, complete inhibition at 48 hr, and over 90% inhibition at 48 hr. The lowest antibiotic concentration inhibiting growth according to each of these criteria was noted as the MIC according to that method.

As the antibiotic concentration producing greater

TABLE 1. Sources and characteristics of classical and El Tor strains studied^a

Biotype	Strain	Place and year isolated	Hemolysis		V-P ^b 22°C	Phage IV ^c	CCA ^c	Poly B ^d	Serotype
			Plate	Tube					
Classical	VC 12	E. Pakistan, 1960	0	0	0	+	0	+	Ogawa
	B1307	E. Pakistan, 1965	0	0	0	+	0	+	Inaba
	CRC 31/64	India, 1964	0	0	0	+	0	+	Inaba
	369	India, 1964	0	0	0	+	0	+	Inaba
	395	India, 1964	0	0	0	+	0	+	Ogawa
El Tor	HK-1	Hong Kong, 1961	+	+	+	0	+	0	Ogawa
	M-27	Malaya, 1963	+	0	+	0	+	0	Ogawa
	PI 1551	Vietnam, 1965	+	0	+	0	+	0	Ogawa
	Iran-46	Iran, 1965	+	0	+	0	+	0	Ogawa
	3083	Vietnam, 1965	+	0	+	0	+	0	Ogawa
	CRC 9933/64	India, 1964	+	0	+	0	+	0	Ogawa

^a Tests performed as described in references 10 and 5.^b Voges-Proskauer.^c Chicken red blood cell hemagglutination.^d Polymixin B susceptibility.

than 90% inhibition at 24 hr was often less than, for example, the concentration producing complete growth inhibition at 48 hr, the highest and lowest MIC of each drug were noted for each strain of *V. cholerae*. Combination studies were carried out at a 1:5 ratio of trimethoprim to sulfamethoxazole, according to the same technique. The trimethoprim (Burroughs-Wellcome & Co., Inc.) was supplied as lactate at 20 mg/ml. Sulfamethoxazole (Hoffman-La Roche, Inc.) was dissolved in water at 40 mg/ml with the use of 1 N NaOH to increase solubility, after which the solution was adjusted to pH 7.4 with 1 N HCl.

RESULTS

Two classical and two El Tor strains were studied in the microdilution liquid media system (Table 2). The classical strains were 5 to 30 times less susceptible to trimethoprim than were the El Tor strains, and over 1,000 times less susceptible to sulfamethoxazole. End points were easily determined for trimethoprim, with which no inhibition was separated from complete inhibition by only one or two dilutions. Sulfamethoxazole, however, caused some inhibition even at low concentrations, so that complete inhibition was separated from no inhibition by as many as seven two-fold dilutions. This phenomenon was less marked with the more susceptible El Tor strains. The studies in which a combination of trimethoprim and sulfamethoxazole were used exhibited sharp end points and showed substantial reductions in the concentration of the two agents required for inhibition. This was particularly marked with both classical strains, against which as little as 0.05 µg of sulfamethoxazole/ml produced growth inhibition when used in combination with trimethoprim.

TABLE 2. Minimal inhibitory concentrations against *V. cholerae* in liquid media

Strain	Minimal inhibitory concn (µg/ml)		
	Tri- methop- rim ^a	Sulfame- thoxazole ^a	T/S combination ^b
<i>Classical</i>			
B1307.....	7.9	5,000	0.04/25-1.2/0.05
VC 12.....	50	7,000	0.3/100-4.7/12
<i>El Tor</i>			
CRC 9933.	1.7	4.4	0.04/0.4-0.08/0.1
PI 1551....	2.0	8.8	0.04/0.4-0.08/0.2

^a Each value represents the geometric mean of two to four MIC determinations.^b Expressed as trimethoprim concentration/sulfamethoxazole concentration. The range of combination MIC values is given for each strain; the intermediate concentration combinations also produced inhibition.

These results clearly demonstrated a marked increase in potency when the drugs were used in combination. They also showed a significant difference in the susceptibility of the El Tor and the classical strains to both agents, but in particular sulfamethoxazole. The diffuse end points in the studies with sulfamethoxazole alone, however, suggested that more accurate results could be obtained by an agar-dilution method. These results are presented in Table 3. The MIC of trimethoprim ranged from 0.39 to 1.56 µg/ml, and that of the combination ranged from 0.08 to 0.15 µg of trimethoprim/ml in combination with 0.4 to 0.8 µg of sulfamethoxazole/ml. Little

TABLE 3. Susceptibility of *V. cholerae* strains to trimethoprim and sulfamethoxazole individually and in a 1:5 combination, by an agar-dilution method

Strain	Trimethoprim			Sulfamethoxazole			T/S combination ^b	
	Highest MIC ^a	Lowest MIC	Highest / lowest	Highest MIC	Lowest MIC	Highest / lowest	Highest MIC	Lowest MIC
Classical								
VC 12.....	0.78 (=) ^c	0.78 (=)	1	1,000 (D)	25 (B)	40	.16/.8 (A, C, D)	.08/.4 (B)
B1307.....	1.56 (D)	0.78 (A, B, C)	2	400 (D)	25 (B)	16	.16/.8 (D)	.08/.4 (A, B, C)
CRC 31/64.....	1.56 (A, D)	0.78 (B, C)	2	1,000 (D)	12.5 (B)	80	.08/.4 (=)	.08/.4 (=)
369.....	0.78 (=)	0.78 (=)	1	2,000 (D)	100 (B)	20	.16/.8 (D)	.08/.4 (A, B, C)
395.....	1.56 (D)	0.78 (A, B, C)	2	4,000 (A)	100 (B)	40	.08/.4 (=)	.08/.4 (=)
El Tor								
HK-1.....	0.78 (C, D)	0.39 (A, B)	2	12.5 (D)	3.13 (A, B)	4	.08/.4 (=)	.08/.4 (=)
M-27.....	0.78 (C, D)	0.39 (A, B)	2	25 (D, D)	3.13 (B)	8	.08/.4 (=)	.08/.4 (=)
PI 1551.....	0.78 (C, D)	0.39 (A, B)	2	25 (D)	3.13 (B)	8	.08/.4 (=)	.08/.4 (=)
Iran-46.....	0.78 (C, D)	0.39 (A, B)	2	12.5 (D)	3.13 (A, B)	4	.08/.4 (=)	.08/.4 (=)
3083.....	0.78 (C, D)	0.39 (A, B)	2	12.5 (D)	3.13 (B)	4	.08/.4 (=)	.08/.4 (=)
CRC 9933/64.....	0.78 (C, D)	0.39 (A, B)	2	12.5 (D)	1.56 (B)	8	.08/.4 (=)	.08/.4 (=)

^a Minimal inhibitory concentration in micrograms per milliliter.^b Combination of trimethoprim and sulfamethoxazole in a 1:5 ratio. The trimethoprim concentration is given before the diagonal, and the sulfamethoxazole concentration is given after.^c The designations in parentheses show the method used to determine the MIC given, as follows: A, complete inhibition at 24 hr; B, 90% inhibition at 24 hr; C, 90% inhibition at 48 hr; D, complete inhibition at 48 hr; =, all determinations the same.

difference was noted either between classical and El Tor strains, or between the more and the less demanding methods of determining MIC values (48 versus 24 hr, complete versus 90% inhibition).

In contrast to this, the results with sulfamethoxazole alone showed substantial MIC differences both between the classical and El Tor strains, and between methods of defining the MIC. The highest MIC values were from 400 to 4,000 $\mu\text{g/ml}$ for classical strains, and from 12.5 to 25 $\mu\text{g/ml}$ for El Tor strains; the lowest MIC values were 12.5 to 100 $\mu\text{g/ml}$ for classical strains and 1.56 to 3.13 $\mu\text{g/ml}$ for El Tor strains.

As in the microdilution plate studies, the combination of trimethoprim and sulfamethoxazole substantially reduced the concentration of both agents required to produce inhibition. This reduction was particularly marked in comparison with sulfamethoxazole used alone against the more resistant classical strains (up to 10,000-fold), was less marked in comparison with trimethoprim used alone (5- to 10-fold), and was least marked in comparison with sulfamethoxazole used alone against the more susceptible El Tor strains (as little as 4-fold), although the last result depended upon the method used to define the MIC of sulfamethoxazole alone.

DISCUSSION

There has never been substantial agreement among investigators as to an ideal method of determining bacterial susceptibility to sulfonamides. Because of incomplete inhibition even in the best of liquid media, most workers have utilized an agar-dilution method for sulfonamide testing. The optimal composition of the test medium, however, has varied in different laboratories. Bauer and Sherris (1) used Mueller-Hinton agar, and found a large number of other media unsatisfactory; i.e., they gave a larger MIC. Leming and Flanigan (6), on the other hand, found a higher level of susceptibility for a variety of organisms when using Trypticase Soy Agar rather than Mueller-Hinton agar. Waterworth (11), using a peptone agar to which had been added 4% lysed horse blood, tested the susceptibility to trimethoprim and sulfamethoxazole of a number of organisms known to be clinically susceptible to the agents. The MIC values she cites are in fact very similar to those we obtained using our agar-dilution method on Trypticase Soy Agar, and are well within the range of concentrations obtainable in patients after average doses of the drug. Thus, it seems likely that our values do represent actual MIC values in an appropriate noninterfering medium.

The determination of four different end points in our agar-dilution studies, obtained by reading

degrees of inhibition at two different times, accentuated the difference between classical and El Tor susceptibility to sulfa, and also permitted the demonstration of a change in the action of sulfa against bacterial growth when used alone or when used in combination with trimethoprim. As demonstrated in Table 3 by the difference between the highest and lowest MIC values, the inhibition by sulfamethoxazole of bacterial growth tended to be incomplete except at high concentrations, and decreased with the passage of time. With trimethoprim alone or when sulfamethoxazole was used with even small amounts of trimethoprim, inhibition tended to be complete when it occurred, and did not decrease between 24 and 48 hr. This growth pattern difference was particularly marked with the more resistant classical strains, suggesting that the *p*-amino-benzoic acid-folic acid metabolic pathway may be different in the two *V. cholerae* biotypes.

To be consistent with the work of other investigators (1), the antibiotic concentration producing 90% inhibition at 24 hr could be selected arbitrarily as the MIC (method B in Table 3). By this criterion, the MIC noted for every strain was the lowest MIC among the four we observed, and appears as the lowest MIC in Table 3.

The difference between the susceptibility of El Tor and classical strains to sulfamethoxazole either in the agar-dilution method or in liquid media is striking, and suggests that, like susceptibility to polymixin B (5), susceptibility to sulfonamides may be used as a differentiating characteristic of the two biotypes. It is of interest that the susceptibility pattern seen in our results is opposite to that seen with polymixin B; i.e., classical strains are susceptible to polymixin, but were resistant to sulfa, and El Tor strains are resistant to polymixin, but were susceptible to sulfa. A similar pattern was observed in 56 classical and 29 El Tor strains studied in East Pakistan by R. Rahman and colleagues (*unpublished data*). Polymixin is thought to act as a cationic detergent on the phospholipids of the bacterial cell wall (8). Thus, susceptibility to this agent may be related to cell wall structural characteristics which might also produce chicken red blood cell hemagglutination and sensitivity to phage type IV, other criteria used to define the El Tor biotype (3). As sulfonamides act on metabolic steps distinct from those having to do with the cell wall, it is thus not unexpected that the sulfa susceptibility pattern would be different than that seen with polymixin.

The combination of trimethoprim and sulfamethoxazole markedly reduced the concentration of each agent required to produce inhibition. Yet, was the combination in fact synergistic, or merely

additive? Smith and his co-workers (9) defined synergy as occurring when the MIC of each of the drugs in a combination was one-quarter or less of the MIC of each drug alone. Using the microdilution method, a technique similar to the checkerboard dilution method they employed, we obtained clear evidence of synergy by their definition. In the agar-dilution studies, the fixed 1:5 ratio between trimethoprim and sulfamethoxazole limited the range of concentrations of each of the agents in the combination. Nevertheless, when the more demanding highest MIC figures were used, the combination was clearly synergistic against all 11 strains of *V. cholerae*; when the lowest MIC figures were used, the combination was definitely synergistic against 10 strains, and showed borderline synergism with regard to sulfamethoxazole against the remaining strain (CRC 9933, Table 3). Thus, it seems reasonable to conclude that the combination of trimethoprim and sulfamethoxazole does act synergistically in vitro against both the classical and El Tor biotypes of *V. cholerae*.

The clinical studies cited earlier suggest that the combination is likely to prove effective in controlled studies of the treatment of patients with cholera. Cholera is an ideal disease in which to test antibiotics, as the success of therapy can be determined objectively, not only by the observation of a reduction in the duration of stool cultures positive for *V. cholerae*, but also by a reduction in the volume and duration of diarrhea. It is hoped that future studies which will clearly differentiate between clinical synergy and an additive effect of trimethoprim and sulfamethoxazole in this disease can be carried out.

ADDENDUM IN PROOF

The report of a controlled study of trimethoprim-sulfamethoxazole (T-S), tetracycline (Te), and placebo

in Nigerian cholera patients (Francis et al. 1972. Effect of chemotherapy on the duration of diarrhea and in vibrio excretion by cholera patients. *J. Trop. Med. Hyg.* 74:172-176) was received by the authors after preparation of this manuscript. It concluded that the duration of diarrhea is reduced equally by T-S and Te, but that Te more effectively shortens vibrio excretion.

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