Action of Polymyxin of Cholera Vibrios. Techniques of Determination of Polymyxin-Sensitivity. (30329)

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Gan and Tjia(1) reported that classical cholera and El Tor vibrios could be differentiated by their susceptibility to polymyxin B, *Vibrio cholerae* strains being sensitive and El Tor strains resistant to bacto-sensitivity discs containing 50 units of polymyxin B. In confirmation of this report, Powell and Finkelstein(2) found that all the El Tor strains that they tested on meat-extract agar plates containing graded concentrations of polymyxin possessed a high degree of tolerance for the antibiotic in contrast to classical cholera vibrios. We present here the results of a study on the polymyxin susceptibility of the 2 types of choleragenic vibrios in a large series of strains isolated from different geographical areas at different times.

Materials and Methods. Strains: The classification of vibrio strains of this series into classical and El Tor types was based principally on the results of tests of susceptibility to group IV cholera phage(3) and chicken erythrocyte agglutination(4). All 5 phage-types of *V. cholerae* strain isolated in different epidemics in India, Pakistan, Nepal, Burma and Thailand between 1958 and 1965 were included in this study. The El Tor strains tested were obtained mostly from the South and South East Asian countries and West Pacific islands which have been involved in the cholera El Tor pandemic since 1961. Strains representing outbreaks of cholera El Tor in the Celebes island from 1938 onwards were also screened. In addition to these, El Tor vibrios isolated in the Middle East countries and water sources in Calcutta in the absence of cholera El Tor in these areas were examined. The Ubol strains were from a mild outbreak of El Tor gastro-enteritis in Thailand in 1960. One El Tor strain from a cholera-like case in a Middle East country in 1964, obtained through the courtesy of Dr. Patricia Carpenter of the International Shigella Centre, London, was also examined.

Polymyxin B: "Aerosporin" brand polymyxin B sulphate, (6 units/μg) manufactured by Burroughs Wellcome & Co. (India) Private Ltd. was used. Some of the polymyxin B discs (50 units) were obtained commercially from Difco Laboratories and some were prepared in the laboratory.

In cases of atypical reactions the tests were repeated at least 3 times.

Screening of strains: (a) *Disc-method:* Two-hour broth cultures of the test strains were spotted on nutrient agar plates and were allowed to dry for a few minutes. A bacto-sensitivity disc containing 50 units of polymyxin B was placed on each spot. After overnight incubation the plates were examined. A clear zone of lysis more than 1 mm wide around the disc was taken to indicate sensitivity of the vibrio strain (Dr. Tjia Soe Khie, personal communication). In cases of narrower zones or crescent shaped clearings, the strains were considered partially sensitive.

(b) *Well-method:* A lawn of the test strain was prepared by plating a mixture of 4 ml of 0.7% agar in nutrient broth at 45°C and 0.05 ml of a 2-hour growth culture in nutrient broth. At the center of the lawn a well was dug with a cork-borer. A portion of polymyxin solution (5 μg/ml) was placed in the well. The plate was incubated at 37°C for 20 hours and then examined for a zone of lysis around the well.

(c) *Plate-method:* Nutrient agar media containing 10, 15 or 20 μg/ml polymyxin B was plated in Petri dishes of about 11 cm diameter. In each plate about 30-40 strains were spotted separately on previously marked zones by applying loopfuls of 2-hour cultures in nutrient broth. The plates were then incubated at 37°C for 20 hours after which they were examined for growth of the vibrios. Strains showing no growth or growth of less than 10 colonies in the spotted areas were taken as sensitive, those showing more than 10 colonies but less than confluent growth as partially sensitive or partially resistant, and

TABLE I. Comparative Sensitivity of *V. cholerae* and *V. el tor* Strains to 50 Units Polymyxin B Discs.

Type of vibrios	Total No. of strains tested	No. of resistant strains	No. of partially sensitive/resistant strains	No. of sensitive strains
V. cholerae	388	0	0	388
V. el tor	625	563	59	3

those with confluent growth, similar to the growth in polymyxin-free media, were taken as resistant.

(d) *Turbidimetric-method:* A 24-hour culture from a Roux flask was washed in saline, then suspended in nutrient broth to give a reading of 25 in a Klett-Summerson photoelectric colorimeter with a filter of 540 mμ, corresponding to 10^8 cells/ml approximately. Generally, 20 ml of this suspension were incubated with 5 μg/ml polymyxin B at 37°C for 5 hours. The growth was measured every hour by reading the optical density of the incubation mixture at 540 mμ.

In experiments with resting cells, a saline suspension giving a Klett reading of 200 with the 540 mμ filter was used. To this was added polymyxin B solution of the required concentration.

Results. Disc-method: It may be seen from Table I, that all the *V. cholerae* strains were found sensitive to 50 units-polymyxin B discs while 99.5% of the El Tor strains were either fully or partially insensitive. Of the 3 fully sensitive *V. el tor* strains, Mak 37/59 was isolated in Makassar island in 1959, Taipei 6/63 from Taiwan in 1962, and Middle East 699/64 from a cholera-like case in the Middle East in 1964. The partially sensitive El Tor strains came from 6 countries where the majority of the El Tor strains were insensitive. A partially sensitive reaction by the disc method was at times difficult to read and in some of the strains appeared unstable. On repetition of the tests inconsistent results were obtained in many cases.

The partially sensitive El Tor strains were further examined by plating them and testing the individual colonies with polymyxin discs. Each of these strains appeared to be composed of a mixture of sensitive and resistant colonies.

Well-method: On screening the classical cholera and El Tor vibrio strains by the well method, the results obtained were similar to those found by the disc method. The well method yielded more consistent results especially with partially sensitive El Tor vibrios, but was not studied with a larger series, as it was considered too laborious for routine use.

Plate-method: The results are given in Table II. It appears that when *V. cholerae* are grown in plates containing 10 μg/ml of polymyxin, none of the strains was found fully resistant. However, 35 out of 447 strains proved partially resistant. At 15 μg/ml all *V. cholerae* strains were found completely sensitive. On the other hand, all but 3 *V. el tor* strains representing over 99.5% proved either partially or completely resistant at 10 or 15 μg/ml. At 20 μg, however, 9 El Tor strains proved completely sensitive. The most suitable concentration for use in differentiating the two choleragenic vibrios by the plate-method thus appeared to be 15 μg/ml.

Turbidimetric-method: On testing the ac-

TABLE II. Comparative Sensitivity of *V. cholerae* and *V. el tor* Strains in Polymyxin Plates.

Concentration of polymyxin B in the plate	*V. cholerae*				*V. el tor*			
	Total No. tested	No. of sensitive strains	No. of partially resistant strains	No. of resistant strains	Total No. tested	No. of sensitive strains	No. of partially resistant strains	No. of resistant strains
10 μg/ml	467	432	35	Nil	517	3	9	505
15 "	502	502	Nil	"	544	3	49	492
20 "	474	474	"	"	518	9	54	455

TABLE III. Action of Polymyxin on Resting Cells of Cholera Vibrios.

Type of vibrio	Strain No.	Sample No.	Incubation mixture: Cell suspension in ml	Incubation mixture: Polymyxin per ml	Klett-readings at 540 mμ at time-intervals: 0 hr	1 hr	2 hr	3 hr	4 hr
V. cholerae	154	A	10	0	200	190	190	190	190
		B	10	10	200	155	147	140	145
		C	10	50	200	120	98	93	90
		D	10	100	200	100	85	90	88
V. el tor	Philippine 42/63	A	10	0	202	198	198	196	200
		B	10	10	202	177	173	162	155
		C	10	50	195	155	140	130	125
		D	10	100	185	162	155	148	123

tion of polymyxin B on the choleragenic vibrios by the turbidimetric method(5) certain interesting features were revealed. With resting cells, there was a gradual fall in the optical density of cultures, both of classical cholera and El Tor vibrios (Table III).

With growing cells, a difference in the action of polymyxin B on these 2 types of vibrios was noted, *V. cholerae* being sensitive and *V. el tor* resistant to the antibiotic. However, the growing cells of El Tor vibrios exhibited an initial phase of inhibition of growth in the presence of the antibiotic. This difference in the response of the 2 types of vibrios is illustrated in Fig. 1.

Discussion. The results of tests by the disc, well, plate and turbidimetric methods of the sensitivity to polymyxin of a large series of *V. cholerae* and *V. el tor* strains have generally been in conformity with the findings of Gan and Tjia(1). All *V. cholerae* strains were found to be fully sensitive to polymyxin and all but 3 El Tor strains were found either partially and fully resistant. The plate method was superior to the disc method in differentiating the two types of vibrios as it yielded more clear-cut results with partially sensitive El Tor strains. Other advantages of the plate technique are that (1) polymyxin is readily available, whereas the commercially prepared bacto-sensitivity polymyxin discs are not available in many places and it may not be possible for individual laboratories to prepare the proper type of discs themselves; (2) the potency of the discs was found in many instances to be progressively lost on storage even in the refrigerator and results of tests using such discs proved inconclusive. Polymyxin plates have to be made with freshly prepared solutions to give consistent results. The well and turbidimetric methods were found reliable but they are more laborious than the plate method and not particularly suitable for routine screening of strains. The plate method appeared to be most satisfactory.

The partially sensitive El Tor cultures were found to be composed of resistant, partially resistant and sensitive strains. On the other hand, some of the *V. cholerae* strains yielded a few colonies on the polymyxin plates at an antibiotic concentration of 10 and 15 μg/ml which proved resistant in further tests using the antibiotic in higher concentrations. If selective multiplication of this type of mutant

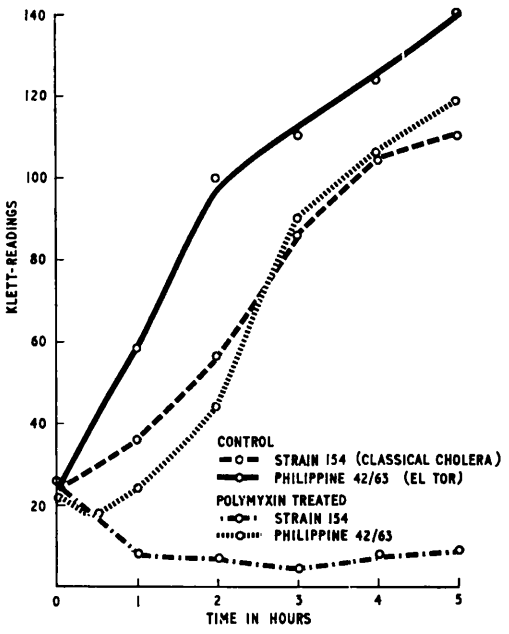

FIG. 1. Effect of polymyxin B on the growth-curve.

takes place in nature, polymyxin-resistant *V. cholerae* strains may be isolated, as well as polymyxin-sensitive *V. el tor* strains.

Of the available tests for differentiating *V. cholerae* and *V. el tor* strains phage-typing(3) has been found to yield the most consistent results. However, this test requires a somewhat specialized technique and is therefore not suitable for all laboratories. Polymyxin-sensitivity test by the plate method appears to be the next best among available methods and may be adopted either alone or as a confirmatory test with the phage-typing procedure.

The turbidimetric method of testing polymyxin sensitivity showed up interesting differences in the growth patterns of the two types of vibrios in the presence of the antibiotic. The results will be published later.

Summary. A comparative study has been made of 4 methods, namely, disc, well, plate and turbidimetric methods for testing polymyxin-sensitivity of the choleragenic vibrios. The plate method using 15 μg/ml of polymyxin B in agar medium is recommended as the method of choice for routine screening tests for differentiating *V. cholerae* and *V. el tor* strains. By this method all the 502 strains of classical cholera vibrios tested were found sensitive and 99.5% of the 544 strains of El Tor vibrios completely or partially resistant to polymyxin B. With the turbidimetric method, resting cells of both *V. cholerae* and *V. el tor* showed evidence of progressive lysis in the presence of polymyxin. But when vibrios were tested in their growing phase *V. cholerae* strains appeared sensitive to polymyxin while the *V. el tor* cultures proved resistant. El Tor strains, however, showed an initial phase of growth inhibition before their normal growth pattern was restored.

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New Surface Antigen in Cells Transformed by Simian Papovavirus SV40.* (30330)

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Transformation of hamster cells *in vivo* and *in vitro* by papovavirus SV40 results in the appearance of new cellular antigens. The specificity of these antigens has been demonstrated by transplantation rejection procedures(1-4). A new antigen in these cells can also be detected by complement-fixation tests (5,6) and by immunofluorescent methods(7, 8) using sera from hamsters bearing large tumors induced by SV40-transformed cells; the latter method has located this "tumor" or T antigen in the nucleus. Since antibodies against the intranuclear T antigen cannot be detected in hamsters immunized with live SV40 and proven resistant to SV40-transformed cells, it is unlikely that the T antibody is involved in rejection of the tumor cells. This paper describes the presence of new and specific antigens at the surface of SV40-transformed hamster cells and the synthesis in hamsters of antibody against the antigens.

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Materials and methods. Cell lines. The H-50 cells were originally derived from a