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Serological Studies in Cholera

2. The Vibriocidal Antibody Response of Cholera Patients Determined by a Microtechnique *

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A microtechnique is described for the determination of vibriocidal antibodies to Vibrio cholerae, *using 0.025 ml of fingertip blood or venous serum per test. This test could be used in epidemiological surveys, or as a routine test on patients admitted to hospital.*

Fourfold or greater rises in vibriocidal titre were noted for 96.5% of 370 bacteriologically confirmed cholera patients in an endemic area of East Pakistan (91.5% for the 94 children under 5 years in the study group). It is necessary to test sera against both Ogawa and Inaba serotypes of V. cholerae, *as a significant titre rise was found against the homologous serotype only in 11% of the cases, and against the heterologous serotype only in 2%.*

Six serologically positive but bacteriologically negative persons were found to be household contacts of confirmed cholera patients (and 3 of the contacts had been vaccinated against cholera within 1 week before testing); 3 other cases (1.6%) were considered to be false positives.

The vibriocidal test using fingertip blood was compared with daily rectal swabbing for the detection of cholera carriers among 153 household contacts of cholera patients, who had not received cholera vaccine within 2 weeks before testing; 16% gave a positive vibriocidal titre, while V. cholerae *was isolated from 13% only.*

The earliest serological studies in cholera were performed with bacteriolysis as the end-point. Originally the peritoneal cavity of the guinea-pig was used for this purpose (Pfeiffer's test), but as early as 1906, Serkowski (quoted by Pollitzer & Burrows, 1959) mixed saline dilutions of serum with constant quantities of cholera vibrios and of complement, incubated the mixture for 4 to 6 hours, then poured agar plates which he incubated for 24 hours. "The absence of growth, or the number of colonies which had by then developed, indicated to what extent the serum dilutions in question possessed bacteriolytic properties."

In a study of bacteriologically confirmed cholera patients, a rise in the titre of bacterial agglutinins 6 days or more after admission was not observed in 10% of the cases (Benenson et al., 1968).[2] The present study was undertaken to determine whether a more sensitive complement-dependent test system would detect additional antibody rises and, more important, whether the technique could be simplified to permit routine use for diagnostic purposes and for mass surveys using fingertip blood. Finkelstein (1962) had shown the great sensitivity of a test equivalent to Serkowski's; he incubated a mixture of complement and serum dilution with a bacterial suspension to give approximately 40 vibrio colonies per drop and noted the number of colonies growing from drops of the incubated mixture placed on the surface of an agar plate; technical difficulty was experienced in getting the plates properly dried. McIntyre & Feeley (1964) determined vibriocidal antibodies by a simplification of the broth assay method of Muschel & Treffers (1956); in this procedure, culture medium is added to the reaction mixture to stop the complement-dependent reaction and support the multiplication of surviving vibrios.

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[2] See the article on p. 267 of this issue.

TABLE 1

PROTOCOL FOR VIBRIOCIDAL ANTIBODY DETERMINATION BY MICROTECHNIQUE [a]

Material or operation	Dilution	Amount (ml)							
		Test preparation		Positive control		Complement (C') control		Anti-complementary (A/C) control	
Test samples									
Serum	1 : 5	—	—	—	—	—	—	0.025	0.025
Fingertip blood	Field dilution								
Fingertip blood	1 : 10 — 1 : 1280	0.025	0.025	—	—	—	—	—	—
Positive control serum	1 : 10 — 1 : 1280	—	—	0.025	0.025	—	—	—	—
Equal parts of:									
complement	1 : 5	0.025	—	0.025	—	0.5	—	0.025	—
Ogawa suspension	1.0 opacity unit								
Equal parts of:									
complement	1 : 5	—	0.025	—	0.025	—	0.5	—	0.025
Inaba suspension	1.0 opacity unit								
Saline		—	—	—	—	0.5	0.5	—	—
Incubate 1 hour at 37°C in water-bath									
Heart infusion broth		0.15	0.15	0.15	0.15	3.0	3.0	—	—
Sensitized sheep cells		—	—	—	—	—	—	0.025	0.025
Incubate at 37°C in water-bath									
Preliminary reading		When C' controls have reached 60 %–70 % transmittance (2–4 hours)						After 30 minutes' incubation	
Final reading		After overnight refrigeration							

[a] The contents of a given column represent the constituents of one test or control preparation and the operations which this undergoes, in chronological order from top to bottom.

The present paper reports that it was possible to adapt this technique to a modified Takatsy microtechnique (Csizmas, 1960; Sever, 1962). The pattern of vibriocidal antibody found with this technique on admission and during convalescence of patients with cholera-like disease was investigated, and the feasibility of using fingertip blood for this procedure was explored.

MATERIALS AND METHODS

The Microtiter kit (Cooke Engineering Co., Alexandria, Va., USA), with loops and pipette droppers calibrated to deliver 0.025 ml and nondisposable plastic plates with "U" cups, was used. The test sera, the criteria for categorizing their donors as confirmed cholera cases or as negative, and the technique used for fingertip blood collections are described in the preceding paper (Benenson et al., 1968).[1]

The vibriocidal test technique described by McIntyre & Feeley (1964), an adaptation of the Muschel & Treffers (1956) bacteriocidal antibody technique, was the basis for the procedure outlined in Table 1.

Commercial complement (Nutritional Biochemicals Corp., Cleveland, Ohio, USA) was used when available. This was reconstituted with chilled 0.85 % saline solution (instead of the reconstituting solution provided, which contained an antibacterial agent) and diluted 1 : 5 with cold saline for routine use. When commercial complement was not available, individual guinea-pig sera were tested at a 1 : 5 dilu-

[1] See the article on p. 267 of this issue.

tion for vibriocidal activity. Only those free of vibriocidal activity were pooled, and the pool was divided into aliquots which were held at −70°C.

Vibrio cholerae Ogawa strain CRL 465 and *V. cholerae* Inaba strain CRL 22463, were used as the test cultures in the routine test. (*V. cholerae* Inaba strain CRL 466, used in the routine vibrio agglutinin test, gave reaction patterns and titres identical with those of strain 22463 in a group in parallel tests.) For the test, overnight (14–16 hours) growths of the Ogawa and Inaba strains on heart-infusion agar were transferred to heart-infusion agar slants which were incubated at 35°C–37°C for 4 hours, suspended in sterile saline and adjusted photometrically to 10 opacity units of the International Opacity Standard. These suspensions were further diluted with 9 parts of cold (4°C) saline to provide the working suspensions with an opacity of 1 unit. The bacterial suspensions were mixed with equal volumes of the chilled 1 : 5 complement dilution, and the two mixtures were held in an ice-bath.

Microtiter plates were prepared with 0.025 ml of serial twofold saline dilutions (1 : 10–1 : 1280) of the test sera and equal volumes of the same dilutions of the positive control serum, 2 rows for each serum, across the 8-cup rows as described in the preceding paper (Benenson et al., 1968).[1]

To ensure that a low vibriocidal titre was not obscured by anticomplementary factors in the test serum, a Microtiter plate was prepared for the anticomplementary (A/C) control tests (Table 1) with a 1 : 5 dilution of each serum or the field dilution of each fingertip blood sample. A tube was used for the complement controls, 0.5 ml of saline being placed in two test-tubes, one labelled "Ogawa", and the other "Inaba".

With the Microtiter pipette, 0.025 ml of the chilled complement–bacterial-suspension mixtures was added to the serum dilutions; one row of each serum received the Ogawa and the other row the Inaba suspension mixture. The A/C control plate was treated similarly, and 0.5 ml of the appropriate mixture was added to each test-tube for the complement controls. The plates were sealed with the plate-sealing tape, tightly applied with the tape roller, and all the plates and controls were placed in the water-bath at 37°C (no leakage occurs even if the plates are submerged). After 1 hour of incubation, 0.15 ml of Bacto heart-infusion broth (Difco) was added to each cup of the test plates and 3 ml to each of the 2 complement control tubes (with a 1-ml Cornwall pipetting syringe). 0.025 ml of sensitized sheep red blood cells was added to each reaction mixture on the A/C control plate. (Sensitized sheep cells were prepared by mixing 2% washed sheep erythrocytes with an equal volume of a 1 : 400 dilution of antisheep haemolysin with a titre of 8000.)

The plates were resealed and returned, with the complement control tubes, to the 37°C water-bath. After 30 minutes, the A/C control cups were examined and if haemolysis was not observed, serial dilutions of the anticomplementary sera were titrated for A/C activity. If the anticomplementary dilutions were in a range which might mask vibriocidal activity, the test was repeated using complement at a 1 : 2 dilution.

The test plates were incubated at 37°C, and when the complement control tubes reached an optical transmittance of 60%–70% (usually after 2–4 hours), the Microtiter plates were examined by transmitted light with the aid of the Microtiter test reading mirror against a black background. A clear distinction was evident between the turbid fluid in the cups in which the vibrios had grown out and the crystal-clear yellowish fluid in those cups in which the organisms had been killed and lysed; the end-point was sharply defined. The vibriocidal titre was recorded as the reciprocal of the original serum dilution in the last cup in which growth was definitely inhibited. The final readings were made after overnight refrigeration, preferably by another observer. Usually the same titres were obtained on both readings, but the end-points were clearer after refrigeration since both turbidity and buttons of sedimented organisms were present in the negative cups, contrasting with the crystal-clear fluid throughout the cups containing the serum dilutions positive for vibriocidal activity.

RESULTS

Reproducibility

This test produced clear-cut objective end-points, so that different observers, reading the tests independently, recorded the same titres. With the heavy bacterial inoculum which was used, bacterial overgrowth from the nonsterile plates was no problem. Dilutions of some contaminated sera, which were turbid at the preliminary reading, were found after refrigeration to have buttons which looked different from the buttons in non-vibriocidal dilutions of

[1] See the article on p. 267 of this issue.

other sera on the same plate (usually broader), and the surrounding fluid was clear, rather than turbid. Such cases were read as positive. Doubt was resolved, if needed, by subculturing a loop of fluid from the questionable cups in a vibrio-selective medium such as tellurite-taurocholate-gelatin agar (Monsur, 1963).

An initial serum dilution of 1 : 10 was selected because this disclosed titre rises among bacteriologically positive cholera cases which were missed when the starting dilution was 1 : 80. The reproducibility of titres to be expected from serum pairs within one protocol was assessed by comparing the titres obtained for the admission sera of 270 bacteriologically negative patients with those on follow-up sera taken from the same patients in the course of hospitalization. (The second samples were drawn 6 days or more after the onset of symptoms in 169 cases, and earlier—often only 1 day after the onset of symptoms—in 96 cases; in 5 cases, the first samples were obtained later than the seventh day.) Cases in which there was dubious evidence of cholera infection (a single positive bacteriology report, or a titre rise in any other serological test if bacteriologically confirmed cholera existed in the home environment) were excluded from this analysis. In 94% of the tests the titres of the 2 sera were the same or differed by 1 dilution only (Table 2). Fourfold titre rises against the Ogawa serotype were noted in the second sample in 3 pairs, and in 5 pairs against the Inaba serotype; in 5 of these 8 pairs the second serum had been drawn less than 4 days after the onset of symptoms and in only 1 test was the rise greater than fourfold. Thus, in the 540 tests, 1.5% showed a rise which might be considered diagnostic. However, in 24 tests (4.4%), a fourfold or greater drop in titre was noted, and in 4 cases this fall was 16-fold. A fall was also noted in the agglutinin titre of a number of patients bacteriologically negative for *V. cholerae* (Benenson et al., 1968), and may be attributable at least in part to the severe dehydration present when the first serum was collected.

TABLE 2. VIBRIOCIDAL TITRES FOR PAIRED SERA IN 270 VIBRIO-NEGATIVE CASES

Late serum titre	Admission serum titre								
	<10	10	20	40	80	160	320	640	≥1 280
Ogawa suspension									
≥1 280	—	—	—	—	—	—	—	3	5
640	—	—	—	—	—	—	3	5	3
320	—	—	—	—	1	3	8	3	—
160	—	—	—	—	4	8	4	1	—
80	—	—	1	8	21	8	—	—	—
40	—	—	7	31	12	3	—	—	—
20	1	5	17	9	1	1	—	—	—
10	5	14	10	2	1	—	—	—	—
<10	56	3	1	1	1	—	—	—	—
Inaba suspension									
≥1 280	—	—	—	—	—	—	1	2	6
640	—	—	—	—	—	—	2	4	1
320	—	—	—	—	1	1	9	1	—
160	—	—	—	—	6	16	2	1	—
80	—	1	—	9	23	5	1	—	1
40	—	—	9	21	11	2	—	—	—
20	2	6	25	7	2	—	—	—	—
10	4	15	3	2	—	—	—	—	—
<10	58	7	—	1	2	—	—	—	—

The inter-test consistency of the procedure (i.e., the reproducibility of the titre obtained in independent tests with different bacterial suspensions) was estimated by comparing the titres obtained on the goat serum which was included as a positive control in 76 consecutive routine tests (Table 3). The range

TABLE 3

INTER-TEST CONSISTENCY OF MICROTITER VIBRIOCIDAL TEST [a]

Serotype of test vibrios	Cups positive						No. of tests	Mean cup No.	Standard deviation
	8	9	10	11	12	13			
Ogawa	—	10	26	27	11	2	76	10.59	0.98
Inaba	2	10	30	26	7	1	76	10.38	0.97

[a] Titres of one lot of control goat serum in consecutive tests.

of titres was so broad that differences in titre can be regarded as meaningful only when two sera are tested concurrently with the same bacterial suspensions.[1]

Vibriocidal response to infection with V. cholerae

In sharp contrast to the results on vibrio-negative patients was the experience with serum pairs from patients with bacteriologically confirmed *V. cholerae* infections. Among 222 confirmed Inaba cases with a second serum drawn 6 or more days after onset and no history of prior anti-cholera vaccine, a fourfold or greater rise in vibriocidal titre occurred with 94.1% of those tested against a suspension of Inaba vibrios; the mean titre rise was 35-fold (Table 4)!

In the routine determination of vibriocidal antibodies on all patients admitted to the ward, the use of serum dilutions from 1 : 10 to 1 : 1280 was found to be acceptable for diagnostic purposes (Table 5). On admission, about 30% of the patients possessed no vibriocidal activity at the 1 : 10 level; after infection (predominantly by the Inaba serotype during the period in question) over half developed titres of 1280 or more against the Inaba serotype and one-third did so against the Ogawa serotype. The end-point was determined in 23 sera which were vibriocidal across the entire 8-cup dilution range to get an approximation to the whole titre distribution; the modal titre against both vibrio serotypes was found to be 1280, and the highest titre observed was 20 480.

The relation between the anti-Inaba and anti-Ogawa titres was examined for 311 admission sera in vibrio-negative cases and 383 sera taken during convalescence from Inaba infection (Table 6). Identical titres were found against both antigens in over half of the admission sera, while in 85.5% of these sera the titres against the two serotypes differed by not more than 1 tube, with an anti-Ogawa : anti-Inaba mean titre ratio of 1 : 0.93. Among sera from patients convalescent from *V. cholerae* infections the titre rises were much greater against the infecting serotype; in fact 6 of the 383 convalescent sera from Inaba cases had anti-Inaba vibriocidal titres ranging from 1 : 40 to 1 : 1280 with no anti-Ogawa activity demonstrable at the 1 : 10 level (Table 6); 110 of these sera (28.7%) had anti-Inaba titres fourfold or more greater than the corresponding anti-Ogawa titres; the reverse was the case in only 10 sera (2.6%) and the anti-Ogawa: anti-Inaba (homologous) mean titre ratio was 1 : 2.02. The probability of this difference occurring by chance is less than 1 in 10 000 (Student's $t = 7.12$; 764 degrees of freedom).

Prospective studies to determine when vibriocidal antibody first appears in the course of illness were not carried out. Retrospective analysis of 49 bacteriologically and serologically positive cases (7 Ogawa and 42 Inaba) in which multiple sera were available indicated that this occurred at the end of the first week for the majority of patients. A fourfold rise to both antigens was noted in 1 case on the third day after the reported onset of illness; by the

TABLE 4
DISTRIBUTION OF ANTI-INABA VIBRIOCIDAL LEVELS ON ADMISSION AND DURING CONVALESCENCE [a]

Cup No. positive	Anti-Inaba vibriocidal titre [b]	Number of cases	
		On admission	During convalescence
0	<10	88	2
1	10	37	4
2	20	38	1
3	40	25	8
4	80	15	6
5	160	11	28
6	320	8	25
7	640	—	37
8	≥1 280	—	111
Total		222	222
Mean cup No.		1.58	6.72
Standard deviation		1.73	1.75
Standard error of mean		0.12	0.12
Geometric mean titre		14	488
95 % confidence limits		12–16	419–605

[a] For 222 patients in Dacca, East Pakistan, with *V. cholerae* Inaba infections and no history of cholera vaccination.

[b] For the purpose of calculations, sera negative in all cups were taken as having a titre of 5 (corresponding to cup No. 0), and sera positive in cup No. 8 or higher were taken as having a titre of 1280 (corresponding to cup No. 8).

[1] Because differing test strains or laboratory conditions may produce differences in the sensitivity of the test and in the resulting vibriocidal titre, a reference serum is essential for interlaboratory comparisons. Information on the potential availability of such a reference serum can be obtained from the Chairman, National Institutes of Health Cholera Advisory Committee, Bethesda, Md. 20014, USA.

TABLE 5. PERCENTAGE DISTRIBUTION OF VIBRIOCIDAL TITRES AMONG PATIENTS FROM THE DACCA AREA

Serotype of test vibrios	Vibriocidal titre									Total No. of cases
	<10	10	20	40	80	160	320	640	≥1 280	
Admission sera — all cases										
Ogawa	28.5	11.4	15.7	14.6	13.2	8.6	4.0	3.0	1.0	699
Inaba	30.0	13.6	14.5	13.9	13.4	7.3	4.5	1.3	1.4	697
Convalescent cholera cases										
Ogawa	3.5	3.5	4.5	6.4	8.3	9.3	17.3	12.8	34.1 [a]	375
Inaba	2.1	1.3	0.5	2.9	3.5	10.9	13.3	14.6	50.8 [a]	376

[a] Extrapolation of the percentage distribution for titres ≥1280, based on the end-point observed for 23 sera:

Serotype	*Titre*					*Total %*
	1 280	2 560	5 120	10 240	20 480	
Ogawa	20.2	9.3	3.1	—	1.6	34.1
Inaba	26.5	6.6	15.5	2.2	—	50.8

TABLE 6. RELATION OF INABA TO OGAWA VIBRIOCIDAL TITRES FOR ADMISSION SERA IN 311 VIBRIO-NEGATIVE CASES AND CONVALESCENT SERA IN 383 INABA-POSITIVE CASES [a]

Ogawa cups positive	Inaba cups positive								
	0	1	2	3	4	5	6	7	≥8
Admission sera (311) [b]									
≥8							1	1	7
7						1	3	4	2
6					4	5	4	3	
5			2	5	8	8	4		
4	—	2	6	8	21	7	3		
3	1	3	14	18	14	4	1		
2	3	11	10	10	2	1			
1	6	9	4	4	2				
0	78	7							
Convalescent sera (383) [c]									
≥8				1	—	2	2	6	106
7						3	3	12	32
6						4	17	18	26
5				1	2	11	15	6	8
4				3	5	9	11	5	6
3	—	1	1	2	3	8	2	3	5
2	—	1	4	6	—	4	2	2	4
1	—	1	2	4	1	1	1	2	3
0	—	—	—	1	—	3	1	—	1

[a] Initial serum dilution 1 : 10.
[b] Geometric mean titres (and 95% confidence limits): anti-Ogawa—29 (27–32); anti-Inaba—27 (24-33).
[c] Geometric mean titres (and 95% confidence limits): anti-Ogawa—240 (208–294); anti-Inaba—484 (431–567).

fifth day half of the 10 sera examined had developed a significant rise averaging 16-fold, 7 out of 9 had converted by the sixth day, 10 out of 11 by the seventh, and 11 out of 12 by the eighth day. A significant rise had occurred in all sera taken on the ninth day or later.

Efficacy of titre rise for retrospective diagnosis of cholera

The efficacy of the vibriocidal test on paired sera for the retrospective diagnosis of cholera was evaluated by analysis of the routine serological testing of all cases with serum pairs whose second sample was taken on the sixth or subsequent day after onset of illness. A significant rise in vibriocidal antibodies was detected for 96.5% of 370 bacteriologically positive cholera patients—91.5% of patients under 5 years of age and 98.2% of patents aged 5 years or over (Table 7). The rise in titre generally occurred against both antigens, but in 11.1% of the cases the diagnostic rise occurred only with the homologous serotype; the reverse was true in 1.9% of the cases. The titre rise to the homologous vibrio serotype only occurred significantly more frequently among children under 5 years of age (17%, *versus* 9% for those 5 years of age or over).

Among 191 bacteriologically negative persons, significant rises in titre occurred in 6 persons who proved on further investigation to be close relatives (sibling, parent, or child) of a patient whose infection with *V. cholerae* was confirmed by the isolation; 3 of them had also received cholera vaccine. Apart from these cases, in only 3 cases (1.6%) would the serological results suggest the possibility of infection with cholera vibrios.

TABLE 7
DIAGNOSTIC EFFICACY OF VIBRIOCIDAL TESTING OF SERUM PAIRS [a]

Age-group (years)	Titre rise ≥4-fold				No. without titre rise	Total No. of cases
	No.			Total (%)		
	Inaba and Ogawa	Homologous serotype only [b]	Heterologous serotype only [c]			
Bacteriologically confirmed cholera cases						
0-4	68 (1)	16	2	91.5	5 (1)	94 (2)
5-14	102 (4)	10 (1)	2	99.1	—	115 (5)
≥15	139 (5)	15	3	97.5	1	161 (6)
Total	309 (10)	41 (1)	7	96.5	6 (1)	370 (13)
Cases bacteriologically negative for *V. cholerae*						
0-4	1 [d]	1	—	(2.2)	40	45
5-14	3 [d]	1 [d]	—	—	14	24
≥15	1 [d]	1	1	(1.6)	81	122
Total	5	3	1	(1.6)	135	191

[a] The second serum sample was taken 6 or more days after the onset of the disease. The figures in parentheses give the number of Ogawa cases found.
[b] For vibrio-negative cases, Inaba only.
[c] For vibrio-negative cases, Ogawa only.
[d] Six persons were household contacts of cholera patients, and 3 of them had been vaccinated within the past week. These persons are not included in the percentage positive.

Use of fingertip blood samples

The feasibility of using fingertip blood samples in the vibriocidal test was studied. Satisfactory reactions and sharp end-points were obtained with either saline or distilled water as the diluent; saline was preferred because readings were easier when there was the same colour tone and hue in all cups. Comparable titres were obtained when fingertip blood and sera obtained simultaneously were run in the same test. The vibriocidal test on fingertip blood was then compared with the bacteriological method as regards efficacy in detecting infection among household contacts of cholera patients. Fingertip blood samples were collected at the first visit to the home and again 3 weeks later; the diluted plasma was held in the frozen state and both samples examined in the same test. Rectal swabs were collected for bacteriological culture daily for 5–10 days. In all, 153 household contacts were studied (individuals who were given cholera vaccine were excluded); 127 were negative by bacteriology and showed no rise in vibriocidal titre, 19 were positive by both techniques, 6 were positive serologically only and 1 bacteriologically only. Thus the bacteriological technique recognized infection in 13.1% of the cases, the serological in 16.3%; in 95.4% of the cases, the two methods gave the same result; in 3.9%, the serological method alone indicated infection, and in only 0.7% did the serological method fail to indicate vibrios which were isolated.

DISCUSSION

The bacteriocidal technique of Muschel & Treffers (1956), as adapted by McIntyre & Feeley (1964) for *V. cholerae* studies, can be performed by the modified Takatsy microtechnique (Csizmas, 1960; Sever, 1962), making it possible to process large numbers of sera with speed and accuracy, using so small an amount of test material that blood samples collected from the fingertip are adequate for repeated tests. In many cultures, folklore and superstition make it exceedingly difficult to perform venepunctures on healthy individuals; such reluctance does not apply with as much force to fingertip punctures.

The mean vibriocidal titres obtained were several tubes lower than those reported by Feeley (1965).

The sensitivity of the test is dependent on the bacterial density used; a 10-fold dilution of the bacterial suspension raises the end-point by 3–4 tubes. Since the equipment used in the microtechnique was clean but not sterile, the vibrio inoculum had to be heavy enough not to be overgrown by any contaminants in the short period used. For practical purposes, the test proved to be sufficiently sensitive. For special purposes, if greater sensitivity is desired, the bacterial suspensions can be diluted and the end-point of the vibriocidal reaction recognized by transferring a standard droplet to a selective medium such as tellurite–taurocholate–gelatin agar.

The vibriocidal test did prove to be more sensitive than the agglutinin test for the retrospective diagnosis of cholera. Of the 370 proven cholera patients whose sera were tested by both procedures, 330 were positive by both procedures and 11 were negative by both, giving diagnostic agreement in 92% of the cases. However, 27 (7.3%) had a vibriocidal but no agglutinin rise; for only 2 (0.5%) did the reverse obtain. Among the vibrio-negative cases, the two procedures agreed in 99.5% of the cases; in almost 200 cases, there were 2 cases with false positive results in the vibriocidal test and only 1 in the agglutination test. Thus, the vibriocidal test is not only clearly more sensitive but not significantly less specific; these results are comparable with those reported by Sack et al. (1966), who used the plate technique of Finkelstein (1962). From the technical point of view, the vibriocidal procedure is well within the capabilities of any competent laboratory.

The broad range of titres obtained with the positive control serum is convincing evidence that rises in titre can have diagnostic significance only if the serum pairs were tested simultaneously so that the results are internally controlled. When the titres are being determined as part of population surveys, aberrant titres for the control serum serve as a signal for repetition of the series of tests involved. The inter-test variation is attributable to differences in the bacterial density of the test vibrio suspension; when the tests are being run for inter-test comparison, as in an epidemiological survey, these variations can be minimized by harvesting the organisms from the slants with chilled sterile saline and maintaining the bacterial suspensions at all times in an ice-bath, as recommended by McIntyre & Feeley (1964); the effect of the residual variations can be reduced by randomizing the samples in the successive tests.

In routine use, the vibriocidal titration technique proved to be a feasible, relatively simple procedure, highly accurate in its diagnostic capabilities, recognizing well over 90% of infections with less than 2% false positive results. It must be recognized that errors in a serological system may not be due to the test itself, but may enter by misidentification of samples at some point from the time of collection from the patient through the laboratory manipulations involved in storage and final testing. In addition, in an endemic cholera area a serological rise may represent a bacteriological failure rather than a serological error. In this group of patients, the serological method recognized at least 3 cases in whom one must presume unrecognized bacteriological infection since contact with a known patient was established, and 3 others with contact but whose rise might possibly have been the results of vaccine administered after cholera had been diagnosed in the family. Using fingertip blood, the technique proved to be a very sensitive and simple field method for detecting subclinical infections among contacts of cholera patients. However, since the vibriocidal antibody responds to vaccination, a rise can only be construed as indicating infection if no vaccine has been administered within the past few weeks (Finkelstein, 1962; Basaca-Sevilla et al., 1964).

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RÉSUMÉ

Dans ce deuxième article consacré aux recherches sérologiques sur le choléra, on montre l'intérêt d'une microtechnique ne nécessitant qu'un prélèvement de 0,025 ml de sérum pour la mise en évidence des anticorps vibriocides.

La sensibilité de la méthode est supérieure à celle de l'épreuve d'agglutination aux fins de diagnostic rétrospectif du choléra. Dans les sérums couplés prélevés chez 370 malades atteints de choléra bactériologiquement confirmé, une hausse significative du titre des anticorps

vibriocides a été constatée dans 96,5% des cas (chez 91,5% des malades âgés de moins de 5 ans, et chez 98,2% des malades âgés de 5 ans ou plus). La réponse immunitaire s'est manifestée en général envers les deux antigènes utilisés, Ogawa et Inaba, mais dans 11,1% des cas, elle ne s'est révélée qu'en présence de l'antigène homologue et dans 1,9% des cas, uniquement en présence de l'antigène hétérologue. Il est donc indispensable de tester les sérums au moyen des deux sérotypes. L'étude sérologique de 191 sujets chez lesquels les examens bactériologiques étaient restés négatifs a montré une augmentation des titres d'anticorps vibriocides dans 6 cas. Il s'agissait de contacts de malades et 3 d'entre eux avaient été vaccinés contre le choléra; 3 autres cas (1,6%) doivent être considérés comme faussement positifs.

Les résultats de la recherche des anticorps vibriocides ont été comparés à ceux des cultures d'échantillons de selles pour le dépistage du choléra parmi les contacts familiaux de malades. Les techniques bactériologiques ont permis de découvrir 13,1% de cas d'infection cholérique, alors que par la méthode sérologique, 16,3% de résultats positifs ont été obtenus.

En pratique normale, la microtechnique de titrage des anticorps vibriocides apparaît comme un procédé sûr, relativement simple, capable de déceler plus de 90% des infections à *Vibrio cholerae* avec moins de 2% de résultats faussement positifs.

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