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Serological Studies in Cholera

1. Vibrio Agglutinin Response of Cholera Patients Determined by a Microtechnique *

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An agglutinin test for the determination of antibody responses to Vibrio cholerae, *requiring only 0.025 ml of serum, has been developed. This microtechnique permits the determination of agglutinin titres using fingertip blood, with results comparable with those obtained using venous blood taken at the same time.*

Among 364 serum pairs in bacteriologically confirmed cases of cholera from an endemic area of East Pakistan, the second serum sample being obtained 6 days or more after the onset of symptoms, a fourfold rise in agglutinin titre occurred in 77.6% of children under 5 years and in 93.2% of persons 5 years of age or older. This titre rise was quite often demonstrated only against a bacterial suspension of the same serotype as the infecting organism. Among 198 serum pairs in bacteriologically negative cases, a fourfold titre rise against the Inaba suspension only was found in 1 case; 5 other persons with fourfold titre rises proved to be household contacts of cholera patients and are taken to represent bacteriological failures or responses to cholera vaccine rather than false positive serological responses.

The development of immune substances during convalescence from cholera was documented very early in the bacteriological era. In their excellent review, Pollitzer & Burrows (1959) refer to a report by Lazarus in 1892, and they quote Metchnikoff's report of a rise in bacteriolytic titre during convalescence, measured by Pfeiffer's reaction in the peritoneal cavity of the guinea-pig. The use of the simpler test of the ability of convalescent sera to agglutinate cholera vibrios, the agglutination reaction, was first recommended in 1897 by Achard and Bensaude (quoted by Pollitzer & Burrows, 1959) and this has become the most widely used serological technique.

As a result of extensive studies with the agglutination reaction, Goodner and his co-workers (Goodner, Smith & Stempen, 1960; Goodner, Stempen et al., 1960; Smith & Goodner, 1965) found that the most specific and sensitive antigen for use in this reaction is the living cholera vibrio. The sensitivity of the test varied with the strain used as antigen; with appropriate strains, excellent correlation between the serological diagnosis of infection and bacteriological confirmation of *Vibrio cholerae* as the infecting organism was obtained (Goodner, Smith & Stempen, 1960). Despite these encouraging results, the volume of material and equipment and the glass-washing competence involved when 16 or more test-tubes are used in testing each individual serum, coupled with the difficulty of obtaining venous blood from healthy people, seriously limit the use of this procedure for epidemiological purposes.

It was observed in the standard tube-agglutination test that agglutinated vibrios form a shield over the bottom of the tube after overnight refrigeration, while nonagglutinated living vibrios form a sharply defined button at the bottom of the tube. This suggested that the modified Takatsy microtechnique, which had been used effectively in the complement-fixation and haemagglutination procedures (Csizmas, 1960; Sever, 1962), might be usable, in which case fingertip blood could be used for serological testing, and large numbers of samples could be processed

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with minimal requirements on glassware and material. This paper describes the microtechnique which was developed, compares the results obtained with those with the standard tube-agglutination test, and describes our experience with the vibrio agglutinating antibody of patients admitted to a cholera ward.

MATERIALS AND METHODS

Equipment and reagents

The Microtiter kit for serial dilutions (Cooke Engineering Co., Alexandria, Va., USA) was used with the accompanying pipette droppers and loops calibrated to deliver 0.025 ml of saline per drop. Microtiter plates with round-bottomed cups (" U " plates) and conical-bottomed cups (" V " plates) were tried; since the patterns were clearer in the round-bottomed cups, the " U " plates were used. Reactions were read with the aid of the Microtiter test reading mirror. A " tetrazolium kit " containing various tetrazolium salts for use as indicators was procured from the Nutritional Chemical Corp., Cleveland, Ohio. Capillary tubes with a volume of 25 μl and 50 μl (0.025 ml and 0.05 ml) (" Microcaps ", Drummond Scientific Co., Broomall, Pa., USA) were used for the collection of fingertip blood.

Sera

Blood was collected on admission and at various times during the course of illness from all patients admitted to the ward facilities of this laboratory; these were patients either with bacteriologically confirmed cholera or with severe diarrhoea from whose daily rectal swabs cholera vibrios were not isolated. The sera were separated, then stored in the frozen state.

Cases were considered bacteriologically confirmed only if *V. cholerae* were identified from at least 2 different rectal swabs; the sera from cases with only a single positive bacteriology report were not included in analyses of titre rise. The bacteriological techniques were those previously reported from this laboratory (Monsur, 1961; Greenough et al., 1964; Benenson et al., 1964).

Fingertip blood

Capillary blood (0.025 ml or 0.05 ml) was drawn into the appropriate capillary tube from the standard puncture in the warm fingertip. This blood was immediately ejected into 0.475 ml or 0.45 ml, respectively, of sterile saline in a screw-cap vial and the capillary tube rinsed once with the diluted blood, giving a 1 : 20 or 1 : 10 dilution of the whole blood. The supernatant diluted plasma was removed from the red cells before the sample was frozen for storage.

Bacterial antigens

A variety of *V. cholerae* strains, including the NIH strains Inaba 35A3 and Ogawa 41 as well as local isolates, were tested for suitability as living test suspensions. The greatest sensitivity and sharpness of agglutination were obtained with 2 local strains, *V. cholerae* Ogawa, CRL 465, and *V. cholerae* Inaba, CRL 466; both had been isolated from cholera patients in Khulna, East Pakistan, in 1962. *V. cholerae* Inaba, strain J89, provided by Dr Goodner, was comparable to Inaba 466.

Tube-agglutination tests

These tests were performed essentially as outlined by Goodner, Smith & Stempen (1960) and Goodner, Stempen et al. (1960) using living organisms as antigen. Cultures were incubated overnight in T_1N_1 (trypticase 1% and NaCl 1%) at 37°C, and adjusted to the turbidity of McFarland Standard No. 1 (equivalent in turbidity to 10 units of the International Opacity Standard) by dilution with sterile broth or saline. Half-millilitre volumes of serum dilutions and test suspensions were mixed and then incubated in a water-bath at 40°C–42°C for 1 hour; tubes were examined for agglutination at this time and again after overnight refrigeration at 4°C. The initial serum dilution tested in this system was 1:40; titres were recorded as the initial serum dilution of the last tube in which clear-cut agglutination was seen.

Microtitre agglutination tests

The bacterial suspensions for the test were overnight cultures of the selected Ogawa and Inaba strains in 50-ml volumes of T_1N_1 broth in 125-ml Erlenmeyer flasks, adjusted to an optical transmission of 74%–80% (against water as 100%) at a wavelength of 515 nm in the Coleman Junior spectrophotometer. When coloured vibrios were desired, a 0.01% solution of tetrazolium violet in 1% alcohol was added to make a final concentration of 0.001%–0.0025% dye; after 15 minutes at room temperature, the suspension was ready for use.

Tetrazolium violet was the most suitable of the various salts of the " tetrazolium kit " for this purpose. Tetrazolium blue also produced coloured organisms when added after overnight incubation, but the buttons of unagglutinated vibrios were less sharp than with tetrazolium violet. When present

at a 0.00001 % concentration at the time the culture media were inoculated with vibrios, triphenyl tetrazolium chloride and neotetrazolium blue were also effective in colouring the organisms, and vibrio growth was unaffected by any of these salts at a final concentration of 0.001 %. Nitroblue tetrazolium and the formazans were ineffective.

Serial twofold saline dilutions of each serum in a 0.025-ml volume were prepared in duplicate across the 8-cup rows using the Microtiter loops, covering the range from 1:10 to 1:1280. With fingertip samples, the dilution made at the time of collection (1:10 or 1:20) was the starting dilution placed in the first cups. When the titre was not defined within the 8-cup row, the test was repeated in the 12-cup row covering a dilution range from 1:10 to 1:20 480.

A positive control serum over the appropriate dilution range was included in each protocol; in this work the serum of an immunized calf and later a positive goat serum were used.

The bacterial test suspensions (0.025 ml) were added to the serum dilutions with the Microtiter pipette; the Ogawa suspension to one row and the Inaba to the second row of dilutions for each serum. The contents were gently mixed; the plate was securely sealed with plate sealing tape, incubated at 40°C for 1 hour in the water-bath (no leakage occurred if the plates were submerged) and then held in the refrigerator overnight.

Reactions were read using the Microtiter test reading mirror. The white buttons of unagglutinated vibrios, after overnight refrigeration, stood out sharply and clearly against a black background when the light was projected through the plastic plate at right angles to the visual axis. The black background was easily provided by laying a mat black cardboard on top of the plate; the patterns were so clear that the reactions could be read without removing the sealing tape from the plate. When tetrazolium salts were used the coloured buttons stood out most clearly against a white background. The last serum dilution in which no button of unagglutinated vibrios was present, an objective observation on which all observers agreed, was recorded as the end-point. Partial agglutination surrounding a small button at higher serum dilutions was noted, but ignored in tabulations and calculations. Positive cups presented a diffuse shield or irregular pattern of agglutinated organisms; inactivation of the sera was found not to influence the titre but the positive cups containing uninactivated sera were often completely empty (bacteriolysis). Alternatively, the reaction could be read under the stereoscopic microscope which clearly revealed static agglutinated shields or scintillating unagglutinated buttons.

The Microtiter plates and other equipment were cared for according to the manufacturer's directions. Decontamination of the plates was ensured by a 30-minute soak in 0.5% sodium hypochlorite; the recommended care of the loops and pipettes effectively destroys vibrios. Thorough rinsing was found to be critical; the organisms were lysed in dirty cups, giving an irregular pattern of empty cups. This was avoided by cleaning each cup with a rotating brush and then prolonged rinsing under running tap-water.

RESULTS

Comparison of microtechnique with the tube agglutination method

The titres obtained in the Microtiter system were compared with those obtained by the standard tube-agglutination procedure by testing consecutive routine clinical samples by both techniques, using the same suspension of vibrios as the antigen for the 2 procedures. In a comparison of 527 tests (Table 1), in only 1.5% did the titre differ by more than twofold, while in 71% of the determinations identical titres were obtained. Since serological diagnostic tests are most meaningful when they demonstrate

TABLE 1
CORRELATION OF VIBRIO AGGLUTININ TITRES OBTAINED BY MICROTECHNIQUE AND TUBE TEST ON 527 ROUTINE SERUM SAMPLES

Tubes positive	Microtiter cups positive [a]									
	0	1	2	3	4	5	6	7	8	9
9	—	—	—	—	—	—	—	—	—	1
8	—	—	—	—	—	—	1	—	1	—
7	—	—	—	—	—	—	2	5	—	—
6	—	—	—	—	—	5	10	1	—	—
5	—	—	—	1	9	38	4	—	—	—
4	—	—	2	18	36	10	—	—	—	—
3	—	—	12	39	8	—	—	—	—	—
2	1	5	47	8	—	—	—	—	—	—
1	11	41	15	—	—	—	—	—	—	—
0	157	36	3	—	—	—	—	—	—	—

[a] Initial dilution tested was 1:40.

TABLE 2
CORRELATION OF VIBRIO AGGLUTININ TITRES BY MICROTECHNIQUE AND TUBE TEST IN 149 SERUM PAIRS

Titre rise, in tubes [a]	Titre rise, in Microtiter cups [a]									
	0	1	2	3	4	5	6	7	8	9
9	—	—	—	—	—	—	—	—	—	1
8	—	—	—	—	—	—	1	—	1	—
7	—	—	—	—	—	—	2	5	—	—
6	—	—	—	—	—	2	7	1	—	—
5	—	—	—	—	4	21	2	—	—	—
4	—	—	—	6	24	2	—	—	—	—
3	—	—	7	22	6	—	—	—	—	—
2	—	3	25	3	—	—	—	—	—	—
1	—	1	3	—	—	—	—	—	—	—
0	—	—	—	—	—	—	—	—	—	—

[a] For example, a titre rise from 1 : 40 to 1 : 160, i.e., from tube or cup 1 to 3, would be expressed as a 2-tube or 2-cup rise.

an increase in antibody content, the titre rises found by the two systems in 149 serum pairs from cholera patients were compared (Table 2). The results of the two methods were identical in 72% of the cases; and in only 1 instance was there a 2-tube disagreement.

The consistency of titres within and between the two test systems in repeated tests is illustrated by the results obtained with the positive calf serum controls (Table 3). The geometric mean titres were lower with the microtechnique than in the tube test, which is consistent with the difference in reading method in that the titre in the tube test was taken as the initial serum dilution in the last tube showing visible agglutination, while that in the Microtiter test was considered to be the initial serum dilution in the last cup in which there was no button of unagglutinated vibrios—which may still correspond to quite a high degree of agglutination. The range of titres obtained was broader with the Microtiter than the tube test, but, in 72 Microtiter tests performed later in which a goat serum was used as control, the standard deviations were of the same magnitude as those of the tube test, suggesting that experience with the technique may result in better inter-test consistency.

Evaluation of the use of fingertip blood

A comparison of agglutinin titres obtained by the microtechnique on 264 samples of fingertip blood, collected in saline, with those of the serum taken at the same time tested by the tube method, using the

TABLE 3
INTER-TEST CONSISTENCY OF VIBRIO AGGLUTININ TITRE BY MICROTECHNIQUE AND BY TUBE TEST

Titre, in tubes or cups	No. of sera					
	Calf serum				Goat serum	
	Ogawa suspension		Inaba suspension		Ogawa suspension	Inaba suspension
	Tube test	Micro-technique	Tube test	Micro-technique	Micro-technique	Micro-technique
11	—	—	—	—	1	—
10	—	—	—	—	3	1
9	—	2	—	1	51	10
8	21	11	4	5	15	49
7	17	15	29	13	2	10
6	1	11	5	16	—	—
5	—	—	1	4	—	—
Total No. of tests	39	39	39	39	72	70
Mean tube or cup No.	7.51	7.10	6.92	6.56	8.81	8.03
Standard deviation	0.56	0.88	0.58	0.94	0.62	0.50

TABLE 4
CORRELATION OF VIBRIO AGGLUTININ TITRES BY THE MICROTECHNIQUE ON FINGERTIP BLOOD WITH THOSE BY THE TUBE TEST ON VENOUS BLOOD [a]

Tubes positive	Microtiter cups positive									
	0	1	2	3	4	5	6	7	8	9
9	—	—	—	—	—	—	—	—	1	—
8	—	—	—	—	—	—	—	—	1	—
7	—	—	—	—	—	—	4	—	—	—
6	—	—	—	—	—	5	1	—	—	—
5	—	—	—	1	12	6	—	—	—	—
4	—	—	—	18	6	—	—	—	—	—
3	—	1	11	12	—	—	—	—	—	—
2	—	5	16	—	—	—	—	—	—	—
1	6	9	1	—	—	—	—	—	—	—
0	136	12	—	—	—	—	—	—	—	—

[a] The samples of fingertip blood and venous blood from the same person were collected at the same time. The initial dilution used for all tests was 1 : 40.

same vibrio antigens, showed excellent correlation (Table 4); 71% had identical titres, 23% a titre one tube lower and 5% one tube higher. Only 2 cases showed a deviation beyond twofold. The frequency of lower titres with fingertip blood is attributable in part to the space occupied by the formed elements in the measured volume of whole blood.

Comparable results were obtained when the fingertip blood was diluted in distilled water and frozen and tested without centrifugation. Haemolysis did not interfere with the readings, but it was necessary to use tetrazolium-violet-stained vibrios to differentiate a button of unagglutinated vibrios from one of red cell stromata and debris in the first cup or two.

Use of disposable plates

The only technical problems encountered were those due to failure to remove all disinfectant or detergent during the cleaning process mentioned above, and difficulty in reading the reaction in old scratched plates. It was hoped that both problems could be resolved by using disposable Microtiter plates. However, in these plates, the cups are not encased in plastic as in the nondisposable plates but are surrounded by air; it rapidly became evident that visualization of the uncoloured buttons of non-agglutinated vibrios depends on conduction of light by the plastic. The reaction in the disposable plates could be read with a dissecting microscope; but this considerably slowed the process. A comparison of 222 tests done simultaneously in disposable and nondisposable plates showed a fourfold or greater difference in titre in 17 sera (7.7%). However, rises in titre were comparable, so that disposable plates were reserved for diagnostic uses, where titre rise was the principal criterion.

Vibrio agglutinin titres of patients with cholera symptoms

In early studies using a 1 : 40 serum dilution, no significant serological rise was observed in many of the patients from whom *V. cholerae* was recovered on 2 or more occasions; when a 1:10 dilution was the initial serum dilution tested, a significant rise in titre was observed in most of these cases. The range of dilutions used (1:10 to 1:1280) covered the distribution of usual titres observed in our patient group; when all admission sera assayed by the microtechnique are considered (Table 5),

TABLE 5
PERCENTAGE DISTRIBUTION OF SERUM VIBRIO AGGLUTININ TITRES AMONG PATIENTS FROM THE DACCA AREA

Vibrio used as antigen	Percentage with agglutinin titre									Total No. of patients
	<10	10	20	40	80	160	320	640	≥1 280	
All patients on admission to hospital										
Ogawa	47.1	29.8	12.5	7.3	3.0	0.4	0.3	—	—	735
Inaba	48.7	28.9	13.3	5.0	2.9	0.8	—	—	—	742
Convalescent cholera patients										
Ogawa	9.0	11.7	12.5	12.7	13.8	13.0	11.2	8.8	7.2	376
Inaba	5.3	4.5	10.9	12.7	17.2	14.3	15.1	8.5	11.1	377

TABLE 6
AGGLUTININ TITRES FOR PAIRED SERA IN 227 VIBRIO-NEGATIVE DIARRHOEA CASES

Late serum titre	Admission serum titre								
	<10	10	20	40	80	160	320	640	1 280
				Ogawa suspension					
1 280	—	—	—	—	—	—	—	—	—
640	—	—	—	—	—	—	—	1	
320	—	—	—	—	—	—	—	—	—
160	—	—	—	—	2	1	1	—	—
80	—	—	—	3	3	1	—	—	—
40	—	—	—	10	—	—	—	—	—
20	—	2	22	6	—	—	—	—	—
10	14	46	9	—	—	—	—	—	—
<10	83	20	2	—	1	—	—	—	—
				Inaba suspension					
1 280	—	—	—	—	—	—	—	—	—
640	—	—	—	—	—	—	—	—	—
320	—	—	—	—	—	1	—	1	—
160	—	—	—	—	1	—	—	—	—
80	—	—	—	1	5	—	—	—	—
40	—	—	2	6	2	—	—	—	—
20	1	5	24	4	—	—	—	—	—
10	17	43	10	—	—	—	—	—	—
<10	85	16	2	—	1	—	—	—	—

about 50% of over 700 had no detectable antibody at the 1:10 level; among convalescent sera in approximately 376 confirmed cholera cases, the modal titre was 80. Only 11% had titres of 1280 or higher; additional dilutions had to be tested if the precise end-points were desired. For practical purposes, the end-point was not required; no admission sera were positive beyond the sixth cup so that all fourfold rises were evident.

The question remained whether, at a 1:10 dilution, nonspecific agglutination might confuse the results. Analysing the early and late titres of vibrio-negative cases, it was evident that serologically false positive results were relatively rare. Two hundred and twenty-seven serum pairs were available (Table 6) from patients from whose daily rectal swabs vibrios were *not* isolated during hospitalization; in 182 the second sample was drawn 6 days or more after the onset of illness; in the other 45, the second serum was obtained earlier. In only 7 of the 454 tests (1.5%) performed with the 2 antigens did the serum pairs differ by fourfold or more, and in only 1 test (0.2%) was this difference in titre a rise. It is pertinent to note that the second sera in general showed a drop in titre more often than a rise. This was also seen not infrequently in cholera cases when the second sample was obtained within the first 2 or 3 days after the onset of disease, and might be a reflection of the haemoconcentration at the time the initial blood was drawn, with an apparent fall in titre when rehydration was effected within the first few hours after admission.

However, 1 case in this group gave an admission titre of 80 against both Ogawa and Inaba suspensions, but no detectable agglutinin at a 1:10 dilution in the second member of the pair; this was con-

sidered to be due to an error in serological routine, which involves the proper identification of the patient, labelling of the collected blood, maintenance of the identification of the blood during centifugation, proper labelling for storage and correct identification during the testing procedures.

The vibrio agglutinin titre against organisms of the Ogawa serotype was compared with the titre against Inaba organisms in admission sera from vibrio-negative cases (including those with unpaired sera) and in convalescent sera from patients with confirmed infections with *V. cholerae* Inaba (Table 7). In only 10 of the 326 admission sera (3.1%) did the titres against the two antigenic suspensions differ by more than one dilution, and in 7 of these, the anti-Ogawa titre was higher. On the other hand, among the 388 sera obtained from patients convalescing from Inaba infections there was a clear excess of anti-Inaba over anti-Ogawa agglutinins, with 18% differing by fourfold or more. Of interest are 16 cases with no detectable antibody against the Ogawa suspension at a 1:10 level but anti-Inaba titres ranging from 20 to 160, suggestive of type-specific responses (Smith & Goodner, 1965).

TABLE 7

CORRELATION OF INABA AGGLUTININ TITRES WITH OGAWA AGGLUTININ TITRES FOR 326 ADMISSION SERA OF VIBRIO-NEGATIVE PATIENTS AND CONVALESCENT SERA OF 388 INABA-POSITIVE PATIENTS [a]

Ogawa cups positive	Inaba cups positive								
	0	1	2	3	4	5	6	7	⩾8
Admission sera (326)									
⩾8									1
7								—	
6							1	—	
5				1	2	—	—	1	
4		—	1	3	7	1	—		
3	—	3	7	11	5	1			
2	2	11	27	2	—				
1	22	65	11						
0	124	16	1						
Convalescent sera (388)									
⩾8					2	—	3	2	15
7					—	1	10	11	6
6					3	7	17	7	3
5					7	20	13	1	5
4			2	9	28	16	2	2	3
3	—	—	5	30	17	7	2	2	1
2	1	4	17	18	9	2	—		
1	5	10	14	6	6	2	1		
0	10	8	7	3	4	2	—		

[a] The initial dilution used in all cases was 1 : 10.

The time after the onset of disease at which vibrio agglutinins appear was assessed in two ways. First, 163 consecutive vibrio-positive patients who had had no demonstrable agglutinin at the 1:40 level in the tube test on admission and developed a titre of 80 or higher during convalescence were selected, and the titres obtained on all sera tested were charted (Table 8). A significant rise in antibodies was first observed on the fourth day after the onset of symptoms; by the eighth day essentially all had developed an antibody rise. The geometric mean titre was at its highest level in the 11–15-day period and had fallen significantly one month after the onset of symptoms. Secondly, a prospective study was set up in which fingertip blood was taken daily except at weekends from 88 patients; all blood samples from one patient were tested by the Microtiter test at the same time, starting at the 1:10 level. Two patients failed to develop antibody by the tenth day, but among those in whom a rise occurred during consecutive daily bleedings, half showed the first (twofold) rise by the fifth day and over half had attained a fourfold rise by the sixth day (Table 9).

In routine use of the Microtiter bacterial agglutinin test, a fourfold rise in titre occurred in paired sera in 89.6% of 364 consecutive bacteriologically confirmed cholera cases for which a second sample was available on the sixth or subsequent day after the onset of symptoms (Table 10). The diagnostic sensitivity of the method varies with age; among children under 5 years of age, only 78% of confirmed cholera cases showed a significant rise in titre, while 93% of 279 cholera patients 5 years of age or over were serologically positive. It is notable that, in the younger age-groups, half the cases with serological responses only showed a rise against the homologous serotype, while in the older age-groups, a fourfold rise occurred with both antigens in 95% of the cases. Among 198 vibrio-negative persons studied during the same period, 6 showed a fourfold or greater rise in titre. However, 5 of these proved to be family members of vibrio-positive cholera patients and 3 of them had received cholera

TABLE 8

TIME DISTRIBUTION OF VIBRIO AGGLUTININ TITRES IN TUBE AGGLUTINATION TESTS OF ALL SERA EXAMINED FOR 163 SEROLOGICALLY POSITIVE PATIENTS WITH NO AGGLUTININ ON ADMISSION [a]

Tubes positive	Period after onset of symptoms														
	Days										Months				
	1	2	3	4	5	6	7	8-10	11-15	16-30	1	2	3	4	5
8								1	1						
7								3	2	1					
6							1	11	3	2					
5					1	3	9	14	4	1					
4					3	4	12	19	12	4	2				
3				1	2	6	13	13	7	3	6	3			
2				5	2	11	10	11	—	1	3	—			
1				4	1	5	3	1	2	1	4	1	—	—	1
0	65	69	35	28	14	4	2	1	—	—	9	9	5	—	1
Total No. tested	65	69	35	38	23	33	50	74	31	13	24	13	5	—	2
Mean tube No.	0	0	0	0.4	1.2	2.3	3.2	4.1	4.2	4.0	1.5	0.8	0	—	0.5

[a] Initial serum dilution 1 : 40.

vaccine within 1 week. These must be considered as serologically positive cases, representing either response to vaccination or bacteriological failure. One single case remained which was bacteriologically negative but showed a fourfold rise only in the Inaba titre, representing a false positive rate of 0.8%.

TABLE 9

DAY OF APPEARANCE OF VIBRIO AGGLUTININS IN DAILY SAMPLES OF FINGERTIP BLOOD [a]

Days after onset of symptoms	No. tested	Titre rise ≥2-fold [b]		Titre rise ≥4-fold [b]	
		No.	%	No.	%
1	48	0	0	0	0
2	82	0	0	0	0
3	88	2	2.3	0	0
4	87	13	14.9	6	6.9
5	84	41	48.2	23	27.4
6	82	67	75.9	47	57.3
7	86	79	90.8	71	82.6
8	82	81	97.6	80	97.6

[a] Initial serum dilution 1 : 10.

[b] The results shown are cumulative, i.e., the figures for a given day refer to samples showing a 4-fold titre rise on the day in question or on a previous day, or both.

DISCUSSION

Routine determination of the vibrio agglutination titre in serum pairs by the microtechnique proved to be a valuable retrospective diagnostic tool; it confirmed 90% of the bacteriologically proven infections in residents of an endemic area, a diagnostic sensitivity comparable to that reported by Felsenfeld et al. (1964), Smith & Goodner (1965), Feeley (1965), and Sack et al. (1966). The procedure was less sensitive in young children; only 79% of those under 5 years showed a rise in titre as opposed to 93% of those older than 5 years. The rise in antibody usually occurred by the end of the first week after the onset of symptoms, was maximal in the second week and fell rapidly later; findings similar to those of Barua & Sack (1964). Among Inaba infections the rise in titre was largely against the homologous bacterial suspension and in some cases was demonstrated only with the Inaba antigen; in 3 of the 13 Ogawa infections, the rise was demonstrable only with the Ogawa suspension. Although absorptions were not done, this suggests that in these cases the predominant rise was in type-specific antibody (Smith & Goodner, 1965), and it is

TABLE 10

DIAGNOSTIC EFFICACY OF BACTERIAL AGGLUTININ TESTING OF SERUM PAIRS [a]

Age-group (years)	Titre rise ≥4-fold				No. without significant titre rise	Total No. of cases
	No.			Total (%)		
	Inaba and Ogawa	Homologous serotype only [b]	Heterologous serotype only [c]			
Bacteriologically confirmed cholera patients						
0-4	32	33 (1)	1	77.6	19 (1)	85 (2)
5-14	88 (4)	20 (1)	1	94.8	6	115 (5)
>14	127 (4)	20 (1)	4	92.1	13 (1)	164 (6)
Total	247 (8)	73 (3)	6	89.6	38 (2)	364 (13)
Bacteriologically negative for *V. cholerae*						
0-4	1 [d]	1	—	2.2 [d]	44	46
5-14	3 [d]	—	—	—	20	23
>14	1 [d]	—	—	—	128	129
Total	5 [d]	1	—	0.5 [d]	192	198

[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] These persons were household contacts of cholera patients, and 3 had been vaccinated within the past week. They are not included in the percentage positive.

evident therefore that both serotypes must be used as antigens in a diagnostic test. The specificity of this serological diagnostic test was high, with only 1 of 198 bacteriologically negative cases showing a possibly false-positive fourfold rise in titre; 5 others with titre rise proved to be close contacts of confirmed cholera patients who very likely had been infected, and 3 of them had received cholera vaccine within one week.

This study has shown that vibrio agglutinin can be assayed as effectively and consistently by the Microtiter system as in the standard tube test. The microtechnique requires so little test material that serological surveys can be carried out on population groups using fingertip blood. With this method, a few technical personnel can process large numbers of sera with rapidity and accuracy. Field surveys can be carried out using fingertip blood diluted either in distilled water or in saline. However, when distilled water was used tetrazolium violet was needed to differentiate a button of nonagglutinated vibrios from one of cellular debris. When saline was used as diluent, it was necessary to separate the supernatant from the red cells before the sample could be frozen for storage. Despite this added step, saline was preferred as diluent by the technical staff because of the sharper patterns. The collection of blood samples on filter-paper discs was discarded because of the problems of completely eluting the antibodies (Schmidt et al., 1966).

The titres obtained by this microtechnique were slightly lower than those found by tube agglutination. This is to be expected, since the end-point in the tube agglutination test was the last serum dilution which produced definite agglutination of the organisms, while the end-point in the microtechnique was defined as the last tube in which a button of nonagglutinated vibrios was *not* present. The difference in the titres obtained by the two methods is not significant. Inter-test reproducibility is better with the experienced observer in the tube agglutination test; there is a suggestion that, with greater

experience, improved inter-test reproducibility can be achieved in the microtechnique. The Microtiter system has all the merits and demerits of any agglutination system with living vibrios, involving the problems of daily antigen preparation and standardization and like all such systems it does not differentiate between group-specific and type-specific antibodies.

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RÉSUMÉ

Une microtechnique pour la mesure de la réponse immunitaire à l'infection par *Vibrio cholerae* a été mise au point. Elle n'utilise qu'une très petite quantité de sérum (0,025 ml) et les échantillons de sang peuvent donc être prélevés par simple piqûre du doigt. Elle permet de titrer les agglutinines sériques avec une précision comparable à celle de la technique classique d'agglutination en tubes.

Cette méthode a été éprouvée au Pakistan oriental sur 364 sérums couplés prélevés chez des malades atteints de choléra bactériologiquement confirmé. Le premier échantillon a été recueilli lors de l'admission à l'hôpital, le second 6 jours au moins après l'apparition des premiers symptômes. On a utilisé comme antigènes diverses souches de *V. cholerae* des types Inaba et Ogawa. Une augmentation des titres d'agglutinines de quatre fois a été notée dans 89,6% des cas. La méthode s'est montrée sensible chez 85 enfants de moins de 5 ans, une hausse significative des titres d'agglutinines se manifestant dans 77,6% des cas, alors que chez 279 malades âgés de plus de 5 ans, une évolution analogue était observée dans la proportion de 93,2%. Dans les groupes d'âge inférieurs, 50% des réponses immunitaires concernaient uniquement le sérotype homologue. Dans les groupes d'âge supérieurs, en revanche, une hausse significative des titres envers les deux antigènes Inaba et Ogawa a été notée dans 95% des cas.

Des sérums couplés ont été également prélevés chez 198 personnes indemnes de choléra. Une augmentation de quatre fois des titres d'agglutinines pour *V. cholerae* a été décelée chez 6 d'entre elles. Il s'agissait dans 5 cas de contacts de cholériques dont 3 avaient été vaccinés contre le choléra. On doit considérer ces réactions comme des échecs de l'examen bactériologique ou des réponses à la vaccination anticholérique, le dernier cas seul représentant une réaction faussement positive.

Les avantages de cette microtechnique sont évidents et son emploi permet l'examen rapide d'un grand nombre de sérums. Les titres d'agglutinines qu'elle met en évidence sont légèrement inférieurs à ceux que l'on décèle par la technique d'agglutination en tubes, mais la différence n'est pas significative.

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