CHARACTERISTICS OF NON-CHOLERA VIBRIOS ISOLATED
FROM PATIENTS WITH DIARRHOEA

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THE vibrios that have similar biochemical reactions to those of *Vibrio cholerae* and are not agglutinable by *V. cholerae* O-subgroup I antiserum have been designated non-cholera vibrios (NCV), non-agglutinable or non-agglutinated vibrios (NAG), choleriform vibrios (Pollitzer, 1959) and water vibrios (McIntyre and Feeley, 1965). In India and Pakistan, the classical home of cholera, NCV have been associated with the "full-blown cholera syndrome" (Carpenter *et al.*, 1965; Lindenbaum *et al.*, 1965). The clinical features of NCV-diarrhoea range from a cholera-like disease with rice-water stools to mild gastro-enteritis (McIntyre and Feeley; Aldová *et al.*, 1968). However, the aetiological relation remains to be proven. Infection with *V. cholerae* is known to cause a rise in the serum agglutinins in 94 per cent. of cases (Sack *et al.*, 1966). On the other hand, NCV infection does not consistently give rise to a detectable immunological response (Lindenbaum *et al.*; McIntyre *et al.*, 1965; Aldová *et al.*).

Heiberg (1936) classified NCV into six groups, and two additional groups have recently been proposed by Smith and Goodner (1965). Heiberg groups are conventionally determined on the basis of fermentation reactions with the sugars mannose, sucrose and arabinose. However, several other biochemical characters of the Heiberg groups have not been described in the literature. The present work was planned to investigate the biological characters of NCV strains associated with choleraic diarrhoea in Calcutta according to their Heiberg groups.

MATERIALS AND METHODS

During 1967-68 we investigated patients with acute diarrhoea suggestive of cholera who had not received prior antibiotic therapy and were admitted to the Johns Hopkins Unit of the Infectious Diseases Hospital, Calcutta. Shortly after admission, portions of a faecal sample from each patient were plated on TCBS Agar (BBL), bile salt agar and MacConkey's Agar (Difco); the plates were incubated overnight at 37°C. The TCBS Agar is a highly selective medium for the isolation of vibrios. It contains yeast extract 5.0 g, peptone 10.0 g, sodium citrate 10.0 g, sodium thiosulphate 10.0 g, ox gall 5.0 g, sodium cholate 3.0 g, sucrose 20.0 g, sodium chloride 10.0 g, iron citrate 1.0 g, thymol blue 0.04 g, bromthymol blue 0.04 g and agar 14.0 g in a total volume of 1 litre at pH 8.6±0.2. The colonies of vibrios are yellow in the case of sucrose fermenters {i.e., for Heiberg groups (HG) I, II} and green in the case of non-fermenters of sucrose (i.e., for HG V, VII, VIII). On TCBS medium the growth of other organisms such as coliforms and members of the *Comamonas*, *Pseudomonas* and *Aeromonas* genera is inhibited. On bile salt agar plates, NCV colonies appear slightly more turbid and a little more elevated than those of *V. cholerae* and *V. eltor*. We also attempted to find vibrios by examining slow-fermenters of lactose as well as lactose non-fermenting colonies that grew on MacConkey's Agar.

In 38 cases NCV were detected as the only pathogen; faeces from nine other patients yielded NCV in association with *V. eltor*. Other enteric pathogens were not detected in any of these patients. After checking their colonial morphology we subjected the 47 strains of NCV isolated from the 47 patients to two series of tests.

The preliminary examinations included observations on the Gram-staining reaction, motility test (Cruickshank, 1965), oxidase activity (Kovacs, 1956) and the string test (Smith, 1958); the smoothness or roughness of colonies was determined from the stability of

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bacterial suspensions in 0.3, 0.5, 0.7 and 0.85 per cent. NaCl in distilled water and also in 1 in 1000 acriflavine (test modified from that of Wilson and Miles, 1964); we also used the O-F test (Hugh and Leifson, 1953) and we studied H₂S production by NCV grown in TSI medium (Cowan and Steel, 1966); Møller's (1955) tests for decarboxylase reactions with arginine, lysine and ornithine were done with Difco base. The strains were tested for agglutinability with *V. cholerae* O-subgroup I antiserum.

The NCV identified by the above methods were subjected to a further battery of tests. Fermentation of glucose, mannitol, lactose, mannose, sucrose, arabinose, melibiose, cellobiose, salicin, dulcitol, inositol, sorbitol, rhamnose and raffinose was tested for in Broth Base Phenol Red (Difco) with the addition of 1 per cent. of the appropriate sugar. The strains were allocated to Heiberg groups (HG) when they were freshly isolated according to the modified procedure of Smith and Goodner. Other tests included formation of pellicle in

TABLE I

Heiberg groups of the 47 NCV strains isolated in Calcutta and the significant sugar fermentation reactions observed

Heiberg group	Result of fermentation test with			Number of strains
	sucrose	mannose	arabinose	
I	+	+	—	14
II	+	—	—	16
III	+	+	+	0
IV	+	—	+	0
V	—	+	—	2
VI	—	—	—	1
VII	—	+	+	13
VIII	—	—	+	1

overnight broth culture, indole test (after culture in peptone water for 48 hr), methyl red (MR) reaction (after 48 hr), Cook's (1950) modification of the nitrate-reduction test, Voges-Proskauer (V-P) test (after 48 hr), utilisation of citrate in Simmons' citrate medium (Difco), *o*-nitrophenyl- β -D-galactopyranosidase (ONPG) test (Cowan and Steel), Kauffmann-Petersen's (K-P) tests for the fermentation of the organic acids citrate and mucate (original method as modified by Ellis, Edwards and Fife, 1957), the appearance of visible turbidity in 1 per cent. peptone water at pH 9.2, haemolysin and haemagglutination tests with 1 per cent. sheep red cells (Barua and Mukherjee, 1964, 1965). Gelatin degradation was tested for by the method of Smith and Goodner. For determination of antibiograms, the conventional agar-diffusion method was employed with "Multodisks" (Oxoid) placed on a dried seeded plate of Mueller-Hinton (Difco) medium. The disks separately held 10 μ g each of chloramphenicol, tetracycline and streptomycin, 25 μ g of ampicillin, 5 μ g kanamycin and 100 units of polymyxin B. Disks containing 50 units of polymyxin B (Difco) were also used separately. Sensitivity to 2,4-diamino-6,7-diisopropyl pteridine ("Vibriostatin" 0/129 supplied by Allen and Hanbury Ltd, Ware, Herts, England) was tested on nutrient agar plates with crystals of the reagent (Shewan, Hodgkiss and Liston, 1954). The sensitivity of the strains to Mukerjee's (1963) cholera group-IV phage was also tested.

RESULTS

Non-cholera vibrios (NCV) of Heiberg groups (HG) II, I and VII were isolated more frequently than NCV of the other groups, and no HG-III and HG-IV strains were obtained during this investigation (table I). Each patient yielded only one biotype of NCV, though this differed from one case to another.

TABLE II

Results produced by the NCV of different Heiberg groups (HG) in various tests

Test	Number of strains of the stated group giving positive test results*						Total number positive
	HG I (14)	HG II (16)	HG V (2)	HG VI (1)	HG VII (13)	HG VIII (1)	
String test	14	16	2	1	12	1	46
Stability in saline	14	16	2	0	10	1	43
Stability in acriflavine	14	16	2	0	4	1	37
Gelatin degradation	14	15	2	0	13	1	45
Nitrate reduction	11	13	2	0	13	1	40
Indole production	14	16	2	1	6	0	39
Methyl red (MR)	3	2	1	1	0	0	7
Voges-Proskauer (V-P)	11	13	0	0	0	0	24
Simmons' citrate utilisation	13	15	1	0	0	0	29
Fermentation of organic acids (K-P tests):							
citrate	14	15	2	0	0	0	31
mucate	14	10	1	0	0	0	25
ONPG	14	16	2	1	0	0	33
Sugar fermentation tests:							
glucose (no gas)	14	16	2	1	13	1	47
lactose	11	15	2	0	0	0	28
mannitol	14	16	2	1	13	1	47
mannose	14	0	2	0	13	0	29
sucrose	14	16	0	0	0	0	30
arabinose	0	0	0	0	13	1	14
melibiose	0	0	0	0	2	0	2
cellobiose	3	0	0	0	0	1	4
salicin	0	0	1	0	0	0	1
Pellicle formation	13	12	1	0	0	0	26
Haemagglutination	9	12	0	0	2	0	23
Haemolysin production	9	9	1	1	0	0	20
Decarboxylase reactions:							
arginine	0	0	0	0	0	0	0
lysine	14	16	2	1	13	1	47
ornithine	14	16	2	1	11	1	45
Antibiotic resistance tests:							
chloramphenicol (10 µg)	0	0	0	0	0	0	0
tetracycline (10 µg)	0	1	1	0	0	0	2
streptomycin (10 µg)	1	2	0	0	0	0	3
ampicillin (25 µg)	0	3	1	0	13	0	17
kanamycin (5 µg)	1	0	0	0	0	0	1
polymyxin B (100 units)	0	0	0	0	0	0	0
polymyxin B (50 units)	9	10	1	0	0	0	20

* Figures in brackets indicate total numbers of strains tested.

All 47 strains were Gram-negative motile rods, giving positive results in tests for oxidase and catalase, producing acid without gas in fermentation tests with glucose and mannitol, giving positive decarboxylase test results with lysine but negative with arginine, and sensitive to "Vibriostatin" 0/129. The organisms belong to the genus *Vibrio* and, being inagglutinable with *V. cholerae* O-subgroup I antiserum, were regarded as NCV.

TABLE III
Comparison of the biochemical reactions of NCV strains isolated in three investigations

Test	Results of tests reported in the series of					
	McIntyre and Feeley (1965) 69 strains		Sakazaki <i>et al.</i> (1967) 118 strains		Present study (1967) 47 strains	
	Number positive	Number negative	Number positive	Number negative	Number positive	Number negative
Oxidase	69	0	118	0	47	0
O-F test (fermentative) . .	69	0	118	0	47	0
H ₂ S production	0	69	0	118	0	47
Indole production	68	1	118	0	39	8
Haemolysin production . .	68	1	117	1	20	27
Fermentation of organic acids (K-P tests):						
citrate	...	...	117	1	31	16
mucate	...	...	118	0	25	22
Decarboxylase reactions:						
lysine	65	4	117	1	47	0
ornithine	65	4	117	1	45	2
Sugar fermentation reactions:						
lactose	48	21	118	0	28	19
melibiose	...	...	0	118	2	45
cellobiose	...	...	29	89	4	43
arabinose	4	65	0	118	14	33
sucrose	66	3	115	3	30	17

The majority of the HG-I and HG-II strains fermented lactose and gave positive results in the indole, V-P, ONPG, Simmons' citrate and K-P organic acid tests; NCV of HG VII all gave negative results in these tests. This distinction could also be made according to the presence or absence of a pellicle (table II). Pellicles appeared either in flimsy or membranous forms; five of the HG-I strains, eight of HG-II, and one of HG-V produced membranous pellicles, and the other pellicles were of the flimsy variety. More than half of the HG-I and HG-II NCV revealed haemolytic and haemagglutinating properties that were not possessed by HG-VII organisms. Unlike the other NCV, those of HG VII produced a faint purplish discoloration in the control tube set up for the decarboxylase test. Two strains of HG VII were ornithine decarboxylase-negative; a single strain in the same group gave a negative string test. Nine of the 13 HG-VII strains produced rough colonies as judged by their reactions in slide tests with saline or acriflavine. In tests of all of the NCV isolated in the present study, acid production was rarely observed in salicin, melibiose or cellobiose. Results of fermentation tests with dulcitol, inositol, sorbitol, rhamnose and raffinose were

DISCUSSION

The HG classification of our NCV isolates provided potentially useful information. NCV of HG I and HG II resemble each other in fermenting lactose and in giving positive reactions in the MR, V-P, Simmons' citrate, and K-P organic acid fermentation tests; on the other hand, HG-VII strains gave negative results in all these tests. The HG-I and HG-II strains appear to be biochemically active and may be related to each other, whereas NCV of group VII are biochemically inactive. Moreover, HG-VII organisms do not produce pellicle or a haemolysin and are sensitive to polymyxin B (50 units). The HG-VII strains are notably resistant to ampicillin (25 μ g). These features may serve as useful markers for the detection and tracing of NCV.

SUMMARY

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ANTIBODY TO ONCOGENIC VIRUS IN CANCER PATIENTS

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THE discovery that some human adenoviruses are oncogenic for hamsters (Huebner, Rowe and Lane, 1962; Trentin, Yabe and Taylor, 1962) and that animal oncogenic viruses, such as the simian virus SV40, are not necessarily species-specific (Koprowski *et al.*, 1962) led to a search for evidence of infection with these viruses in cancer patients (Larson, Gasnell and Hilleman, 1967; Rowe and Lewis, 1968; Shah, 1969).

In the present study we looked for complement-fixing antibodies against adenovirus type-12 tumour (T) antigen and SV40 tumour (T) antigen in both cancer patients and control subjects. In the hamster adenovirus-tumour model the presence of complement-fixing antibody against T antigen has been shown to reflect current antigenic stimulation; the antibody level drops on removal of the tumour (Larson *et al.*). The presence of this antibody in human serum probably indicates, therefore, either recent infection with the virus or a cancerous condition induced by the virus.

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