Vibrio parahaemolyticus and Diarrhoea Associated with Non-Cholera Vibrios

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Vibrios that do not agglutinate with Gardner & Venkatraman's (1935) O subgroup I antiserum have been associated with outbreaks of acute gastroenteritis (Aldová et al., 1968; Carpenter, Mitra & Sack, 1965; Lindenbaum et al., 1965; Yajnik & Prasad, 1954). These organisms have been collectively designated as NAG (non-agglutinating) vibrios or NCV (non-cholera vibrios). In 1936 Heiberg proposed a classification of these bacteria into 6 groups on the basis of reactions in mannose, sucrose and arabinose. Two additional groups have recently been added (Smith & Goodner, 1965). The serotypes of non-cholera vibrios have not been of great practical importance, however, because of their extreme antigenic heterogeneity (Gallut, 1963). Sakazaki, Gomez & Sebald (1967) studied the taxonomy of the non-cholera vibrios and some other related organisms and also proved the pathogenicity of non-cholera vibrios in infant rabbits. Although the association of these microbes with diarrhoea appears to be strong, the serological response following infection, when studied with paired sera, has so far been irregular (Lindenbaum et al., 1965; McIntyre et al., 1965; Aldová et al., 1968). This is in contrast to infection with *Vibrio cholerae*, where uniform increases in antibody titre have been recorded in 94% of the infected persons (Sack et al., 1966).

During recent years, *Vibrio parahaemolyticus*, a halophilic marine bacterium, has been reported to cause bouts of gastroenteritis in Japan. There the disease is communicated to man by consumption of contaminated sea fish (Sakazaki, 1965).

The present report deals with the characterization of the *Vibrio* strains isolated from patients with diarrhoea, in whom they are thought to have played a causative role on the basis of quantitative examination.

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Materials and methods

Patients. During 1968, a total of 37 patients with acute diarrhoea who had not received prior antibiotic therapy was admitted to the Johns Hopkins Unit of the Infectious Diseases Hospital, Calcutta. Shortly after admission, faecal samples were collected for bacteriological examination.

Microbiological techniques. The fluid samples of faeces were serially diluted with sterile saline in 10-fold steps up to 10^8. All dilutions, and the undiluted specimen, were spread with a glass spoon, in 0.1-ml quantities, on the surfaces of the following plates: TCBS agar (BBL), bile salt agar, MacConkey's agar (Difco), SS agar (Difco), bismuth sulfite agar (Oxoid), brain-heart infusion agar (Difco) with 7% sheep blood and 25 μg per ml of neomycin (anaerobic), *Mitis salivarius* agar (Difco), mannitol salt agar (Difco), Nagler medium with 50 μg per ml of neomycin (anaerobic). Sabouraud agar (Difco) and Rogosa SL agar (aerobic and anaerobic; Difco) were used as poured plates after mixing with the diluted specimens. All plates were incubated at 37°C. The period of incubation depended on the type of organism isolated. MacConkey's agar (for coliforms), SS agar (for *Salmonella* and *Shigella*), bile salt agar and TCBS agar (for vibrios), and Nagler neomycin (for lecithinase-positive clostridia) cultures were incubated for 1 day. *Mitis salivarius* (for faecal streptococci), and mannitol salt agar (for staphylococci and micrococci) cultures were read after 2 days. Cultures on Sabouraud agar (for fungi) and Rogosa SL agar (for aerobic and anaerobic lactobacilli) were counted after 3 days and those on neomycin blood agar (for *Bacteroides*) were observed after 5 days. Enrichment in alkaline peptone water (for 6 hours) and Selenite F broth (for 18 hours) was also carried out.

For a preliminary study of the genus *Vibrio* the tests included Gram staining, a motility test, oxidase and catalase tests, acidity in glucose, oxidation-fermentation (O-F) test, decarboxylase reactions in arginine, lysine and ornithine (Cowan & Steel, 1965) and slide agglutination in cholera antiserum.

For characterization of non-cholera vibrios the tests comprised: reactions to mannose, sucrose and arabinose, mannitol, lactose, dulcitol, adonitol, halose, rhamnose, raffinose, sorbitol, inositol, salicin and cellobiose (prepared by adding 1% sugar in phenol red broth base (Difco): acidity was observed daily for 5 days), gelatin degradation (Smith & Goodner, 1965), production of indole, nitrate reduction, methyl red (MR) test, Voges-Proskauer (V-P) reaction, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) test and Simmons' citrate test (Cowan & Steel, 1965). Kauffmann & Petersen's (1956) test (K-P) with organic acids, using citrate and mucate, and the test for haemolysin (Barua & Mukherjee, 1964) were also performed. For the categorization of the halophilic vibrios, overnight growths at 37°C in peptone water (pH 7.6) containing 0%, 0.5%, 3%, 6%, 8% and 10% sodium chloride were studied. Overnight growth in peptone water (pH 7.6) at 42°C was also observed (Sakazaki, personal communication, 1969). Ampicillin 25 μg (using Oxoid Multodisks) and 50 IU polymyxin-B discs (Difco) were selected for antibiotic sensitivity tests.

Results

The episodes of diarrhoea occurred suddenly and frequent loose stools followed at intervals. In 18 out of 37 cases, the quantitative study showed the presence of 10^6–10^9 non-cholera vibrios per ml of faeces: 8 of these had sucrose-fermenting and 10 non-sucrose-fermenting colonies on the TCBS agar. The growth of all 18 strains was slightly inhibited on MacConkey's agar. Counts of *Escherichia coli* ranged between 10^3–10^5 organisms per ml, while those of *Bacteroides* and streptococci were less than 10^3. Other normal faecal flora such as lactobacilli (aerobic and anaerobic), staphylococci, clostridia and fungi occurred infrequently.

All the 18 strains appeared as actively motile Gram-negative rods. These fermented glucose in O-F medium, produced acid without gas in glucose, were positive in oxidase and catalase tests and for lysine and ornithine decarboxylases. None produced arginine dehydrolase and none agglutinated in cholera antiserum (not shown in the table).

Some further characteristics of the non-cholera vibrios have been analysed in the accompanying table under the different Heiberg groups (Smith & Goodner's modification). On the basis of biochemical reactions, the organisms appear to be distributed in 2 broad groups: (1) halophilic *Vibrio parahaemolyticus*, non-sucrose fermenters (Heiberg groups, VII and VIII) and (2) non-halophilic, sucrose and non-sucrose fermenters (Heiberg groups, I, II and V). The former group showed growth in 0.5%, 3%, 6%, and 8% sodium chloride peptone water media; no turbidity was, however, observed at 0% and 10%. The same group of strains was ampicillin-resistant, polymyxin-B sensitive and was negative to lactose, V-P, MR, ONPG, Simmons' citrate, K-P organic acids, and to haemolysin tests. The second group could grow in 0%, 0.5%, and 3% sodium chloride concentrations; some (4 out of 10 strains) grew in the 6% concentration but no growth was detected in the 8% and 10% concentrations. All these strains were lactose and ONPG positive; most of them were positive to V-P, Simmons' citrate, K-P organic acids and haemolysin tests.

All strains were positive for gelatin degradation, nitrate reduction and indole production (except 1 indole-negative strain in Heiberg group VIII), they produced acid in mannitol and showed growth at 42°C. None of them produced acid in inositol, dulcitol, adonitol, rhamnose, raffinose, salicin, cellobiose and sorbitol.

Only 1 biotype was isolated from each individual patient although the strains differed in different patients. No *Shigella*, *Salmonella* or agglutinating vibrios were isolated from any of these cases.

Discussion

In recent years, the importance of quantitative bacteriological study has been emphasized in a number of infectious conditions. The enumeration of viable organisms in the urine is valuable in the diagnosis of urinary infection (O'Sullivan et al., 1960). Efficacy of antibiotics under the conditions of lung sepsis can be precisely assessed by undertaking periodic checks on the viable count of pathogens in the sputum, while ordinary qualitative study remains inconclusive (Louria, 1962). Similarly, Thomson (1955a, 1955b) observed that such methods are very informative in several types of diarrhoeic disease, for instance, *Salmonella* food-poisoning, enteric fever, infantile gastroenteritis and cases of symptomless excreters and carriers.

During the present study, the detection of 10^6–10^8 non-cholera vibrios per ml of faeces in 18 patients, the isolation of only 1 biotype from each case, and significant suppression of the normal intestinal flora, are evidence to suggest that the pathogens (non-cholera vibrios) succeeded not only in implanting but also in multiplying freely in the gut and in causing diarrhoea. While it is true that the results of sero-

NUMBER OF NON-CHOLERA VIBRIOS, BY HEIBERG GROUP, SHOWING POSITIVE REACTIONS IN CHARACTERIZATION TESTS

Tests	Heiberg group (in parentheses: no. of strains tested)					
	I (3)	II (5)	V (2)	VII [a] (7)	VIII [a] (1)	Total (18)
Growth in peptone water with:						
0 % NaCl	3	5	2	0	0	10
0.5 % NaCl	3	5	2	7	1	18
3 % NaCl	3	5	2	7	1	18
6 % NaCl	0	3	1	7	1	12
8 % NaCl	0	0	0	7	1	8
10 % NaCl	0	0	0	0	0	0
Gelatin degradation	3	5	2	7	1	18
Indole production	3	5	2	7	0	17
Nitrate reduction	3	5	2	7	1	18
Sugar reactions: [b]						
Mannose	3	0	2	7	0	12
Sucrose	3	5	0	0	0	8
Arabinose	0	0	0	7	1	8
Lactose	3	5	2	0	0	10
Trehalose	1	2	2	7	1	13
MR	1	0	1	0	0	2
V-P	2	5	0	0	0	7
ONPG	3	5	2	0	0	10
Simmons' citrate	3	5	1	0	0	9
K-P organic acids:						
Citrate	3	5	2	0	0	10
Mucate	3	4	1	0	0	8
Haemolysin	3	2	1	0	0	6
Antibiotic resistance:						
Ampicillin, 25 µg/disc	0	3	1	7	1	12
Polymyxin-B, 50 IU/disc	3	2	1	0	0	6
Growth at 42°C	3	5	2	7	1	18

[a] *Vibrio parahaemolyticus.*

[b] Reactions to other sugars, inositol, dulcitol, adonitol, cellobiose, salicin, raffinose, rhamnose, sorbitol were negative for all strains: all strains produced acid in mannitol.

logical study with paired sera from the patients are equivocal (McIntyre et al., 1965; Aldová et al., 1968), the evidence of pathogenicity in infant rabbits (Sakazaki, Gomez & Sebald, 1967) helps one to correlate indirectly these pathogens with human diarrhoea. Furthermore, the total absence of non-cholera vibrios in normal faeces, as observed by Smith & Goodner (1965) during an elaborate survey in 1 cholera endemic area is significant. The pathogenicity of *Vibrio parahaemolyticus* is well documented in the literature (Sakazaki, 1965), and has been further corroborated by the present observations.

During the present investigation, non-cholera vibrios were differentiated from Enterobacteriaceae by the oxidase test, from *Pseudomonas* by the fermentative breakdown of glucose in the O-F medium and from *Comamonas* by their capacity to attack glucose. *Plesiomonas* and *Aeromonas* were excluded by the decarboxylase reactions.

The halophilic vibrios isolated from cases of diarrhoea were investigated by Sakazaki (1965). He considered them under a single species, *Vibrio parahaemolyticus* having 2 biogroups (subgroups). Subgroup 1 is sucrose and V-P negative and grows in peptone water having 7% NaCl (recently said to be 8% NaCl), but not at the 10% NaCl concentration. In contrast, subgroup 2 is sucrose and V-P positive and grows in concentrations of NaCl up to 10%. Acid production in arabinose is another feature of subgroup 1. The strains of *Vibrio parahaemolyticus* isolated in this study seem to fit the description of subgroup 1.

During the present investigation, 5 out of 18 cases had stools similar to those of persons with bacillary dysentery. In 2 cases they were caused by *Vibrio parahaemolyticus*. The same pathogen was associated with rice-water stools in 1 patient. In 1969, a total of 8 cases of diarrhoea that showed heavy growth of *Vibrio parahaemolyticus* was studied. Of this total 6 manifested dysenteric symptoms with features of dehydration, nausea, pyrexia, abdominal pain and frequent evacuations of blood and mucus. The disease, though first reported here, seems to be common in Calcutta. Other cases of diarrhoea with non-cholera vibrios (non-halophilic) commonly showed signs of dehydration. In all cases the disease was self-limited and did not last beyond 48 hours. The possible sources of infections were not investigated during this study.

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