

## NON-AGGLUTINABLE VIBRIOS CAUSING CHOLERA-LIKE DISEASE IN SINGAPORE

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### SYNOPSIS

Five strains of non-agglutinable or NAG vibrios have been isolated from stool cultures of patients with cholera-like disease. They resembled the true cholera vibrios culturally, but were inagglutinable by the cholera O-group antisera or the rough anti-cholera serum. The vibrios were sensitive to most of the antibiotics tested. Immunological studies showed that the five strains belonged to three serotypes, although on sugar fermentation reactions, they could be placed into two Heiberg's groups (I and II).

### INTRODUCTION

Besides the true cholera vibrios which cause severe diarrhoea in man, other members of the *Genus Vibrio* have been found to give similar clinical symptoms. Among these pathogens are the halophilic bacteria *Vibrio parahaemolyticus* and *Vibrio alginolyticus* of Sakazaki (1963 and 1968), *Vibrio fetus* of King (1962) and a large heterogeneous group which are non-agglutinable with the cholera O-group I antisera hence designated as NAG (non-agglutinable group of vibrios). These NAG vibrios are widely distributed in nature and differ from *Vibrio cholerae* serologically and in certain biochemical reactions. In 1960, Ewing *et al* suggested that the differentiation between the true cholera and the so-called NAG vibrios should be based on the decarboxylation of the amino-acid lysine, arginine and ornithine along with other biochemical and serological tests. Sakazaki and his associates (1967) studied their collection of NAG vibrios and found that some were true vibrios while others belonged to the *Aeromonas* and *Plesiomonas* groups. They recommended that the differentiation should be based on the fermentation of glucose and mannitol, production of hydrogen sulphide, oxidase test and lysine decarboxylation.

It has been shown by early workers in the 1930's and 1940's that besides the true cholera vibrio, NAG, El Tor and other vibrios can also produce cholera-like symptoms in rabbits and man. In 1954, Yajnik and Prasad reported an outbreak of gastro-enteritis among pilgrims in Allahabad, India, and believed the NAG vibrios, which formed predominant growth of the stool cultures, were responsible for the infection. Two years later (1956), Gupta *et al* showed that the vibrios isolated from the 1954 gastro-enteritis outbreak in Allahabad could cause similar reactions on ligated rabbit gut as those of the

true cholera vibrios. This was confirmed by De in 1957. Dutta and his workers (1963) experimented on infant rabbits with Ogawa, El Tor, NAG and water vibrios, with enteropathogenic *Escherichia coli* and *Salmonella typhi* as controls. They proved that the NAG vibrios could produce diarrhoea in the rabbits identical with that produced by Ogawa strains. In 1965, McIntyre and Feeley isolated NAG vibrios from patients with diarrhoea in East Pakistan, and demonstrated high titres of agglutinating antibodies in these patients against the recovered organisms. The pathogenicity of NAG vibrios was also observed by Aldova *et al* in 1968 in Czechoslovakia during an outbreak of gastro-enteritis among a group of young men.

It is the purpose of this paper to present the characteristics and antibiotic sensitivity of the five strains of NAG isolated in Singapore from patients with cholera-like disease. All the patients had watery diarrhoea which was clinically indistinguishable from cholera.

### MATERIALS AND METHODS

The five strains of NAG vibrio (NAG 1-5) were isolated from patients with cholera-like symptoms from whom no other stool pathogens were cultured.

#### 1. Cultural reactions:

The watery stools were inoculated into 2% NaCl-peptone water and on thiosulphate citrate bile-salts sucrose agar (TCBS) and incubated at 37°C overnight. After isolation, the NAG vibrios were tested together with a local strain of *V. cholerae* biotype El Tor on solid media.

#### 2. Biochemical tests performed at 37°C:

Indol production in 1% tryptone; methyl red test (Cowan and Steel); citrate utilization (Simmons); nitrate reduction (Cook's); urease activity (Christensen's); oxidase test (Kovacs); hydrogen sulphide production in Kligler's iron agar; motility test (Edwards and Bruner); string test (Smith); decarboxylation of lysine, arginine and ornithine

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(Moeller); salt tolerance in 1% peptone water; growth in 1% peptone water at pH 4.5, 7.0 and 8.5; carbohydrate fermentations.

3. Serological tests:

Slide agglutination against *V. cholerae* Polyvalent, Ogawa, Inaba and rough anti-cholera antisera.

4. Immunological studies:

Immune sera were prepared in rabbits using the five strains of NAG vibrios as antigens.

Preparation of antigen—the bacterial cells were harvested from an overnight plate culture in normal saline. The suspension was heated at 100°C for one hour, washed twice, and resuspended in saline to give a turbidity corresponding to Brown's opacity tube No. 2 (circa  $2.8 \times 10^9$  cells per ml.). Sodium azide was added to give a final concentration of 0.1%. This suspension was used to prepare antiserum and as the antigen for the tube agglutination test.

Preparation of antiserum—rabbits were immunized with two 1 ml. antigen suspension, spaced one week apart. Ten days after the second immunization, blood was collected and the serum used in the agglutination test.

5. Antibiotic sensitivity:

The method of Kirby-Bauer was followed, using "Sensi-Disc Microbial Susceptibility Test Discs" (Baltimore Biological Laboratory—BBL). *Escherichia coli* (ATCC 25922) was included as a control.

RESULTS

1. The NAG 1—5 and El Tor vibrios produced similar cultural reactions on the different solid media tested. After overnight incubation, the

colonies were smooth, medium-sized and yellow on TCBS agar. All strains grew well on eosin methylene blue, Hektoen enteric (Difco) and MacConkey (BBL) media. They did not grow on desoxycholate citrate (BBL), Salmonella-Shigella (BBL) and bismuth sulphite (BBL) media.

2. The biochemical and other characteristics of the five strains were:

- (a) Indol +
- (b) Methyl red —
- (c) Citrate +
- (d) Nitrate to nitrite +
- (e) Urease —
- (f) Oxidase +
- (g) Hydrogen sulphide —
- (h) Motility +
- (i) String test +
- (j) Decarboxylases—lysine +
- arginine —
- ornithine +
- (k) Salt-tolerance ( 0% NaCl) +
- ( 3% NaCl) +
- ( 7% NaCl) —
- (10% NaCl) —
- (l) Growth at pH 4.5 —
- pH 7.0 +
- pH 8.5 +
- (m) Carbohydrate fermentation (Table I)

3. Slide agglutination tests:

None of the NAG vibrios was agglutinated by the cholera O-group I antisera or by the rough anti-cholera serum.

4. Results of the antigenicity study are recorded in Table II:

The agglutination studies showed that there were cross-reactions between strains 1 and 2 and 3 and 5.

TABLE I  
CARBOHYDRATE FERMENTATION IN ANDRADE PEPTONE WATER

	NAG 1	2	3	4	5	El Tor
Glucose	+	+	+	+	+	+
Lactose	(+)	(+)	—	—	—	—
Mannitor	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Salicin	—	—	—	—	—	—
Adonitol	—	—	—	—	—	—
Inositol	—	—	—	—	—	—
Dulcitol	—	—	—	—	—	—
Sucrose	+	+	+	+	+	+
Mannose	—	—	—	—	—	+
Arabinose	—	—	—	—	—	—
Heiberg' group	II	II	II	I	II	I

Acid production = + (positive)  
— (negative)

Strain 4 behaved differently from the rest in fermenting mannose.

TABLE II  
CROSS-AGGLUTINATION TITRES OF  
THE NAG VIBRIOS

Antigen Antiserum	NAG				
	1	2	3	4	5
1	128	64	—	—	—
2	64	64	—	—	—
3	—	—	32	—	32
4	—	—	—	32	—
5	—	—	32	—	64

differentiate between the true cholera and the non-agglutinable vibrios.

In a taxonomical study of the cholera and the non-agglutinable vibrios, Sakazaki *et al* (1966) divided the vibrios into thirty-three O-antigenic groups. The true cholera vibrios, including the El Tor vibrios, belonged to group I except for very rare strains (Sen *et al*, 1967; Pesigan *et al*, 1967), and the NAG vibrios which were inagglutinable with the O-antisera of *V. cholerae*, were put in other groups. Rough variants of *V. cholerae*, usually from carriers, are difficult to separate from other members of the vibrios. To identify such a rough strain, the

TABLE III  
ANTIBIOTIC SENSITIVITY (INHIBITION ZONE DIAMETER IN MM.)

			NAG 1	2	3	4	5	E. coli	Sensitive zone size
Penicillin	(BBL)	10 IU	20	20	18	18	18	0	22
Chloramphenicol	"	30 mcg	28	31	30	28	27	25	21—27
Tetracycline	"	30 mcg	22	21	22	22	21	21	18—25
Neomycin	"	30 mcg	18	19	18	17	17	17	17—23
Ampicillin	"	10 mcg	23	24	20	20	19	18	15—20
Septin	"	25 mcg	23	25	25	26	26	30	16
Carbenicillin	"	50 mcg	23	26	22	22	23	23	23
Kanamycin	"	30 mcg	21	23	20	19	20	23	17—25
Erythromycin	"	15 mcg	22	22	20	18	19	11	8—14
Triple-sulpha	"	0.25 mcg	16	16	20	17	15	27	17
Cephaloridine	(Oxoid)	30 mcg	19	20	19	18	19	21	18—23
Gentamycin	(Difco)	10 mcg	19	21	18	17	18	21	19—26
Polymyxin	(Difco)	50 IU	0	0	0	0	0	9	12—16

The five NAG strains were all resistant to 50 IU of Polymyxin B.

The agglutinins in these sera could be completely absorbed out by the cross-reacting antigens. This showed that 1 and 2 were similar, and 3 and 5 were also similar strains.

5. Antibiotic sensitivity results (Table III) showed the NAG strains were sensitive to a wide variety of antibiotics. No significant difference in sensitivity was observed between the strains. Like *V. cholerae* biotype El Tor strain, they were all resistant to 50 IU of Polymyxin B.

#### DISCUSSION

In accordance with a report by Ranjit Sen (1970), the NAG vibrios under investigation culturally and biochemically resembled *V. cholerae*. Using the grouping schema by Heiberg (1934) with the two additional groups by Smith and Goodner (1965) based on the fermentation of sucrose, mannose and arabinose, the five strains of NAG vibrio belonged to Group I (NAG 4) and Group II (NAG 1, 2, 3 and 5). NAG 1 and 2 were late lactose fermenters. Generally, the Heiberg's fermentation grouping is useful only in the classification of the vibrios. It cannot

colony is tested by slide-agglutination with a rough anti-cholera serum. The five NAG strains failed to agglutinate with any of the cholera O-group I antisera or the rough anti-cholera serum.

Although the five NAG strains were found to belong to only two biochemical (Heiberg) groups, immunological studies showed that they could be divided into three serotypes based on results obtained from cross-agglutination tests. The use of cross-agglutination tests might be useful in epidemiological studies.

For sensitivity testing, the method of Kirby-Bauer was adopted. All the strains gave fairly identical results. They were all resistant to Polymyxin B. The reaction of the vibrios to Polymyxin B is a useful test in differentiating between the true cholera and the El Tor vibrios. It would be useful to know if other NAG vibrios are similarly resistant to Polymyxin B.

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