THE ANTIGENS OF THE CHOLERA GROUP OF VIBRIOS

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CONTENTS

During the last few years a suspicion has arisen in the minds of medical officers in the East that the agglutinating sera with which they are provided for the diagnosis of *Vibrio cholerae* are not always able to distinguish it from other vibrios.

This question was brought to the notice of the Health Bureau of the League of Nations, which thereupon undertook to organise an international research with the object of producing a standard agglutinating serum of unexceptionable reliability.

For the solution of this problem a sound knowledge of the antigenic structure of *V. cholerae* and its antigenic relationship to other vibrios is clearly necessary. Supposing, for example, some of the sera in use contain an antibody common to *V. cholerae* and related organisms they may well be responsible for the incredibly high proportion of healthy carriers that has been reported in Syria and elsewhere.

The antigenic structure of vibrios of the cholera group has recently been investigated by a number of workers, amongst whom Balteanu (1926), Abdoosh (1932), Gohar (1932) and Shousha (1931) have differentiated the heat-stable from the heat-labile antigens of the vibrio. The non-specific nature of the thermolabile (H) antigen has been demonstrated by Shousha, Abdoosh and Bruce White (1934 *a*), though their work has hardly been on a wide enough scale to give a sense of finality. Greig (1915–16) established a serological classification of the group that has stood the test of improved methods; and Mackie and Storer (1918) confirmed Castellani's (1916) observation of "paracholera" vibrios serologically distinct from *V. cholerae* and apparently capable of causing choleraic diarrhoea. The reasons why these authors were successful in spite of a deficient knowledge of the antigenic structure of the vibrios are discussed later.

Japanese bacteriologists have also made important contributions to the subject, which have hitherto not been recognised in Europe (Gardner and Venkatraman, 1935). Thus Kabeshima (1913, 1918) discovered serological variants of the cholera vibrio, which were further investigated by Nobechi (1923) and Inouye and Kakihara (1925) (see Takano *et al.* 1926). The exact nature of the antigenic difference, however, was not established; in fact Inouye and Kakihara attributed it erroneously to the heat-labile antigens.

Aoki and Oshiro (1934) have claimed that a single culture can give rise to all the "types", which they consider to be expressions of a specific-non-specific alternation comparable to that of the Salmonella group. Their results, however, are based on the behaviour of a single strain, and could be explained by the assumption of an originally mixed culture. Further work on the question is desirable.

The important biochemical work of Linton and Mitra (1934) on the polysaccharide and protein components of vibrios of the group is not yet complete. The classification it indicates does not fully agree with the serological classification, since in certain cases identity of the protein and carbohydrate fractions is recorded in vibrios with different O antigens (El Tor 20 and 67 and El Tor D49).

Although a generally true picture of the constitution of the group may be drawn from these various records, we found sufficient uncertainties and disagreements to make a complete reinvestigation desirable.

For this purpose about 100 races of cholera and cholera-like vibrios have been collected from various sources and subjected to antigenic analysis. A list of these appears in Table I, which also contains information as to the biochemical characters, haemolytic properties and antigenic structure, in so far as these have been ascertained. This latter information anticipates the main thesis of our investigation, and study of it should therefore be postponed until later.

The arrangement is in batches, as we received them, and corresponds roughly with the supposed clinical significance of the vibrios.

A number of our cultures were lost during the investigation, and others were examined only for limited purposes. Hence the blanks scattered throughout the various columns.

SEROLOGICAL METHODS

Agglutination tests were done by Dreyer's method (Medical Research Council, 1929–31). The tubes were incubated in a water-bath at 51–53° C. and readings were taken after 18–24 hours. Preliminary readings at 4–5 hours were also taken when desired. The end-point recorded was the last definite trace of agglutination visible with a weak lens by artificial light against a dark background.

Table I

No	Name	Source and date of isolation	Origin	Biochemical characters	Haemolysis of goat erythrocytes	Antigens: Group H	Antigens: Subgroup O
1	Kasauli 11	Cent. Res. Inst. Kasauli, 1932	From case of cholera	Atypical (sacch. 0, C.R. 0)	+	+	Individual
2	Kasauli 73	,,	,,	Typical	+	+	VI
3	Kasauli 77	,,	,,	Atypical (C.R. 0)	+	+	Individual
4	Kasauli 1410/1	,,	,,	—	—	+	I
5	Kasauli 1416/1	,,	,,	—	—	+	I
6	Kasauli 1485	,,	,,	—	—	+	I
7	Kasauli 1486/2	,,	,,	—	—	+	I
8	Kasauli 3205/2	,,	,,	Typical	+	+	II
9	Kasauli 3214/4	,,	,,	,,	—	0 (n.m.)	I
10	Kasauli 3222/1	,,	,,	—	—	+	I
11	Manila 30/529	Cent. Res. Inst. Kasauli, 1930	,,	Typical	0	+	I
12	Manila Ha 10	East. Bur. Health Organ. 1924	,,	,,	+	+	Individual
13	Manila Ha 11	,,	,,	—	—	+	Rough
14	Manila Ha 19	,,	,,	—	—	+	Individual
15	Nyback	*Old stock, Oxford*	,,	*Typical*	*0*	+	I
16	Shillong 1077	Shillong Coll. Morrison, 1934	,,	,,	—	+	I
17	Shillong X	,,	,,	—	—	+	I
18	Shillong 610	,,	,,	Typical	0	+	I
19	Shillong 653	,,	,,	—	—	+	I
20	Kasauli 1617	Capt. Ahuja, 1934	,,	Typical	0	+	I
21	Kasauli 92/1	,,	,,	,,	0	+	I
22	Kasauli 63 A	,,	,,	,,	0	+	I
23	Kasauli 1800	—	,,	,,	0	+	I
24	Pasig O 27/9	East. Bur. 1930	,,	—	+	+	Individual
25	Bulacan 215,530	,,	,,		+	+	III
26	Inaba	Japan, old strain	,,	Typical	0	+	I
27	Wada	,,	,,	,,	0	+	I
28	Hikojima	,,	,,	,,	0	+	I
29	Shukeishin	,,	,,	,,	0	+	I
30	Ogawa	,,	,,	,,	0	+	I
31	Kimura	,,	,,	,,	0	+	I
32	Nanking 32/121	Dr Yang, 1932	,,	,,	+	+	II
33	Nanking 32/123	,,	,,	,,	+	+	III
34	Nanking 32/124	,,	,,	,,	0	+	II
35	Nanking 32/126	,,	,,	,,	+	+	II
36	Nanking 32/127	,,	,,	,,	+	0 (n.m.)	Individual
37	Water vibrio 32/101	Nanking, 1932	Water	,,	+	+	Individual
38	Water vibrio 32/102	,,	,,	,,	—	+	IV
39	Water vibrio 32/103	,,	,,	Non-fermenting	0	0	0°
40	Water vibrio 32/104	,,	,,	Typical	—	+	Individual
41	Water vibrio 32/105	,,	,,	Non-fermenting	0	0	0°
42	Water vibrio 32/106	,,	,,	Typical	0	+	IV
43	Water vibrio 32/107	,,	,,	,,	0	+	III
44	Water vibrio 32/108	,,	,,	,,	—	+	II
45	Water vibrio 32/109	,,	,,	,,	+	+	IV
46	Water vibrio 32/110	,,	,,	,,	+	+	IV
47	El Tor 34–D 9	Quar. Camp, El Tor, 1933	From healthy pilgrim	,,	+	+	I
48	El Tor 34–D 10	,,	,,	,,	+	+	I
49	El Tor 34–D 11	,,	,,	,,	+	+	I
50	El Tor 34–D 12	,,	,,	,,	+	+	I
51	El Tor 34–D 13	,,	,,	,,	+	+	I
52	El Tor 34–D 14	,,	,,	,,	+	+	I
53	El Tor 34–D 15	,,	,,	,,	+	+	I
54	El Tor 34–D 16	,,	,,	,,	+	+	I
55	El Tor 34–D 17	,,	,,	,,	+	+	I
56	El Tor 34–D 18	Quar. Camp, El Tor, 1934	,,	,,	+	+	I rough
57	El Tor 34–D 19	,,	,,	,,	+	+	V
58	El Tor 34–D 20	,,	,,	,,	+	+	I
59	El Tor 34–D 21	,,	,,	,,	+	+	I
60	El Tor 34–D 22	,,	,,	,,	+	+	I
61	El Tor 34–D 23	,,	,,	,,	+	+	V
62	El Tor 34–D 24	,,	,,	,,	+	+	I
63	El Tor 34–D 25	,,	,,	,,	+	+	I
64	El Tor 34–D 26	,,	,,	,,	+	+	I
65	El Tor 34–D 27	,,	,,	,,	+	+	I

https://doi.org/10.1017/S0022172400032265 Published online by Cambridge University Press

Table I (*continued*)

No.	Name	Source and date of isolation	Origin	Biochemical characters	Haemolysis of goat erythrocytes	Antigens: Group H	Antigens: Subgroup O
66	El Tor Doorenbos 6	N.C.T.C. 1929–31	Healthy person	Typical	+	+	I
67	El Tor Doorenbos 20	,,	Case of "malaria"	,,	+	+	V
68	El Tor Doorenbos 47	,,	Healthy pilgrim	,,	+	+	I
69	El Tor Doorenbos 67	,,	Bacillary dysentery	,,	+	+	V
70	El Tor Doorenbos 80	,,	Healthy pilgrim	,,	+	+	I
71	El Tor A.T.C.C. 582	,,	—	,,	+	+	I
72	El Tor 34–W/$_1$	Suez, 1934	Healthy pilgrim	,,	+	+	I
73	Tor 1	Prof. van Loghem, 1933	—	,,	+	+	I
74	Tor 3	,,	—	,,	+	+	I
75	Tor 4	,,	—	,,	+	+	I
76	Tor 8	Prof. van Loghem, 1934	—	,,	+	+	I
77	Tor 9	Prof. van Loghem, 1933	—	,,	+	+	I
78	Tor 10	Prof. van Loghem, 1934	—	,,	+	+	I
79	Tor 47–D	Prof. van Loghem, 1931	—	,,	+	+	I
80	El Tor Doorenbos 49	N.C.T.C. 1931	Bacillary dysentery	,,	+	+	VI
81	Tor A	Prof. van Loghem, 1905	,,	,,	+	+	I
82	Paracholera Martin	N.C.T.C.$_{30}$, 1916	Choleraic diarrhoea	,,	+	+	III
83	Paracholera A. Mackie	N.C.T.C.$_{558}$, 1918	,,	Atypical (malt. 0)	+	+	Not I–VI
84	Paracholera B. Mackie	N.C.T.C.$_{559}$, 1918	,,	Typical	+	+	Not I–VI
85	Paracholera K. Mackie	N.C.T.C.$_{560}$, 1917	,,	Atypical (malt. 0, C.R. 0)	+	+	I
86	Paracholera Forrest Mackie	N.C.T.C.$_{562}$, 1917	,,	Atypical (sacch. 0, dulc. +C.R. 0)	0	0	—
87	Cholera-like vibrio, Glen Liston, 1280	N.C.T.C.$_{686}$ —	—	Typical	+	0 (n.m.)	VI
88	Cholera-like vibrio, Glen Liston, 1406	N.C.T.C.$_{689}$ —	—	Atypical (C.R. 0)	+	+	Not I–VI
89	Cholera-like vibrio, Glen Liston, 129	N.C.T.C.$_{717}$ —	—	Typical	0	0 (n.m.)	I
90	Cholera-like vibrio, H 309	N.C.T.C.$_{3636}$, 1929	Healthy person	,,	0	+	VI
91	Cholera-like vibrio, P 57	N.C.T.C.$_{3646}$, 1929	—	,,	+	+	Not I–VI
92	Cholera-like vibrio, W 832/$_3$	N.C.T.C.$_{3648}$, 1929	India, water	,,	+	+	Not I–VI
93	Cholera-like vibrio, W 835/$_1$	N.C.T.C.$_{3649}$, 1929	,,	,,	+	+	Not I–VI
94	Cholera-like vibrio, P 615	N.C.T.C.$_{3650}$, 1929	—	,,	0	+	Not I–VI
95	Cholera-like vibrio, W 833/$_5$	N.C.T.C.$_{3651}$, 1929	India, water	,,	0	+	? IV
96	Cholera-like vibrio, Dennet	N.C.T.C.$_{2084}$, 1925	—	Non-fermenting	0	0	0°
97	El Tor 1908	N.C.T.C.$_{1548}$, 1908	—	,,	?	0	0°
98	Water-vibrio, Ramnad	Madras	Water supply	Typical	+	+	II
99	Water vibrio, Tanjore	,,	,,	,,	+	+	II
100	Water-vibrio, Trichy	,,	,,	,,	+	+	II
101	Water-vibrio, Coimbatore	,,	,,	,,	+	+	II

+ = positive. 0 = negative. − = not tested or unknown. n.m. = non-motile. I–VI. See Table II.
0° = neither biochemically nor antigenically related to the rest.
Not I–VI = proved not to belong to subgroups I, II, III, IV, V, VI, but not further tested.
C.R. = Cholera Red reaction. N.C.T.C. = National Collection of Type Cultures.

https://doi.org/10.1017/S0022172400032265 Published online by Cambridge University Press

Suspensions. (1) *H-O type.* Veal-broth cultures (*p*H 8) incubated for 24 hours and killed with 0·2 per cent. formalin and 0·2 per cent. chloroform were made in sufficient bulk (200–500 c.c.) to last a considerable time, and were diluted when necessary to the opacity of Brown's tube No. 1.

Comparison of these with living saline suspensions from agar showed no appreciable difference of average sensitiveness, while their sterility, stability and freedom from agglutinative variability make them much more convenient and reliable reagents. Such suspensions generally preserve their properties without appreciable change for many years.

It is noteworthy that formalin does not inhibit the O agglutination of these suspensions, as it does in the Salmonella group.

It is rarely possible to distinguish the two types of clumping, H and O, in these suspensions. In contrast with richly flagellated organisms the vibrios tend to show early O and late H agglutination. Workers who read their tests after a few hours of incubation, especially at relatively low temperatures, may completely miss delayed flagellar reactions.

(2) *O type.* Dense suspensions of 24 hours' agar cultures in saline solution were placed in boiling water for 2 hours, and then diluted to about twice the density of the H-O suspensions. A little chloroform was added as preservative. These suspensions, though not so durable as those in formolised broth, are generally fit for use for 6 months or more. They are often very slightly granular and tend to deposit more rapidly than the broth suspensions. Their agglutinability may be considerably depressed by the prolonged heat; thus a pure O serum showing a titre of 2000 against an H-O suspension, may show only 500 or less with an O suspension. Prolonged boiling was necessary since the suspensions were used also as antigens for injection, and experience has shown that a shorter period of boiling may fail to destroy the antigenic action of the H component. We have, however, recently found that a few minutes' exposure to 95–100° C. is enough to remove the H agglutinability of a suspension; and some experiments have indicated that formolised broth cultures thus treated are excellent reagents for detecting and measuring O agglutinins.

Growth of the vibrios on phenol agar was not found of any help in the production of O suspensions.

Alcohol treatment of the vibrios was tried and abandoned, since it renders the vibrios almost completely inagglutinable. Moreover, as some recent joint experiments with Mr Bruce White have shown, it is difficult or perhaps impossible to destroy the H component completely with either cold or boiling alcohol.

Sera. (1) H-O sera were made by injecting rabbits intravenously with the formolised unheated suspensions.

Two doses of 0·5 and 1·0 c.c. at a week's interval are generally enough to give an *H* titre of 2000, and an *O* titre of a few hundreds. With four doses of concentrated suspension, representing ten or more times the above quantities, sera of 100,000 H titre and 500–1000 O titre can be made. It is curious

that the H can be raised to such heights while the O very seldom goes beyond 2000–5000 whatever the antigen or dosage employed.

(2) Pure O sera were made with saline suspensions from agar boiled for 2 hours. Two doses of 0·5 and 1·0 c.c. at a week's interval generally gave sera of 1000–2000 O titre, and no method of dosage was discovered that would consistently improve on this, though three doses of a fivefold denser suspension appeared sometimes to be a better stimulus. Recent trials of a system of nine doses of fairly dense suspensions at about 3 days' intervals gave no better results than the quicker method.

An O serum was considered pure if it showed no agglutination of vibrios belonging to heterologous O subgroups (Table II) in 24 hours at dilutions of 1 in 25 or over.

Absorption tests. These were done in the usual way with either living suspensions from agar or boiled suspensions. In all fundamental experiments a dose or doses sufficient to remove all demonstrable homologous agglutinins was employed. A control mixture of serum and saline solution was always made at the same dilution as the absorption mixture and subjected to the same conditions and tests.

Biochemical reactions. In Table I the vibrios are described as typical, atypical or non-fermenting. By "typical" we mean producing acid without gas in glucose, mannite, maltose, saccharose; giving the cholera red reaction, and not fermenting dulcite. "Atypical" implies divergence from this list in one or more characters, but a general similarity. The individual points of difference are recorded in the table. "Non-fermenting" means a failure to acidify any of the carbohydrates mentioned. In all the cases observed it was accompanied by other differences, such as a failure to produce indole (cholera red negative) or to liquify gelatin.

Both typical and atypical vibrios generally show a slow fermentation of lactose, but this is not sufficiently regular or powerful to be taken as a standard character. All the typical and atypical vibrios that we have tested liquify gelatin, but not the "non-fermenters".

The general experience of bacteriologists is strongly against the possibility of an accurate biochemical classification of the group (Nobechi, 1923). Heiberg's (1934) claims in that direction have been denied by Doorenbos (1934), and have found little support in our limited study of the question.

CHOLERA GROUP

We apply the term "cholera group" to vibrios possessing the main characters of *V. cholerae*, *i.e.* to the "typical" and "atypical" classes, to the exclusion of the non-fermenting vibrios. As we shall see later, the cholera group vibrios thus defined possess a common heat-labile (H) antigen, whereas the non-fermenting vibrios do not.

Antigenic stability. We have proceeded on the assumption that the vibrios

are antigenically stable for long periods under cultivation, the exceptions to this rule being:

(1) That a change from inagglutinability on isolation to agglutinability immediately after, seems established as a not uncommon phenomenon (Pasricha, 1934). This is well worthy of further investigation, but it does not fall within the scope of our work.

(2) That the rough variation, involving the loss of the smooth O antigens, need only be taken into account when sufficiently pronounced to be detectable in ordinary cultures, *e.g.* by instability in broth or peptone water.

A most careful watch has been kept for the possible effects of partial roughness, and numerous tests with Millon's reagent and acriflavine have been done.

The majority, of culture-stable vibrios show imperfect smoothness to these reagents, but, so far as we have seen, no qualitative antigenic difference from pure smooth variants obtained from them or from other strains of the same type.

No thorough investigation of rough antigens has been attempted, since Mr Bruce White has had this in hand. We have merely confirmed the fact that rough forms, if motile, have the common H antigen, and are deficient in the specific O component (see Tables IV and V).

We have made no thorough investigation of the effects of the very numerous bacteriophages that act upon *V. cholerae*; but such work as we have done, and a careful examination of the literature, have given us no reason to believe that transmutation of species occurs under bacteriophage action—and in this connection the serological subgroups that we are about to describe appear to us to deserve the term species.

The theory advocated by Doorenbos (1934) that *V. cholerae* (typus epidemicus) is transformed by the bacteriophage at the end of epidemic outbreaks into a disequilibrated form (typus endemicus), which has temporarily lost its epidemic potentialities and gained the power of haemolysis, appears to us founded on insufficient evidence. One of its main corollaries, that El Tor and similar vibrios must all contain bacteriophage, has been experimentally disproved by Flu (1934). A more accurate specification of the cholera vibrio and similar organisms, and a clearer understanding of the factors involved in their antigenic identification are necessary before we can rightly interpret the apparent changes of character resulting from bacteriophage or other influences.

Specific O antigens

Table II shows the results of cross-agglutination reactions of a selection of our vibrios, in which O sera and unheated suspensions were employed. The figures given are percentages of the titre of the serum to its homologous organism. Thus, the figure 50 below a serum with a titre of 1 in 1000 means that the vibrio in question was agglutinated up to 1 in 500.

The group clearly contains a great diversity of O antigens, six of which were found in more than one strain, and eight others in single strains. The former we refer to as subgroups I–VI; the latter as "Individual".

It is, however, to be understood that the number of subgroups here given has no final significance, since each separate antigen represents a potential subgroup.

Only a selection of the vibrios tested is given in this table, the results of the grouping tests of the remainder being shown in Table I.

Subgroup I. All the standard stock cholera vibrios that we have received from central laboratories in different countries fall into this group, and also the majority of races isolated from typical cases of epidemic cholera. Ten such strains have been tested for haemolysis, and found negative. The subgroup also includes the greater number of the haemolytic vibrios called "El Tor".

Numerous cross-absorption tests have proved the complete difference of this antigen from those of the other subgroups and "Individual" races in the table.

Subgroup II. This contains four vibrios isolated by Dr Yang from cholera cases at Nanking. Their specific O antigen is shared by a race from Kasauli, No. 3205, originating from a case of cholera, by a vibrio from Nanking water, and four strains from the Madras public water-supply (see Table I, Nos. 98–101). The Kasauli strain had been found agglutinable by certain sera, but "inagglutinable" by others. It appears that at Nanking in 1932 some of these vibrios were for a time believed to be responsible for an outbreak of cholera; but the evidence on which this view was based has not been published. Eight out of nine of these strains have proved to be haemolytic.

Subgroup III. Consists of one of Dr Yang's vibrios from cholera cases, one from Nanking water, and a race from Balacan sent to Prof. Madsen from Singapore as a "non-agglutinable" vibrio "from a cholera case" (further details not obtainable). *V. paracholerae* (Martin), No. 82 in Table I, also falls into this subgroup. Three of the four strains are haemolytic.

Subgroup IV. Contains only vibrios isolated from Nanking water during the cholera epidemic of 1932. Their specific O component distinguishes them clearly from the races in the two preceding subgroups. Some are haemolytic, others not. An Indian vibrio, No. 95 in Table I, has some antigenic relationship to this subgroup, but we have not investigated it thoroughly.

Subgroup V. Consists of a number of haemolytic "El Tor" vibrios from Dr Doorenbos, which do not fall into subgroup I. Here, again, there is no clinical connection with cholera.

Subgroup VI embodies the curious fact of an antigenic identity or similarity between a haemolytic "El Tor" vibrio and a supposedly "agglutinable" cholera vibrio. The latter, however, has proved also to be haemolytic, and is only agglutinable by the non-specific (H) agglutinins in cholera (subgroup I) sera.

Table II. *A selection of vibrios classified by "O" agglutination.*

No. in Table I	H.	ensions ios	O sera (titres given as percentages of the homologous titres) Nyback (titre 900)	Kasauli 3205 (titre 900)	Nanking $32/_{123}$ (titre 3000)	Nanking water $32/_{106}$ (titre 3000)	El Tor 34–D 23 (titre 2000)	Kasauli 73 (titre 500)	Kasauli 11 (titre 2000)	Manila Ha 10 (titre 4000)	Nanking water $32/_{101}$ (titre 2000)	Nanking water $32/_{104}$ (titre 1000)	Char istic ant (s gr
11	Man	^{529}	100	0	0	–	0	–	0	0	0	0	
10	Kas	$22/_1$	100	0	0	0	0	0	0	0	0	0	
6	Kas	85	60	0	0	–	0	–	0	0	0	0	
15	Nyb		100	0	0	0	0	0	0	0	0	0	
16	Shill	)77	100	0	0	0	0	0	0	–	0	0	
51	El T	D 13	50	0	0	0	0	0	0	0	0	0	
52	El T	D 14	50	0	0	0	–	0	0	0	0	0	
32	Nan	$2/_{121}$	0	50	0	0	0	0	0	12	0	0	
34	Nan	$2/_{124}$	0	100	0	0	0	0	0	(5)	0	0	
44	Nan	ater $32/_{108}$	0	40	0	0	0	0	0	8	10	0	
8	Kas	$05/_2$	0	100	0	0	0	0	0	0	0	0	
25	Bul	15,530	0	0	60	0	0	0	0	0	0	0	I
33	Nan	$2/_{123}$	0	0	100	0	0	(16)	0	0	0	0	
43	Nan	ater $32/_{107}$	0	0	100	0	0	0	0	(5)	0	0	
38	Nan	ater $32/_{102}$	0	0	0	50	0	0	0	0	0	0	]
42	Nan	ater $32/_{106}$	0	0	0	100	0	0	0	0	0	0	
46	Nan	ater $32/_{110}$	0	0	0	150	0	0	0	0	0	0	
67	El T	orenbos 20	0	0	0	0	95	0	0	0	0	0	
69	El T	orenbos 67	0	0	0	0	50	0	0	0	0	0	
61	El T	-D 23	0	0	0	0	100	0	0	0	0	0	
2	Kas		0	0	0	0	0	100	0	0	0	0	
80	El T	orenbos 49	0	0	0	0	0	70	0	0	0	0	
1	Kas		0	0	0	0	0	0	100	5	0	0	Indi
3	Kas		0	0	0	0	0	0	0	0	0	0	anti
12	Mar	,10	2	0	0	0	0	0	0	100	0	0	
14	Mar	,19	0	0	0	0	0	0	0	0	0	0	
24	Pas	$/_9$	0	0	0	0	0	0	0	5	0	0	
36	Nar	$2/_{127}$	0	0	0	0	0	0	0	0	0	0	
37	Nar	ater $32/_{101}$	0	0	0	0	0	0	0	0	100	0	
40	Nar	ater $32/_{104}$	0	0	0	0	0	0	0	0	0	100	
39	Nar	ater $32/_{103}$	0	0	0	0	0	0	0	0	0	0	Not
41	Nar	ater $32/_{105}$	0	0	0	0	0	0	0	0	0	0	gro

0 = nc ion at lowest dilution tested, usually 1 in 25. In all cases 0 = less than 10 per cent.; generally less than 5 per cent. of titr

(5) = a f agglutination at 5 per cent. of titre.

– = nc d.

The "*Individual*" residue consists of vibrios each with a different specific O component. With the exception of the two water-vibrios all the strains are reported to have come from acute cases with typical symptoms of cholera. The great majority, however, have proved to be haemolytic.

Vibrios not belonging to the cholera group. The two water vibrios from Nanking shown in Table II, viz. Nos. $32/_{103}$ and $32/_{105}$, and the strains labelled "vibrio Dennet" and El Tor 1908 (see Table I) differ from all the others in failing to ferment any of the carbohydrates, in producing no indole and in not liquifying gelatin. Their O antigens do not correspond with any of the subgroups or "Individual" races examined.

HEAT-LABILE (H) ANTIGEN

(1) All our vibrios that conform to the cultural and biochemical standard of *V. cholerae* have a common H antigen. Thus, Table III shows that H-O sera made with formolised, unheated broth suspensions, agglutinate similar suspensions of homologous and heterologous types. Further qualitative information on this point is recorded in Table I. Table III, which shows general cross-agglutination should be compared with Table II which shows sharp specificity.

In some sera the H agglutinin is strong, giving a far higher titre than the O, in others it is relatively weak. By giving large doses of concentrated H-O suspensions we have obtained sera with H titres of 50,000–200,000 while the O did not go up beyond 1000–2000.

(2) Absorption of an H-O serum with homologous O suspension removes all agglutinins for O suspensions of all species but leaves the common H agglutinin intact. A similar absorption is effected by using unheated suspensions of a homologous non-motile vibrio Kasauli $3214/_4$, as is shown in Table IV, thereby confirming the dependence of the H antigen on the presence of flagella.

(3) On the other hand, the absorption of an H-O serum with an H-O suspension of a different O subgroup removes the H agglutinin for all species, but leaves the O agglutinin intact. Table V shows an experiment in illustration of this effect. Here the absorption of a subgroup I H-O serum with unheated suspension of a motile subgroup III vibrio removes all the group-agglutinin (H), while not affecting the specific O titre. The latter point is also proved by the unaltered titre for the two non-motile vibrios of the homologous subgroup. Several other experiments of the same type with different sera gave similar results.

(4) It is possible that, as Gohar (1932) suggested, the H component is not completely identical in all species. In fact the peculiar behaviour of Kasauli 77 in Tables III and IV may be an indication of this. A serum made with an unheated suspension of that vibrio gave only an incomplete reaction to one-quarter or one-tenth of its titre with various heterologous vibrios, and various H-O sera react similarly with Kasauli 77. The strain is peculiar in several ways, giving no cholera red reaction and producing an unusually intense pig-

Table III. *Common H antigen.*

No. in Table I	Vibrio	O subgroup	H-O sera: Subgroup I, Inaba H titre 20,000 O titre 2,000	H-O sera: Subgroup V, El Tor H titre 20,000 O titre 2,000	H-O sera: Subgroup VI, El Tor Doorenbos 49 H titre 50,000 O titre 2,500
		Formolised broth (H-O) suspensions.			
26	Inaba	I	20,000	20,000	50,000
10	Kasauli $3222/_{1}$		20,000	20,000	50,000
6	Kasauli 1485		10,000	20,000	50,000
15	Nyback		20,000	20,000	40,000
16	Shillong 1077		20,000	20,000	50,000
51	El Tor 34–D13		10,000	10,000 sl. tr.	20,000 sl. tr.
52	El Tor 34–D14		10,000	10,000	20,000
32	Nanking $32/_{121}$	II	10,000	10,000	20,000
34	Nanking $32/_{124}$		20,000	20,000	50,000 sl. tr.
44	Water vibrio $32/_{108}$		10,000	20,000 sl. tr.	20,000
8	Kasauli $3205/_{2}$		20,000	20,000 +	20,000
33	Nanking $32/_{123}$	III	20,000	20,000	40,000
42	Water vibrio $32/_{106}$	IV	20,000 sl. tr.	20,000	20,000
45	Water vibrio $32/_{109}$		20,000	20,000 +	50,000
46	Water vibrio $32/_{110}$		10,000	20,000	20,000
67	El Tor Doorenbos 20	V	20,000	20,000	20,000
69	El Tor Doorenbos 67		10,000 sl. tr.	10,000	20,000
61	El Tor 34–D23		10,000	20,000	20,000
2	Kasauli 73	VI	10,000	20,000 sl. tr.	40,000
80	El Tor Doorenbos 49		20,000	20,000	50,000
1	Kasauli 11	Individual	4,000	4,000	10,000
3	Kasauli 77		2,000	4,000	10,000
13	Manila Ha 11		20,000 +	20,000 +	50,000 +
14	Manila Ha 19		20,000 sl. tr.	20,000	50,000
24	Pasig O $27/_{9}$		10,000 sl. tr.	10,000	40,000
40	Water vibrio $32/_{104}$		20,000 sl. tr.	20,000 sl. tr.	50,000 sl. tr.
36	Nanking $32/_{127}$ (n.m.)		0	0	0
		Boiled (O) suspensions.			
26	Inaba	I	2,000	0	0
15	Nyback		2,000	0	0
44	Water vibrio $32/_{108}$	II	0	0	0
8	Kasauli $3205/_{2}$		0	0	0
33	Nanking $32/_{123}$	III	0	0	0
42	Nanking $32/_{106}$	IV	25	0	0
46	Nanking $32/_{110}$		0	0	25
67	El Tor Doorenbos 20	V	0	2,000	0
61	El Tor 34–D23		0	2,000	0
2	Kasauli 73	VI	0	0	3,000
80	El Tor Doorenbos 49		0	0	2,500
24	Pasig O $27/_{9}$	Individual	0	0	0
14	Manila Ha 19		0	0	0

0 = less than 25, the lower limit of observation.
sl. tr. = slight trace of agglutination.
n.m. = non-motile.

https://doi.org/10.1017/S0022172400032265 Published online by Cambridge University Press

Table IV. *Absorption of an H-O serum with a non-motile (O) strain of the same subgroup.*

No.	Vibrio	O subgroup	Subgroup I (polyvalent) H-O serum: Untreated	Subgroup I (polyvalent) H-O serum: Absorbed with living Kasauli $3214/_4$
	Formolised broth (H-O) suspensions.			
11	Manila $30/_{529}$	I	6000	5500
15	Nyback		4000	3500
16	Shillong 1077 (rough motile)		7000	7000
16	Shillong 1077 (smooth non-motile)*		400	<50‡
9	Kasauli $3214/_4$ (non-motile)†		300	<50
25	Bulacan 215,530	III	3500	3000
3	Kasauli 77	Individual	200§	200
1	Kasauli 11		1000	900
	Boiled (O) suspensions.			
11	Manila $30/_{529}$	I	250	<50
15	Nyback		150	<50
16	Shillong 1077 S.		150	<50
9	Kasauli $3214/_4$		200	<50

* Temporarily non-motile.

† Permanently non-motile.

‡ 1 in 50 the lowest dilution observable after absorption.

§ Kasauli 77 appears to have a peculiar H antigen; see text and Table III.

Table V. *Removal of H agglutinin from an H-O serum by absorption with an H-O suspension of a different O subgroup.*

No.	Vibrio	O subgroup	Subgroup I (polyvalent) H-O serum: Untreated	Subgroup I (polyvalent) H-O serum: Absorbed with living Bulacan (subgroup III)
	Formolised broth (H-O) suspensions.			
11	Manila $30/_{529}$	I	6000	300
15	Nyback		4000	320
16	Shillong 1077 (rough motile)		6000	60*
16	Shillong 1077 (smooth non-motile)		400	400
9	Kasauli $3214/_4$ (non-motile)		300	300
25	Bulacan 215,530	III	3000	<50†
3	Kasauli 77	Individual	200‡	<50
1	Kasauli 11		1000	<50
	Boiled (O) suspensions.			
11	Manila $30/_{529}$	I	250	250
15	Nyback		250	250
16	Shillong 1077 S.		150	125
9	Kasauli $3214/_4$		200	150

* Deficiency of smooth O antigen in rough variant.

† 1 in 50 the low limit of observation after absorption.

‡ ? Abnormal H antigen; see text and Table IV.

mentation. Boiled suspensions do not agglutinate with the homologous H-O serum, and their antigenic power is extraordinarily feeble. A test with Millon's reagent shows it to be largely rough, though it is stable enough in liquid media and 0·9 per cent. NaCl solution. An investigation of possible H differences in the group in general is being made by one of us (K.V.V.) by means of special flagellar suspensions and sera.

Table VI. *To show common or non-specific O agglutination.*
(*Titres expressed as percentages of homologous H and O titres.*)

No. in Table I	Vibrio	O subgroup	H-O serum Subgroup I (polyvalent)		O serum Subgroup I (Nyback)	
			Versus H-O suspensions (H titre 2000)	Versus O suspensions (O titre 400)	Versus H-O suspensions (O titre 1000)	Versus O suspensions (O titre 1000)
11	Manila $30/_{529}$	I	100	300	31	110
10	Kasauli $3222/_{1}$		100	75	100	180
6	Kasauli 1485		120	100	90	180
9	Kasauli $3214/_{4}$ (n.m.)		30	120	95	150
15	Nyback		100	100	100	100
16	Shillong 1077 (smooth n.m.)		17	200	100	100
16	Shillong 1077 (rough m.)		100	<25	15	40
32	Nanking $32/_{121}$	II	90	<10	<2	15
34	Nanking $32/_{124}$		80	<10	<2	15
44	Nanking water $32/_{108}$		90	<10	<2	20
8	Kasauli $3205/_{2}$		150	< 6	<2	20
25	Bulacan 215,530	III	200	< 6	<2	20
33	Nanking $32/_{123}$		70	<10	<2	20
43	Nanking water $32/_{107}$ (n.m.)		<2	<10	<2	25
38	Nanking water $32/_{102}$	IV	110	<10	<2	105
42	Nanking water $32/_{106}$		120	<10	<2	35
45	Nanking water $32/_{109}$		100	<10	<2	33
46	Nanking water $32/_{110}$		100	<10	<2	33
2	Kasauli 73	VI	90	< 6	<2	20
1	Kasauli 11	Individual	40	< 6	<2	20
3	Kasauli 77		7	< 6	<2	6
12	Manila Ha 10		70	< 6	<2	25
13	Manila Ha 11 (rough)		200	< 6	5	50
14	Manila Ha 19		40	< 6	2	6
24	Pasig O $27/_{9}$		100	< 6	<2	25
36	Nanking $32/_{127}$ (n.m.)		<2	<10	<2	20
37	Nanking water $32/_{101}$		100	<10	<2	20
40	Nanking water $32/_{104}$		110	<10	<2	55
39	Nanking water $32/_{103}$	Not cholera group	<2	<10	<2	<3
41	Nanking water $32/_{105}$		<2	<10	<2	<3

m. = motile. n.m. = non-motile.

(5) Those of our vibrios that differ widely from the cholera group in their biochemical characters do not show the common H antigen of the group (viz. Table I, serial Nos. 39, 41, 96, 97).

A non-specific O antigen. This is demonstrated by the action of O sera on boiled suspensions.

In Table VI the last column shows this effect; while the preceding column shows its absence when unheated suspensions are used. The column of H-O

serum versus boiled suspensions also shows no cross-agglutination of the subgroups; while the first column gives the non-specific effects of H-O sera on H-O suspensions.

The non-specific O reaction could be explained in one of two ways: (1) the common flagellar (H) antigen is changed by heat into a new common antigen, or (2) boiling destroys the H and brings out a common component which is inert in the unheated vibrio.

The former hypothesis, however, is ruled out by the behaviour of the two non-motile cultures of subgroup I shown in Table VII. Whereas they possess no common H component, when boiled and tested with a heterologous O serum they show as much common O as ordinary motile vibrios of the group (lowest line of table). Moreover, a serum made with unheated suspension of Kasauli 3214 showed plenty of common O agglutinin, though, of course, no H component.

Table VII. *Non-specific O antigen in motile and non-motile vibrios.*

			Non-motile vibrios		Motile vibrios			
Serum	Subgroup	Type of suspension	I, Kasauli $3214/_4$	I, Shillong 1077 S.	I, Nyback	II, Kasauli 3205	III, Bulacan	Individual, Kasauli 11
Nyback H-O	I	Unheated	100	200	800	900	1200	300
		Boiled	150	100	100	(25)	<25	<25
Nyback O	I	Unheated	950	1000	1000	<25	<25	<25
		Boiled	900	700	650	140	140	150
Kasauli 11 H-O	Individual	Unheated	<40	<40	250	500	200	2500
		Boiled	<40	(25)	<25	<25	<25	2500
Kasauli 11 O	Individual	Unheated	<25	(25)	<25	<25	<25	2000
		Boiled	400	300	150	350	300	2000

(25) etc. = trace of agglutination at 1 in 25.

<40, <25 etc. = no agglutination at 1 in 40, etc., the lowest dilution observed.

Absorption of O sera with heterologous boiled suspensions, in so far as the low common titres allowed any conclusions to be drawn, effected a strong but not always complete reduction of the common O component. *E.g.* a Kasauli 11 O serum ("Individual" subgroup) absorbed with a suspension of Manila $30/_{529}$ (subgroup I), lost all its measurable common O titre for vibrios of various subgroups, viz. reductions from 1/200 or 1/500 to less than 1/25. Several other similar experiments gave similar results.

To test the hypothesis that the common O component is a rough O antigen, a subgroup I (Nyback) O serum was absorbed with boiled suspensions of a rough variant of the same subgroup (Shillong 1077), and of an incompletely rough heterologous vibrio, Manila Ha 11. In both cases reductions varying from 60 to 100 per cent. of the titre for various vibrios were effected, but the results were not clear enough to allow safe conclusions to be drawn.

Some O sera show very little common O reaction, while others react to a large fraction of their specific titre.

Different batches of serum made with the same vibrio may vary considerably in this respect.

With regard to a possible extension of non-specific O agglutination to vibrios or other organisms outside the cholera group, we have little evidence to record. Two races of non-fermenting vibrios, Nos. 39 and 41 of Table I gave no reaction with any of our O sera, but No. 97 showed agglutination at a 1 in 25 dilution of a heterologous serum of subgroup V (El Tor D23). Bruce White's (1934 *b*) demonstration of a widely non-specific "Q" antigen in *V. cholerae* might account for such phenomena.

O VARIATION WITHIN SUBGROUP I. THE JAPANESE "TYPES"

Cultures of the three "types", original, variant and middle, having been obtained from the State Institute for Infectious Diseases in Japan, it was first ascertained that all three fall into our subgroup I, viz. that they are

Table VIII. *Japanese types of* Vibrio cholerae.
Cross-agglutination reactions.

	Vibrios			Sera: Type original, Inaba		Type middle, Hikojima		Type variant, Ogawa	
No.	Race	Type	Suspension	H-O	O	H-O	O	H-O	O
26	Inaba	Original	H-O	50,000	—	100,000	—	50,000	—
			O	—	2000	—	500	—	1500
28	Hikojima	Middle	H-O	50,000	—	90,000	—	60,000	—
			O	—	900	—	300	—	1750
30	Ogawa	Variant	H-O	100,000	—	150,000	—	100,000	—
			O	—	800	—	400	—	4000

agglutinated by O sera of that subgroup (*e.g.* Nyback and Shillong 1077) to full titre or to a large fraction of the titre, and that O sera of the Japanese races similarly agglutinate H-O suspensions of various subgroup I strains. They also possess the common H, as is shown by their partial and slow reaction with H-O sera of heterologous organisms such as Kasauli 11. With O sera of these latter they give no agglutination. They also conform to type in showing a common O effect to a fraction of their titre when O sera (Inaba and Ogawa) are tested on a heterologous boiled suspension (Kasauli 11).

In addition to the main O antigen, which places these vibrios in subgroup I, the original and variant types each has a different, subsidiary O component. Table VIII shows that even in simple cross-agglutination tests the difference between the two is clear enough. Thus the ratio of the titres Inaba: Ogawa with Inaba O serum is 2·2, with Ogawa O serum, 0·4. The figures are reliable since the tests were done at the same time with the same suspensions and sera. Further and final confirmation of the reality of the two components is given by absorption tests (Table X). These were done with O sera and O suspensions, and numerous vibrios were tested with the absorbed and unabsorbed sera, but for the sake of simplicity only two races of each "type"

are given. The figures show that from each type serum the heterologous type vibrio removes all the subgroup agglutinin for itself but leaves a large residue of agglutinins for the other types.

The table does not include absorption tests of middle type serum, since our results have been conflicting and the character of this "type" is at present uncertain. In the hands of one of us (A. D. G.) it agrees with the Japanese specification, *i.e.* the middle type races exhibit both the type-specific antigens of T.O. and T.V. This is indicated, though not proved, in Table IX by the residual agglutination of the middle type strains by both absorbed sera. Experiments by the other author (K. V. V.), however, have thrown doubt on the constancy of these findings, and have tended to identify the two middle type races (Hikojima and Shu Kei Shin) with the original type. Further work on this point is desirable.

Table IX. *Types of subgroup I. Demonstration of two antigens.*

Suspensions (boiled)		O serum Inaba (T.O.)		O serum Ogawa (T.V.)	
			Absorbed		Absorbed
Vibrio	Type	Unabsorbed	Ogawa	Unabsorbed	Inaba
Inaba	Original (T.O.)	2000	700	1100	0
Kasauli $3222/_2$		2000	1000	1300	0
Hikojima	Middle (T.M.)	1000	450	800	170
Shu Kei Shin		2500	1500	1500	100
Ogawa	Variant (T.V.)	900	0	2500	2000
Shillong 1077		1500	0	4000	2000

0 = <1 in 50, the low limit of observation.

Although it has hitherto not been observed elsewhere, the type difference is in no way peculiar to Japanese cholera vibrios. In Table IX a very recent strain from Kasauli and another from Shillong are included, to show that they are just as unlike each other as the Japanese type strains. Of the other strains that we have tested the following correspond to the original type: Kasauli 1617 and 1800, Nyback and six El Tor vibrios (Table X); and to the variant type: Kasauli 92/1 and 63A; Shillong 1077, 610 and 653; and twenty-four El Tor vibrios.

Table X. *El Tor vibrios. Classified according to their O antigens.*

Biochemically typical and possessing group H antigen				Biochemically atypical and no group H antigen. Not cholera group
Subgroup I				
Original type	Variant type	Subgroup V	Subgroup VI	
34–D13, D18, D26, Doorenbos 80, A.T.C.C. 582, Tor A	34–D9, D10, D11, D12, D14, D15, D16, D17, D20, D21, D22, D24, D25, D27, $W/_1$, Doorenbos 6, Doorenbos 47, Tor 1, 3, 4, 8, 9, 10 and 47 D	34–D19, D23, Doorenbos 20, Doorenbos 67	Doorenbos 49	1908 (N.C.T.C. 1548)

https://doi.org/10.1017/S0022172400032265 Published online by Cambridge University Press

Stability of types. The Japanese view is that these "types" are not necessarily stable. Some experiments by Fukushima and Kabeshima quoted by Nobechi (1923) on the transmutation of types met with some success. Growth in immune serum and in the gall bladder of rabbits effected a change of several variant type strains into the middle type. From the account given, this change was concomitant with roughening; but it must not be supposed from this that *the middle type is the rough form, since the middle type strains isolated in* Japan in 1922 are said to have been perfectly smooth, and the ones we have examined are quite normal.

Haemolysis. Method. One c.c. of a 3 days' broth culture of the vibrio was added to 1 c.c. of a 5 per cent. suspension of washed goat erythrocytes (Greig's method); the mixture was incubated for 2 hours at 37° C. and kept overnight in the cold before reading.

The rate of haemolysis is very variable, in some cases being complete within a few minutes at room temperature, in others only partial at the end of the period of test. A number of tests were repeated, and gave consistent results. In all negatives, haemolysis was absent even after several further days' standing at room temperature (an indication of the great stability of goat erythrocytes).

The reports of Flu (1934), Heiberg (1934) and others on the unreliability of the test, made us at the outset sceptical of its value, but without having made any special investigations on the matter, our final impression was that its results were both consistent and illuminating. Zimmerman (1934) finds that the haemolytic power of a given vibrio is constant, not variable under bacteriophage or other influences, as Doorenbos (1934) believes.

Table I, column 5 shows that:

(1) Ten vibrios sent to us as *V. cholerae* and possessing the O antigen of subgroup I (Table II) were tested, and none produced any haemolysis.

(2) Haemolysis was produced by (*a*) all vibrios labelled "El Tor" whether from healthy pilgrims or cases of intestinal disease; and (*b*) most of the vibrios belonging to the cholera group but possessing O antigens other than that of subgroup I, whether purporting to be cholera, paracholera or cholera-like vibrios.

There is little doubt that the absence of haemolytic power for goat cells and the possession of a characteristic O antigen (subgroup I) are the chief distinctive characters of the vibrios most undoubtedly causative in epidemic cholera.

The vibrios called "El Tor" (Tables I, II, X). (1) The majority are biochemically typical and have the group H antigen. One race (No. 97 El Tor 1908) showed neither biochemical nor antigenic relationship to the cholera group.

(2) According to their O reactions they are divisible as shown in Table X. The majority of strains fall into subgroup I, *i.e.* they are antigenically identical with the "true" cholera vibrios; and possess the same range of subsidiary O antigens. Four-fifths of these correspond with the Japanese "variant" type; one-fifth to the "original" type.

(3) Four races have an O antigen different from any in our other divisions of the cholera group (=group V in Table II), and one (El Tor 49) corresponds with Kasauli 73, an organism sent to us as an agglutinable *V. cholerae*, but found by us to be antigenically atypical and haemolytic.

Doorenbos (1934) has attached the name "vibrio cholerae, typus endemicus" to the El Tor vibrios, in the belief that they can mutate into "vibrio cholerae typus epidemicus". We feel, however, that even in the case of the subgroup I haemolytic vibrios the evidence is unconvincing, and that in the light of the serological heterogeneity of the vibrios called "El Tor" demonstrated by Shousha and ourselves, all such terminology is premature. We prefer, with Shousha, to restrict the term El Tor to the subgroup I vibrios, and to be content for the present with the principle that since undoubted epidemic strains appear to be consistently non-haemolytic, haemolytic strains may be considered as non-epidemic.

Paracholera vibrios (Table I). This title is applied to such members of the group as are believed on clinical and bacteriological grounds to have been the cause of limited outbreaks of choleraic diarrhoea. As Mackie and Storer (1918) found, the paracholera A and B vibrios are antigenically distinct from the subgroup I (cholera) vibrios. Our finding of a common H antigen in paracholera B is in disagreement with both Mackie (1922) and Douglas (1921) who found that it gave no cross-reaction with cholera serum. As we shall see in the next section, this is almost certainly a matter of technique. The race called K (Mackie) has all the qualities of a subgroup I "El Tor" vibrio. "Forrest", though motile, appears to have no group H component; it also gives atypical biochemical reactions, both of which facts conflict with Mackie's (1922) report. This arouses a suspicion that an error of labelling has occurred. The "Martin" strain proves to belong to subgroup III, which also contains two supposed cholera vibrios and one isolated from water (all haemolytic).

It seems likely that a large number of different vibrios can give rise to cholera-like diarrhoea, and that both the disease and the microbe are not infrequently mistaken for "true cholera". The term "paracholera" is, we feel, a useful one; but the range of vibrios to which the term is applicable needs further defining.

WORKING SCHEME OF CHOLERA GROUP

Cholera group of vibrios

(Biochemically similar. Common H antigen)

O subgroup I

Non-haemolytic (goat cells) **Cholera vibrios** (Types—original, variant and middle)

Haemolytic (goat cells) **El Tor vibrios** (Types—original and variant (? middle))

O subgroups II, III, IV, V, VI, and Individual races. (Mostly haemolytic.) **Paracholera, cholera-like and some El Tor vibrios.** (Types within subgroups undetermined.)

"*Agglutinability.*" *The importance of technique.* We are now in a position to explain the success of Greig, Mackie and others in classifying vibrios without knowledge of their antigenic structure, and the later differences between Douglas (1921) and Mackie (1922) concerning the "paracholera" vibrios.

The agglutination method used by Greig, Mackie, etc., involving a low temperature (37° C.) and a relatively short period of incubation (2 hours), reveals in general only O agglutination, and so enables a distinction to be made between the various O subgroups. Douglas, however, using formolised broth suspensions, and a temperature of 50–55° C. observed a common reaction between true cholera and certain "paracholera" strains. This was dismissed, after verification, by Mackie as "coagglutination"—a vague word much in vogue at that time. The recent adoption by many workers of an "optimum" technique (as used by Douglas) which reveals all the antigenic components involved in the reaction has thus tended to introduce complication in a situation erroneously believed to be simple.

The resulting confusion is illustrated by the fact that a number of our antigenically diverse vibrios (Kasauli 11, 73, 77, 3205; Bulacan; Pasig; Manila Ha 10, 11 and 19; Nanking $32/_{121}$, $32/_{124}$ and $32/_{126}$) were all classed on isolation as agglutinable cholera vibrios on the strength of tests with sera containing common H agglutinin, prepared in specialist laboratories. The fact that we have found these vibrios to be haemolytic as well as antigenically (and sometimes biochemically) atypical casts much doubt on their aetiological rôle in cholera. Thus, our subgroup III contains vibrios isolated from (1) cases of cholera, (2) a case of paracholera (Martin, Table I) and (3) water. Seeing that all these are easily distinguishable from "vibrio cholerae" by O agglutination tests, and are haemolytic, it is, to say the least of it, difficult to regard the culture from the cholera case (Bulacan) as a true cholera vibrio, in spite of its having been sponsored as "agglutinable". Similar considerations arise in the case of Kasauli 73, also supposed to be an agglutinable cholera vibrio, but which shows a close antigenic and haemolytic correspondence with El Tor 49, while being obviously different from vibrios of subgroup I.

Another example of error due to the use of H sera is found in Doorenbos' (1934) treatise. On p. 76 he classes together as "agglutinable" a range of haemolytic strains (El Tor D 9 to D 27 inclusive, No. 49, etc.), which in fact comprises three different kinds of vibrios (Table X).

Incidentally the vibrios (Nos. 20 and 67 of 1930) which Doorenbos describes as "vibrio El Tor classique", are of our subgroup V, while Gottschlich's original and classical "A" strain of 1905 is of subgroup I.

A STANDARD DIAGNOSTIC SERUM

The term "agglutinable," in so far as it refers to the use of sera containing the non-specific H agglutinin, must clearly be discontinued. All the official diagnostic sera hitherto in use have been of this type, which should no longer be produced.

In order that a serum should be able to identify the indubitable cholera vibrios of subgroup I it must contain the main characteristic antibody of that subgroup, and it must be made or employed in such a way as to avoid altogether the two forms of non-specific agglutination.

This is best achieved by preparing the serum with suspensions boiled or steamed for 2 hours to destroy the common H antigen. Suspensions of cultures under identification should be used in the unheated state, with or without formalin (0·2 per cent.), since heated suspensions tend to show a common O cross-agglutination with these sera.

A possible, but in our opinion less desirable, alternative is to use unheated suspensions for the preparation of H-O sera of subgroup I and to employ only boiled suspensions for the diagnostic test. Although this avoids both group H and non-specific O reactions, it is less efficient than the other combination since the O agglutinability of suspensions is generally greatly depressed by boiling, whereas it is at its maximum in unheated suspensions.

The standard serum will of course react with the El Tor vibrios of subgroup I. These however, can be distinguished from true *V. cholerae* by the haemolytic test with goat erythrocytes, which in our hands has proved a perfectly reliable criterion. (Other writers have found sheep's cells equally reliable, but not human cells.)

As a working rule we suggest that only non-haemolytic vibrios of subgroup I be accepted as proof of cholera or of the carrier state. It is not impossible that the causative vibrios are more various than we suppose, but we feel that such a view should not be adopted without the most stringent proofs.

SUMMARY

1. Previous work on the antigenic structure of the cholera group of vibrios is experimentally reviewed, and the data amplified and systematised.

2. The cholera group is defined as consisting of vibrios with similar biochemical characters and having a common heat-labile antigenic component.

3. The heat-stable antigens are divisible into:

(*a*) A considerable number of specific antigens, best demonstrated by O sera and H-O suspensions, which serve as a basis of classification into O subgroups.

(*b*) A non-specific component, demonstrable with O sera and O suspensions.

4. The first subgroup contains all the standard cholera vibrios from central laboratories, and the majority of other epidemic strains. We consider that it represents the only class of vibrios known for certain to cause epidemic cholera.

5. The races of this subgroup I are further divisible into two (or perhaps three) "types", as established by Japanese workers, according to differences in their subsidiary O antigens.

6. The haemolytic "El Tor" vibrios are serologically diverse. The term

"El Tor" should, as Shousha suggests, be reserved for those that have the same specific O component as the standard cholera vibrios.

7. For the identification of the undoubted cholera vibrios a standard subgroup I O serum is recommended in conjunction with the haemolytic test. The serum should contain both the main and the subsidiary antigens of the subgroup.

8. As a working rule it is suggested that bacteriological proof of "cholera" or a cholera carrier should rest on the isolation of a non-haemolytic vibrio with the specific O antigen of subgroup I.

The studies and observations on which this paper is based were conducted while one of us (K. V. V.) was holding a Fellowship of the International Health Division of the Rockefeller Foundation.

Our thanks are due to Mr P. Bruce White for constant help and advice, and to the numerous bacteriologists in various lands who have kindly supplied us with cultures and sera.

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(*MS. received for publication* 8. IV. 35.—Ed.)