JOURNAL OF BACTERIOLOGY, Mar., 1965

Vol. 89, No. 3
Printed in U.S.A.

Classification of *Vibrio cholerae* (*Vibrio comma*), Including El Tor Vibrios, by Infrasubspecific Characteristics

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Received for publication 15 October 1964

ABSTRACT

FEELEY, JOHN C. (National Institutes of Health, Bethesda, Md.). Classification of *Vibrio cholerae* (*Vibrio comma*), including El Tor vibrios, by infrasubspecific characteristics. J. Bacteriol. **89**:665–670. 1965.—A study of the properties of 220 serotype O group I vibrios indicated striking similarity in most of their properties. However, by using four tests often applied in the identification of the El Tor vibrio, five types were identified and characterized as follows: type 1 strains, phage IV-sensitive, nonhemolytic by tube and plate methods, unable to agglutinate chicken red cells (CCA), and Voges-Proskauer (VP) negative or weakly positive at 22 C; type 2, same as type 1 except for CCA; type 3, phage IV-resistant, CCA and VP usually positive, and strongly hemolytic by tube and plate methods; type 4, same as type 3, except hemolytic only by plate method unless culture has undergone pronounced rugose variation; and Type 5, same as type 3, except stably nonhemolytic. Type 1 and type 3 strains possess the characteristics usually ascribed to classic cholera vibrios and El Tor vibrios, respectively. Geographical and chronological distribution of the types is discussed. The thesis is presented that it is invalid to recognize two species, *V. cholerae* and *V. eltor*, because alleged differences are infrasubspecific. Recognition of a single species, *V. cholerae*, consisting of several types, is recommended.

Renewed interest has arisen in the so-called El Tor vibrios as a result of their emergence in the past 3 years as the principal cause of the widening circle of epidemic cholera in Southeast Asia. Felsenfeld (1964) pointed up the present state of confusion in their taxonomic status; some workers (deMoor, 1939, 1963; Mukerjee, 1963) have placed the El Tor vibrio in a separate species, *Vibrio eltor*, whereas others regard it as a biotype (Hugh, 1962; Feeley and Pittman, 1963) or a variant (Felsenfeld, 1963) of *V. cholerae* (*V. comma*).

Originally, a functional separation of El Tor vibrios and classic cholera vibrios was made by demonstrating hemolytic properties of the former with a tube test employing sheep or goat erythrocytes (Pollitzer, 1959). Difficulties with this method, at least some of which are due to inadequate media and incubation conditions (Feeley and Pittman, 1963), resulted in development of several other differential criteria (*see* Felsenfeld, 1964). Among these, the resistance of El Tor vibrios to group IV phage (Mukerjee, 1963), and their ability to agglutinate chicken erythrocytes (Finkelstein and Mukerjee, 1963), have been widely employed; positive Voges-Proskauer reactivity, also, has been reported (vanLoghem, 1938; Pollitzer, 1959; Felsenfeld, 1964). As a result of application of these methods, strains which are partially or completely lacking in the historic hemolytic property have been described as El Tor vibrios (deMoor, 1963; Roy, Mukerjee, and Tanamal, 1963), and the name *V. tor* var. *anhaemolyticus* has been proposed (deMoor, 1963).

In attempting to differentiate the so-called classic cholera and El Tor vibrios, too often sight has been lost of the fact that basically these vibrios are similar: the vast majority of their biochemical and serological properties are identical (Hugh, 1962), they cause an identical disease in man, and cholera vaccines show equal cross-protection in animals against classic cholera and El Tor vibrios (Pittman and Feeley, 1963).

The present work encompasses a study of properties of 220 representative O group I serotype vibrios, most of which came from recent epidemics. It will be demonstrated that some strains are intermediate between classic cholera

and El Tor vibrios, that alleged differences are infrasubspecific in nature, and that a single species, *V. cholerae*, consisting of a number of subtypes, should be recognized.

MATERIALS AND METHODS

Cultures. All of 220 cultures studied, with the exception of 5 from the El Tor Quarantine Station, were isolated from cases of clinical cholera occurring in the geographical areas shown in Table 1. On receipt they were plated on gelatin-agar (Smith, Freter, and Sweeney, 1961) to determine purity and gelatinase activity. Colonial morphology was examined by low-power stereoscopic oblique light microscopy (Feeley, 1962). All strains were freeze-dried promptly, and working cultures were maintained on Trypticase-agar slants [1% Trypticase (BBL), 1% NaCl, 1.5% agar] at 4 C until all tests were completed. Certain strains were maintained, also, on Trypticase-agar slants at 22 C, with monthly transfers for 4 to 6 months for subsequent comparison with freeze-dried strains.

Characterization of cultures. Heat-fixed smears were stained by Gram's method and by crystal violet. Agglutinability in vibrio O group I serum was tested by both slide and tube methods. The following tests were performed: hydrogen sulfide in Kligler's Iron Agar (Difco); motility and nitrate reduction in semisolid motility-nitrate agar (1% Trypticase, 1% NaCl, 0.1% KNO_3, 0.4% agar); urease activity in Urea Broth (Difco); indole on 1% Trypticase broth with Kovacs' reagent; lysine and ornithine decarboxylase and arginine dihydrolase in Falkow's medium with a mineral-oil seal (Ewing, Davis, and Edwards, 1960); and cytochrome oxidase (Ewing and Johnson, 1960). Fermentation tests were carried out in Purple Broth Base (Difco) containing 0.5% of the following carbohydrates: dextrose, lactose, sucrose, mannose, mannitol, arabinose, and salicin.

TABLE 1. *Geographical distribution of strains studied*

Type	Origin	No. of strains	Year isolated
1	India	2	1941
	Egypt	1	1947
	India	2	1953
	India	4	1957–58
	Thailand	40	1958–59
	East Pakistan	32	1960
	India	5	Unknown
2	Philippines	1	1930's
	Hong Kong	1	1939
	China	10	1945
	West Pakistan	3	1960
	East Pakistan	4	1964
3	Egypt*	1	1905
	Egypt*	4	1930's
	Celebes	13	1957
	Celebes	10	1959
	Thailand	2	1959
	Thailand	3	1960
	Hong Kong	16	1961
3	Philippines	14	1961
	Djakarta	7	1962
	Philippines	3	1963
	Taiwan	3	1962
	South Korea	6	1963
4	Malaya	6	1963
	Thailand	6	1963
	Burma	2	1963
	Viet Nam	6	1964
5	West Irian	4	1962
	Philippines	9	1963

* From El Tor Quarantine Station.

Four tests recommended for differentiation of El Tor vibrios from classic cholera vibrios were performed as follows.

Hemolysis tests. Tube hemolysis tests, done by use of sheep erythrocytes and Heart Infusion Broth (Difco) cultures, were performed as recommended by Feeley and Pittman (1963). Blood-agar plates [Blood Agar Base (Difco) plus 5% defibrinated sheep blood] were incubated aerobically at 35 C, and also anaerobically as recommended by deMoor (1963) to avoid confusion with so-called "hemodigestion."

Bacteriophage susceptibility. Strains were tested for susceptibility to group IV phage at the routine test dilution (RTD), by the method of Mukerjee (1963). Appropriate susceptible and resistant control strains were included in each test.

Chicken-cell agglutination (CCA). Hemagglutination of chicken erythrocytes was determined by by the method of Finkelstein and Mukerjee (1963) using 18-hr Heart Infusion Agar (Difco) slant cultures.

Voges-Proskauer (VP) reaction. Cultures grown in MR-VP medium (Difco) for 48 hr at 35 C and 22 C were tested by the Barritt method (Ewing, 1962).

RESULTS

Common characteristics. Each of the 220 strains produced cloudy zones indicative of strong gelatinase activity on gelatin agar. Colonial morphology by oblique light microscopy was typical of vibrios, but only recently isolated cultures or older cultures maintained continuously in freeze-dried form gave rise to the typical greenish to reddish bronze, finely granular, chromatic colonies seen on direct isolation from patients (Husain and Burrows, 1956; Feeley, 1962). The majority of strains contained variant bluish-gray, whitish-opaque, or rugose colonies.

Microscopically, all strains were composed of gram-negative short rods, with the majority showing some tendency toward cellular curvature. Curvature was more easily determined in smears stained with crystal violet.

All cultures were motile, were oxidase- and indole-positive, and reduced nitrates to nitrites. Urease and H_2S tests were negative. Lysine and ornithine decarboxylase tests were positive, and the arginine dihydrolase test was negative. Acid without gas was formed in fermentation tests with dextrose, lactose (delayed 2 to 6 days), sucrose, mannose, and mannitol. Arabinose and salicin were not fermented. Fermentation of sucrose and mannose but not arabinose places them in the so-called Heiberg fermentation group I.

Serologically, all strains were agglutinated by vibrio O group I antiserum.

Characteristics permitting type differentiation. On the basis of hemolytic activity, phage IV sensitivity, CCA, and VP reactivity, the 220 strains can be divided into five types (Table 2).

Type 1 strains would be regarded as classic *V. cholerae* according to the criteria of Pollitzer (1959), Mukerjee (1963), Finkelstein and Mukerjee (1963), deMoor (1963), and Roy et al. (1963). Included among these strains is the proposed neotype strain of *V. cholerae* (ATCC 14035; NCTC 8021; Hugh, 1964). Although all were nonhemolytic by tube and plate tests, it should be noted that they produced greenish clearing around areas of heavy growth but not around well-isolated colonies on 18- to 24-hr aerobic sheep blood-agar plates. This phenomenon, often described as "hemodigestion," is inhibited by anaerobic incubation (deMoor, 1963). It differs from hemolysis due to the heat-labile hemolysin of the El Tor vibrio and appears to be due to the action of strongly alkaline dialyzable metabolic products (Liu, 1959). All strains were sensitive to group IV phage, and none gave positive CCA reactions. In VP tests at 35 C, 66 of 86 strains were weakly positive; weak reactions were produced by only 9 strains at 22 C. This was probably due to the small amount of acetoin produced by most cholera vibrios (Gallut, 1946).

Type 2 strains differed from type 1 only by giving positive CCA reactions. These organisms would be regarded as El Tor vibrios if the CCA test (Finkelstein and Mukerjee, 1963) were the sole criterion employed.

Type 3 strains were strongly hemolytic by the tube hemolysis test as well as by the plate hemolysis test, in which definite clear-cut zones of hemolysis were observed around well-isolated colonies on both aerobic and anaerobic sheep blood-agar plates. All were resistant to lysis by group IV phage. CCA reactions were positive with 78 of the 82 strains, and VP tests were strongly positive with 79. There is little doubt that strains of type 3 would be considered as typical El Tor vibrios by most workers; in fact, five of these strains were isolated at the El Tor Quarantine Station. Included among these strains is the proposed neotype strain of *Vibrio cholerae* biotype *eltor* [*V. eltor* (*sic*); ATCC 14033; NCTC 8457; Hugh, 1962].

TABLE 2. *Characteristics of 220 O group I serotype vibrio strains*

Type	No. of strains	No. of positive strains				
		Hemolysis		Phage IV sensitivity	CCA	VP†
		Tube	Plate*			
1	86	0	0	86	0	9 (weak)
2	19	0	0	19	19	0
3	82	82	82	0	78	79
4	20	0	20	0	20	16
5	13	0	0	0	13	13

* Reaction around well-isolated colonies on both aerobic and anaerobic blood-agar.

† Reading made on cultures incubated for 48 hr at 22 C.

Type 4 strains differed from type 3 only by failing to show positive tube hemolysis. However, all strains produced weak but detectable hemolysis on aerobic and anaerobic blood-agar plates. The zone of hemolysis was only slightly larger than the colony and was more readily observed when the colony was scraped away with a loop. On repeated platings, colonial variants of much greater hemolytic activity were detected. These were rugose variants which, by the tube hemolysis test, gave positive results. This phenomenon is discussed at greater length below. Strains identified as type 4 have been considered either as nonhemolytic or hemolytic El Tor vibrios by some workers, depending on criteria employed. They appear to be similar to the "apparently nonhemolytic" cultures described by Roy et al. (1963).

Type 5 strains were identical with types 3 and 4, except that they were completely nonhemolytic by each criterion. Cultures of this type correspond to the *V. tor* var. *anhaemolyticus* described by deMoor (1963) and the "stably nonhemolytic" El Tor vibrios described by Roy et al. (1963). In my hands, repeated attempts to obtain hemolytic mutants of these strains have failed.

A key to the differentiation of the types is given:

I. Phage IV sensitive (nonhemolytic, VP reaction negative or weak).
  A. CCA-negative .... Type 1
  B. CCA-positive .... Type 2

II. Phage IV resistant (VP and CCA reactions usually positive).
   A. Hemolytic
      1. Tube and plate methods both positive .... Type 3
      2. Plate method only positive .... Type 4
   B. Nonhemolytic .... Type 5

Stability of type characteristics. Eighty strains maintained by monthly transfers on Trypticase-agar slants at 22 C for 4 to 6 months were compared with corresponding strains freshly reconstituted from the dried state. The greatest change occurred in hemolytic activity; 12 of 20 type 4 strains became capable of hemolyzing red cells by the tube test (Table 3). One type 3 and one type 5 strain lost VP reactivity, and another type 3 strain lost ability to hemagglutinate chicken erythrocytes. Both type 1 and 2 strains remained constant in type characteristics. No cultures changed in phage IV sensitivity.

Conversion to positive tube hemolysis of type 4 cultures, when maintained on agar slants, was accompanied by the appearance of a large proportion of strongly hemolytic colonies on blood-agar plates and a decrease in the proportion of colonies characteristic of fresh isolates. These strongly hemolytic colonies were rugose variants, which were present in only small numbers when the strains were first received and also after maintenance in the dried state. A similar increase in the proportion of rugose variants was obtained by agar passage of type 5 strains, but these were not hemolytic.

TABLE 3. *Stability of type-differentiating characteristics*

Type	No. of strains	Maintenance	No. of positive strains				
			Hemolysis		Phage IV sensitivity	CCA	VP
			Tube	Plate			
1	20	Dried*	0	0	20	0	0
		Slant†	0	0	20	0	0
2	7	Dried	0	0	0	7	0
		Slant	0	0	0	7	0
3	20	Dried	20	20	0	20	20
		Slant	20	20	0	19	19
4	20‡	Dried	0	20	0	20	16
		Slant	12	20	0	20	16
5	13	Dried	0	0	0	13	13
		Slant	0	0	0	13	12

* Culture freshly restored after maintenance in freeze-dried state at 4 C for 6 months (4 months for six type 4 strains).

† Culture maintained on agar slants at 22 C with monthly transfer for 6 months (4 months for six type 4 strains).

‡ Includes six strains maintained on agar slants for 4 months at 22 C, all of which proved to be tube hemolysis-positive.

TABLE 4. *Characteristics of El Tor Quarantine Station strains**

Strain	Year isolated	Phage IV sensitivity	CCA	VP
Tor A	1905	0	0	0
Doorenbos 80	1929–31	0†	0	+
ATCC 14033	1930	0	0	+
34-D9	1933	0	0	+
34-D13	1933	0	+	+

* All strains were strongly hemolytic by tube and plate tests.

† Developed a few tiny plaques when spotted with RTD.

Characteristics of cultures from the El Tor Quarantine Station. The properties of five strains belonging to type 3 and originally isolated from Pilgrims at the El Tor Quarantine Station are shown in Table 4. Each strain was strongly hemolytic by plate and tube tests, and all were resistant to confluent lysis by group IV phage; however, one strain (Doorenbos 80) developed a sprinkling of very tiny plaques when spotted with the RTD. This phenomenon, which was more readily observed when the organisms were incorporated in a soft-agar layer, is under investigation and appears to be due to sensitivity to a small portion of the phage population. It has not been observed with other cultures. The CCA reaction was positive with only one of the five strains, whereas four were VP-positive. However, it should be noted that these old historic cultures undoubtedly have undergone many passages on artificial media before receipt and their characteristics may have changed.

Geographical and chronological distribution of types. Type 1 strains with the characteristics of classic cholera vibrios have remained localized mainly in the longstanding Bengal endemic focus and have caused only limited epidemics elsewhere in recent years, Egypt in 1947 and Thailand in 1958–59 (Table 1). It is of particular interest that epidemics in other areas from which cultures were studied have been caused by strains that were capable of CCA. Recently, the epidemic strains also have been phage IV-resistant and usually hemolytic. Type 2 strains appeared in the Bengal endemic area in East Pakistan in 1964, and it will be of interest to note whether vibrios of types 3, 4, and 5 invade this area.

The present surge of epidemics in Southeast Asia beginning in 1961 was initiated by type 3 strains with hemolytic characteristics of the old El Tor Quarantine Station strains and of those that had been causing localized epidemics in the Celebes since the late 1930's (deMoor, 1949; Tanamal, 1959). By 1962 some of the epidemic strains began to show changes in hemolytic activity. Some strains (type 5) showed no hemolytic activity, whereas others were hemolytic only by the plate test (type 4).

DISCUSSION

Historically, the El Tor vibrio was separated from the classic cholera vibrio on the basis of a tube hemolysis test with washed sheep or goat erythrocytes (Pollitzer, 1959). Difficulties with the tube hemolysis test, some of which were doubtless due to inadequate methods (Feeley and Pittman, 1963), have led to the use of other differential criteria (Felsenfeld, 1964). Among the properties ascribed to the El Tor vibrio are hemolytic activity on anaerobic blood-agar (deMoor, 1963), resistance to group IV phage (Mukerjee, 1963), positive CCA reactions (Finkelstein and Mukerjee, 1963), and usually positive VP reactions (vanLoghem, 1938; Pollitzer, 1959; Felsenfeld, 1964). Although lacking the historic hemolytic criterion, strains which are CCA-positive and phage IV-resistant have been designated as El Tor vibrios (deMoor, 1963; Roy et al., 1963). The taxonomic and nomenclatural problems surrounding the separation of vibrios which agglutinate in cholera antiserum have not been solved, even for frankly hemolytic cultures. Some workers have placed organisms differing from *V. cholerae* by only a few infrasubspecific properties in a separate species, *V. eltor* (deMoor, 1939, 1963; Mukerjee, 1963). The name *V. tor* var. *anhaemolyticus* has been suggested by deMoor (1963) for "nonhemolytic El Tor vibrios." Others have regarded the El Tor vibrio as a biotype (Hugh, 1962; Feeley and Pittman, 1963) or a variant (Felsenfeld, 1963) of *V. cholerae*. Hugh (1964) recently pointed out that biotypes and phagotypes are infrasubspecific divisions of a species, as stated clearly in recommendation 8a (2) of the *International Code of Nomenclature of Bacteria and Viruses*. I strongly support this viewpoint.

By use of tests which have been widely employed to identify the "El Tor vibrio," it has been shown in the present paper that O group I serotype vibrios from various geographical regions may be divided into five types. With the exception of type 4, these types appear to be stable. A satisfactory differentiation of types 3 and 4 can not always be made on cultures, belonging originally to type 4, which have undergone pronounced rugose variation. Differentiation of these types can be made, however, if the culture still contains some of the characteristic parent-colony types seen by the oblique-light method when freshly isolated from patients. With type 3 cultures, colonies of the typical parent type are surrounded by a large and definite zone of hemolysis on blood-agar and, if transferred, will give a positive tube hemolysis test; with type 4, the zone of hemolysis is small and the tube hemolysis test is negative. Unfortunately, the typical parent-type colony is often lost on prolonged cultivation on artificial media.

Each type could be subdivided into either Ogawa or Inaba serotypes by agglutination tests with absorbed sera.

Subdivision of the vibrios which agglutinate in cholera O group I antiserum certainly has epidemiological value, but the described infrasubspecific divisions should not be regarded as a basis for creation of separate species. This seems all the more obvious when one considers the overwhelming similarities of the cultures studied here and the undeniable fact that strains of all types are capable of causing epidemic cholera. Indeed, it should not be forgotten that the species *V. cholerae* is, in effect, a "serotype." Serological methods currently constitute the only satisfactory means of differentiating members of this species from biochemically identical noncholera vibrios of Heiberg fermentation group I, which are ubiquitous in nature. On the other hand, many theoretical taxonomists would object to defining a species solely on a serological basis. The electrotaxonomic survey of Sneath and Cowan (1958) implies that the genus *Vibrio* perhaps corresponds to a species-level ranking when compared with certain other genera. Practical necessity, however, would seem to sanction the use of serological methods to differentiate *V. cholerae* and noncholera vibrios from stool specimens. Viewed from this perspective, the separation of two species, *V. cholerae* and *V. eltor*, becomes absurd. There remains every reason to recognize a single species, *V. cholerae*. This species may be subdivided by hemolytic, phage, hemagglutination, and VP tests, as well as by serological differentiation into Ogawa or Inaba serotypes and by certain other techniques. Recognition of a single species would in no way detract from any value which may be attached to the recognition of subspecies characteristics.

A further pragmatic consideration calling for recognition of a single species is the problem faced by quarantine and public health officials.

At the time of the major Hong Kong and Philippine epidemics in 1961, cholera caused by the so-called El Tor vibrio was excluded from the list of quarantinable diseases. Although International Sanitary Regulations were changed on 23 May 1962 (World Health Organization, 1962), to include cholera due to these organisms, the state of confusion as to their significance continues in many countries. The solution to this problem might be aided materially by the recognition of a single species, *V. cholerae*.

ACKNOWLEDGMENTS

I thank Margaret Pittman for her encouragement, advice, and review of the manuscript, and Roland E. Mills for technical assistance. Contributions of cultures from the following individuals are gratefully acknowledged: A. S. Benenson, W. Burrows, O. Felsenfeld, R. A. Finkelstein, J. D. Gilmore, Mrs. C. Z. Gomez, K. Goodner, D. C. Lahiri, D. J. M. MacKenzie, S. Mukerjee, H. L. Smith, Jr., R. W. Swanson, and S. J. W. Tanamal.

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