PRESENT STATUS OF THE EL TOR VIBRIO PROBLEM

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INTRODUCTION

According to the data presented in the reviews of Linton (66), Pollitzer (98), and Seal (106), El Tor organisms are closely related to the "true" cholera vibrios. Both belong in the first biochemical group of Heiberg; i.e., they ferment mannose and sucrose but not arabinose. True cholera vibrios as well as El Tor vibrios are members of the serological group O-I of Gardner and Venkatraman of the genus Vibrio. El Tor strains differ from the true V. comma vibrios chiefly by their ability to hemolyze sheep and goat red blood cells. Hemolysis, however, is a rather variable characteristic, especially in freshly isolated strains. Thus, a search for further diagnostic means is being carried out in many laboratories. In addition, the role of the El Tor vibrios in cholera was a moot point until recently when a cholera epidemic caused by El Tor organisms swept through southeast Asia. These problems stimulated research on El Tor vibrios, and this survey is a brief summary of recently published results of studies aimed towards the clarification of the status of these organisms.

Geography and Pathogenicity

It is of historical interest that El Tor-like vibrios were described in a number of localities in Bulgaria, Germany, and the Middle East before the El Tor organisms acquired their name from the quarantine station in which they were isolated from pilgrims by Gotschlich (see 98). El Tor vibrios have been recovered in areas where true V. comma strains prevailed in the past and were usually considered nonpathogenic (98). An occasional El Tor strain was isolated also during the recent true V. comma outbreak in Poona (101) and in Calcutta (86). During the declining phase of the true cholera epidemic in Thailand in 1959, Mukerjee and Guha Roy (86) found, among 70 vibrio strains which were submitted to them, 4 hemolytic and 3 nonhemolytic El Tor vibrios.

El Tor vibrios were frequently isolated from water and food during cholera epidemics (2, 12, 39), and also in areas from which cholera had been absent for a long time, e.g., from the Ural River (6) and from the Kola Bay (111). The evaluation of the finding of El Tor organisms in the environment is, therefore, a difficult task.

The pathogenicity of the El Tor vibrios had been questioned, and it was believed that they never cause cholera epidemics (36, 37, 66, 98, 106, 114). When cholera broke out in the Celebes (Sulavesi) in 1937 and the vibrios isolated from patients, carriers, and water were found to be El Tor organisms, the disease was named "para-cholera" or "choleriform enteritis" (98, 114). De Moor and van Loghem, who did most of the investigations in the Celebes, stated that the Celebes-type illness differs from "true" cholera by its low morbidity rate, by its failure to spread to other countries, and by its less-marked tendency to become water-borne.
During and after World War II, only a few instances were described in which El Tor vibrios spread from the Celebes to Java or Singapore (37, 54, 114). A mild diarrheic outbreak caused by El Tor organisms was observed in the city of Ubol, Thailand, in 1960 (12, 39) in the wake of an epidemic caused by true cholera vibrios. The origin of this outbreak could not be traced. In 1961, El Tor cholera spread out of the Celebes and invaded several other parts of Indonesia, the Philippines, Kwantung, Formosa, and, lately, also the southern portion of Malaysia and West Irian (2, 16, 28, 29, 30, 31, 31a, 37, 68, 78a, 80, 87, 103a, 104, 126). During the past months, it also invaded Thailand. The epidemiology and the clinical picture of this El Tor disease does not differ from that observed in cholera caused by true V. comma (37). International regulations did not require the member nations to impose quarantine and other regulations when El Tor disease (called also "paracholera") appeared, because this infection was regarded by the World Health Organization as different from true cholera. In view of the clinical and epidemiological observations made in 1961 and 1962, the World Health Organization decided that El Tor disease should be considered cholera and not a different malady (125). However, the taxonomy of the El Tor vibrios still remains to be decided.

**Taxonomy**

The difficulties encountered in the classification of El Tor vibrios were discussed by Gallut (51), who considered them simply variants of the true cholera vibrios. Szturm-Rubenstein et al. (112) and Felsenfeld (37) suggested that El Tor vibrios be designated as variants of *V. comma*. Hugh (61) recommended the name *V. comma* biotype El Tor. Mukerjee (84) used the terms "V. cholerae" and "V. el tor." Sayamov (105) developed a nomenclature which distinguishes *V. cholerae*, *V. makassari* (pathogenic El Tor vibrios; synonymous with the "Celebes type" of Abdoelrachman, see 37, 98), and *V. el tor* (non-pathogenic El Tor strains; synonymous with the "Zam Zam type" of Abdoelrachman). There is not even a general agreement in the English language literature whether cholera vibrios should be called *V. comma* or *V. cholerae*. Thus, there is little hope that a new designation for the El Tor strains could be found which would be accepted world-wide.

Owing to uncertainties in the nomenclature, the typical nonhemolytic cholera vibrios will be designated in this review as the "true" *V. comma* or the "true" cholera vibrios; El Tor strains will be referred to as El Tor vibrios.

**Biochemical Properties**

Hugh (61) studied 57 El Tor strains and found that the majority of them did not grow on Deoxycholate Citrate Agar. They were gram-negative rods with a polar monotrichous flagellum while motile, but with little suggestion of a somatic curvature. Acid was formed from dextrose (and accumulated anaerobically), fructose, galactose, lactose, maltose, mannitol, mannose, sucrose, and trehalose. The following reactions were positive: indole, Voges-Proskauer, catalase, cytochrome oxidase, gelatin, nitrite, lysine, ornithine, hemolysis of sheep red blood cells in tube tests, and agglutination in vibrio group O-I serum. Acid was not formed from adonitol, esculin, arabinose, dulcitol, inositol, inulin, melezitose, melibiose, raffinose, rhamnose, salicin, and xylose. The following tests were negative: hydrogen sulfide formation in Kligler's medium, nitrate reduction to gas, phenylalanine deaminase, urea, and arginine. Hugh believed that El Tor is a biotype of *V. comma*, is hemolytic, and often forms acetyl methyl carbainol. He recommended strain ATCC 14033 (NTCC 8457) as the neotype of *V. comma* biotype *El Tor*.

When El Tor vibrios were analyzed chemically, no significant difference was observed in the ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content of these and of true cholera vibrios (10). The amino acid composition of both groups appeared to be the same in the studies of Guha and Mukerjee (59); Pustulova (100) found less methionine and tyrosine but more proline and OH-proline in El Tor than in true *V. comma* strains but could not detect statistically significant differences in the nucleic acid content of these organisms (100a).

Chugh et al. (15) studied vibrio mucinase activity. The mucinase was classified as an ovomucinase. It was present both in true and in El Tor cholera vibrios, and was produced also by nontoxic strains. This ovomucinase was insoluble in ethyl ether, was not precipitated by acetone or ethanol, and was unstable at low pH.

Esselman and Liu (32) observed the lecinthase activity of El Tor vibrios. According to Mitra...
and Chatterjee (76) and Chatterjee and Mitra (11), this lecithinase is a lysolecithinase, a phospholipase B, which does not act on purified lecithin. It appeared in broth cultures during the late log phase, i.e., 48 hr after seeding. It could be isolated from the culture filtrates by precipitation with ammonium sulfate saturated to 50 to 75%. Dissolved in a 0.01 M phosphate buffer and after dialysis, it remained active when lyophilized and kept at −15 C for 4 weeks. The optimal pH was 8.0. The lecithinase was not destroyed by heating at 100 C for 15 min, by enzymes, or by metallic ions, except by Zn⁺⁺ to about 40%. Glutathione and cysteine enhanced its activity.

The nutritional requirements of El Tor vibrios were investigated by Goto et al. (58). Ammonium sulfate and other ammonium salts were not utilized as the sole source of N in semisynthetic media. A 0.1% concentration of dextrose and 0.025 N phosphate were adequate for growth support. Addition, per ml of medium, of 100 μg of magnesium sulfate, adenine, guanine, xanthine, and adenosine remarkably enhanced not only growth but also hemolysin production.

El Tor vibrios were more resistant to antibiotics, chemical agents, and physical environmental factors than were true cholera vibrios (2, 37, 40, 55, 98). They survived in water for a longer time than did true cholera vibrios in experiments carried out in the Philippines (2) and in Thailand (40) but not in Calcutta (88). This difference was due to the strains employed and to the water inoculated with them; water used in the Calcutta tests was not brackish.

Antigens and Toxins

Both true and El Tor cholera vibrios belong to the serological group O-I of vibrios. They may be characterized within this group as Ogawa (AB), Inaba (AC), and Hikojima (AB/C) subtypes, according to the time-honored classification of Nobechi (see 98). Aoki and Sakazaki (1) suggested recently that special attention should be paid to the E antigen of Burrows (see 98) appearing in the combination ABE, because of the different geographic origin of the strains containing this antigenic factor.

Before World War II, most El Tor vibrios, especially those isolated in the Celebes, were Inaba strains. In the Ubol outbreak and during the present El Tor epidemic in Southeast Asia, predominantly Ogawa subtypes were diagnosed. Inaba and Hikojima subtypes were encountered only occasionally (2, 37, 39, 43, 86, 87). It may be of interest that at this writing Inaba strains of true cholera vibrios predominate in Calcutta, while the El Tor outbreaks in Malaysia and Thailand still yield Ogawa serotypes.

The analysis of the antigenic and nonantigenic fractions of El Tor vibrios has been continued by Gallut (49, 50, 52), who worked with phenol-treated organisms. El Tor strains, as the true cholera vibrios, yielded a thermostable fraction which was a specific O-agglutinogen and precipitogen poly saccharide, as well as a nonspecific, hemagglutininogenic, and precipitogen protein. The specific O agglutinogen was found in the cell wall of the vibrios. The nonspecific protein was present also in nonagglutinable vibrios. (The term nonagglutinatable or NAG vibrios is the misnomer of strains belonging to serological groups other than O-1.) NAG vibrios may contain variable amounts of a specific O-I antigen and give partial cross-reactions with Ogawa or Inaba sera, or both (98).

Misra and Shrivastava (73, 74, 75), Misra et al. (72), and Shrivastava (107a) separated seven antigenic factors of which one, a heat-stable component having the characteristics of a lipopolysaccharide, was designated the α antigen. This α antigen was responsible for the serological activity of the intact vibrios. It was present, like Gallut's specific O antigen, in the cell wall and perhaps also in the cytoplasmic membrane of the vibrios. The cell wall precipitated the same amount of antibody N as the isolated lipopolysaccharide. Reactions with Ogawa and Inaba antibodies were highly specific. This was confirmed also by N determinations. Singh and Devi (108) agreed with Linton (66) and Gallut (49, 50, 52) that the specific polysaccharide isolated from El Tor vibrios after phenol treatment is identical with that prepared from true cholera vibrios.

The relationship of antigens to the toxins of the cholera vibrios as well as the methods for their preparation were surveyed by Burrows (3), Polli tizer (98), and Seal (107). Burrows (4) and Polli tizer (98) called attention to the fact that chemical and physical manipulations employed in the isolation of the toxins may alter them. To decompose vibrios as gently as possible, Jenkin (62), Jenkin and Rowley (63), and Rowley (102)
used 2.5 m urea and subsequent fractioning with ammonium sulfate. A component was isolated which was perhaps identical with Gallut's toxic polypeptide and with the acid-insoluble vibrio fraction of Freret (see 98). It was part of the Boivin and Mesrobeanu antigen which can be prepared by trichloroacetic acid precipitation (see 98). The toxic protein of Jenkin and Rowley was isolated also from NAG water vibrios. It is destroyed by proteolytic enzymes and by heating at 100 C for 10 min. The O-specific lipopolysaccharide is relatively nontoxic when compared with lipopolysaccharides from other gram-negative organisms. It does not cause diarrhea in susceptible experimental animals but is absorbed from the intestines into the blood stream. This was proven in rabbits by eliciting in them a Shwartzman phenomenon. When the endotoxin was prepared according to Westphal et al. by extracting cholera vibrios with 45% phenol water at 65 C, the lipopolysaccharides in the watery portion of the extract contained only 15% of the toxic substance of the vibrios, whereas _Escherichia coli_ yielded 50% when treated in the same manner. After the vibrios were treated with urea, the protein part contained 50% of the toxin of the vibrios.

Ghosh and Mukerjee (56) and Ghosh and Mukerjee (57) examined the precipitation bands formed in agar gel between crude saline and distilled water extracts of vibrios and the respective antisera. One ring, identical with the α band of Misra and Shrivastava (75), developed. Additional rings appeared when the antigens were heated at 100 C. The latter were caused probably by degraded and masked antigenic components and, perhaps, also by the flagellar antigen which may be more heat-resistant in vibrios than in Enterobacteriaceae.

De and co-workers (17, 21, 22, 23) prepared vibrio "toxin" by culturing the organisms in 5% peptone water, and compared its action with that of saline washings of cells grown on nutrient agar. The toxicity of the vibrios was rapidly lost on serial subculturing. The filtrates of peptone water cultures contained mucinase and enterotoxin but no hemolysin when true cholera vibrios were tested. Filtrates of the saline washings of the cells grown on nutrient agar showed mucinase and hemolytic activity also when true cholera vibrios were examined. The toxin was thermolabile. De et al. (21, 22, 23), and De (18) believed that the thermolabile cholera toxin is an exotoxin. This toxin appeared in 6-hr-old cultures, but the amount began to decline already after 12 hr of incubation. When the toxin was inoculated into the ligated intestinal loop of rabbits according to the technique of De and Chatterjee (20), cholera-like disease developed.

Oza and Dutta (93a) used sonic vibration for the production of a highly active toxin. Ghosh and Mukerjee (56a) found that the ultrasonic extracts of true cholera vibrios showed variable toxicity.

Seal (107) prepared a toxin by growing several vibrio strains in broth cultures containing 0.5% different carbohydrates, under a paraffin seal.

Watanabe and Felsenfeld (121, 122), studying the supernatant fluid of El Tor cultures, found that the toxin causing death in mice and the hemolytic activity of the supernatant vary independently. At least three water-soluble components were distinguished: a thermolabile exotoxin-like, a thermostable bacterial, and a thermolabile bacterial factor. The antilethal toxin but not the antihemolytic activity of the sera prepared against El Tor supernatant fluids could be partly absorbed with bacterial cells. Watanabe and Verwey (124) stated that the polysaccharide antigen present in the supernatant fluid of El Tor cultures gave a single band by ultracentrifugation and by paper electrophoresis. A second faint line appeared after prolonged incubation. This antigen was highly active against El Tor and true _V. comma_ Ogawa, but much less so against Inaba strains. Rabbits responded with the production of protective and bactericidal antibodies but not with agglutinin formation. Absorption with the homologous antigen removed all protective and bactericidal activity from the sera. The polysaccharide displayed a mild tumor-necrotizing action but was, on the whole, less toxic than the endotoxin from most other gram-negative species.

Burrows (4) observed in electrophoretic studies that relatively larger amounts of faster-moving intracellular polysaccharide and cell-wall protein were present in _V. comma_ strains isolated from severe cases than in vibrios cultured from patients with mild cholera. Gallut (48) reported fluctuations in the toxicity of the vibrios isolated during various phases of the disease. De et al. (22) noted that the enterotoxicity of the vibrios decreased with the decline of the epidemic. A corresponding difference was seen in vitro and in vivo when the
toxin production of vibrios from the severe El Tor outbreak in the Celebes and of El Tor strains causing only mild or no symptoms in man were compared (40). This problem needs further study because of the small number of strains tested in these experiments.

Liu (67) studied vibrio hemolysins and concluded that the hemolysis observed on blood-agar plates is due to excess alkalinity, not to an enzymatic activity. Hemolysis in test tubes was ascribed to hypotonicity. Nevertheless, some hemolysin production was seen when the cellophane plate technique was employed at pH 7.0. The hemolysin from true V. comma was nonantigenic and acted only on human red blood cells, while El Tor vibrios produced a heat-labile and antigenic hemolysin.

Martinez-Silva and Caselitz (70) immunized rabbits with 3-week-old supernatant fluids of V. jamaicensis cultures which were converted into a toxoid-like agent by formalization. The serum of the animals neutralized the hemolysin of water vibrios belonging to the V. jamaicensis group but not the hemolysin of El Tor and of a set of NAG vibrios. The authors concluded that the hemolysin of El Tor vibrios differs from that of other vibrios.

Roy and Mukerjee (103) confirmed the observation that the hemolysin production of El Tor vibrios varies with the medium used. They expressed the opinion that the hemolysin is perhaps an adaptive enzyme.

The hemolysin of the El Tor vibrios was purified by Watanabe and Seaman (123) by fractioning with ammonium sulfate, ethanol, and ultracentrifugation. It contained about 95% lipids and was homogeneous when examined with the aid of ultracentrifugation, paper chromatography, electrophoresis, and agar gel diffusion techniques.

No statistically significant difference was found between the hemolysin production of El Tor vibrios from Thailand and from the present El Tor epidemic (40), but the El Tor vibrios isolated in Irian did not produce hemolysin at all (78a).

Phage Susceptibility

Mukerjee and his co-workers (80 to 87) studied vibrio phages and found five phage-susceptibility patterns with the aid of four group-phages. All true cholera vibrios were sensitive to phage IV, while El Tor organisms and NAG vibrios were not. This observation was utilized as a differential diagnostic means for distinguishing true cholera and El Tor strains, especially when dealing with slowly hemolytic or nonhemolytic El Tor vibrios (40, 43, 87).

Newman (89) reported briefly on studies which led him to establish a phage-sensitivity pattern for true cholera and El Tor vibrios, but details have not been published to date.

Nicolle et al. (90, 91) and Gallut and Nicolle (53) examined 213 vibrios with a set of eight phages. Approximately 30% of the true cholera and of the El Tor vibrios but only 5% of the NAG vibrios were lysogenic. The nonlysogenic El Tor strains belonged to phage type I; the lysogenic, to phage type II of the Nicolle-Gallut classification. True cholera vibrios from the Far East were classified as type VI; those from the Near East and the Middle East, as type V of the Nicolle-Gallut scheme.

Animal Experiments

One of the fruitful developments in experimental cholera work was the introduction of the use of rabbits, producing in them a disease resembling, and perhaps even identical with, human cholera. De and Chatterjee (18, 20, 21) injected ligated intestinal loops of rabbits with vibrios and their toxins. Dutta and Habbu (25) recommended suckling rabbits. Disease could be produced not only by injecting the organisms or their toxins into the intestinal loops but also by feeding suckling rabbits, according to Dutta et al. (27), Panse and Dutta (96), and De et al. (23). Dutta and Ozra (26) found that the intestinal enzymes of adult rabbits, especially lipase and phospholylase but not trypsin or pepsin, destroy the cholera toxin. Thus, young animals should be used for feeding experiments. Dutta et al. (26a) were able to produce cholera-like disease by injecting into the intestinal loop of 10-day-old rabbits not only true cholera vibrios but also some El Tor strains. When vibrios were passed on from one group of suckling rabbits to another, their choleragenic capability increased, and all true cholera and El Tor vibrios, as well as some NAG strains, caused disease in these animals.

Nikonov (92) inoculated V. comma into the bile duct or into the gall bladder of guinea pigs. About 25% of the animals died in 1 to 10 days. The vibrios showed S → R and R → S variations in the surviving animals. Carriers of R forms developed frequently. Sayamov (105) continued
these experiments, with rabbits. Water vibrios caused an intensive but nonspecific inflammation of the gall bladder, but the vibrios died in a short time. True cholera and Makassar (Celebes) El Tor but not the Middle East and Near East El Tor (Zam Zam) strains survived in the gall bladder. Sayamov, like Dutta et al. (26a), did not find any difference between the two subtypes of El Tor vibrios in experiments on isolated guinea pig intestinal loops.

**Isolation and Identification**

Burrows and Politzer (7, 98) and Caselitz (9) published extensive studies on media and diagnostic methods useful in cholera work. Several additional media and modifications of plates and methods were reported recently.

Rananavare et al. (101) used a plate containing (per 100 ml): 2.5 g of agar, 2 g of peptone, 0.5 g of sodium taurocholate, 0.3 g of beef extract, 0.5 g of NaCl, 2 g of lactose, and sufficient litmus solution (pH 7.4). Both true cholera and El Tor vibrios grew well on this medium when tested in the Poona outbreak.

Smith et al. (109) modified the Smith and Goodner medium to contain (per 100 ml): 1 g of Trypticase, 3 g of gelatin (Difeo), 1.5 g of agar, 0.1 g of yeast extract, 0.5 g of sodium taurocholate, and 1 g of NaCl. This plate was employed by Finkelstein and Gomez (43) in the Philippines, in comparison with plain meat agar (pH 7.5) and colony selection with the aid of the Lankford modification of the Henry oblique-light technique (65) as well as the fluorescent-antibody method described by Finkelstein and LaBrec (44). The latter two procedures gave better results.

The tellurite-salt-lauryl sulfate-agar plate was modified by Morgan et al. (79). It was satisfactory for the isolation of true and of El Tor cholera vibrios in the following composition: 40 g of BBL or Difeo Blood agar Base, 15 g of NaCl, 15 g of agar, 800 ml of distilled water (pH 8.6). After autoclaving, 50 ml of a watery solution of sucrose, 100 ml of a 0.1% aqueous solution of sodium lauryl sulfate and 2 ml of Difeo or BBL Tellurite Blood Solution were added. This plate was streaked directly with feces, with specimens collected in peptone water of pH 7.8, or, when the samples were expected to be delayed on route (39), with specimens preserved in the fluid of Venkatraman and Ramakrishnan (118).

There is no record in the hitherto available literature on the field use of the recently described tellurite plates of Ouchterlony (94) and Monsur (77) and of the employment of the collecting method of Monsur (78) in the detection of El Tor vibrios. Feeley (33), Soman and Sayyid (110), and Felsenfeld (37) recommended the use of simple, noninhibitory media, because they are easily prepared and well adapted for work in field laboratories.

El Tor vibrios are often inhibited on the highly selective alkaline bismuth agar plate of Wilson and Reilly and its modifications (see 98). Excellent growth has been observed on the Hynes modification of Leifson's Desoxycholate Citrate plate (37). El Tor vibrios may grow faster on Difco Desoxycholate Citrate plates than true cholera vibrios (38). The use of the Hynes plate of the Oxoid Co. has some additional advantages, like the medium of Panja and Ghosh (95). It is not difficult to prepare. Furthermore, not only vibrios but also Salmonella and Shigella grow well on it. This permits the concurrent diagnosis of salmonellosis and shigellosis, which may appear in a considerable proportion of the patients in whom cholera was diagnosed on clinical examination. The efficacy of gelatin and tellurite plates often depends on the quality of the chemicals used as well as on the accuracy with which the recommended procedures for their preparation are followed (37).

Colonies suspected of being cholera vibrios are usually picked into Kligler's medium, in which they form acid in the butt, an alkaline slant and, sometimes, small amounts of hydrogen sulfide (40). For further routine tests, fermentation reactions in manitol, sucrose, mannose, arabinose, and lactose, as well as the inoculation of tubes with broth for indole formation and for the Voges-Proskauer test, are recommended. Neither true nor El Tor cholera vibrios ferment arabinose. Lactose decomposition is usually slow. Some strains do not break down manitol (98). The Barritt method has to be employed for the Voges-Proskauer test, which is expected to be positive with El Tor vibrios, but strains which do not produce acetyl methyl carbinol are not rare among El Tor organisms (40, 87, 98). Agglutination tests with sera of high titer, usually performed with the slide technique, complete the basic diagnostic procedure.

Shortcuts in the diagnostic methods have been
reported (see 98). Recently, Finkelstein and Gomez (43) enriched the specimens in alkaline peptone water and agglutinated the organisms with immunoglobulins tagged with fluorescein isothiocyanate. Reactions were prompt but not strictly type-specific. Finkelstein and Gomez (43) also employed the Henry oblique-light technique to identify El Tor colonies on alkaline meat agar (65). The results were excellent. El Tor vibrios, like other vibrios, undergo colony variations quite frequently (98), especially when sulfonamides or antibiotics are given to the patient (93, 115). This rapid method was, therefore, recommended only for use by well-trained laboratory personnel (37).

The lack of hemolysin production by some freshly isolated strains, and the slow development or the total absence of this activity in a number of the El Tor strains, caught some laboratory workers unaware during the present El Tor epidemic (82). The interest that followed the rediscovery of these facts, which are not included in all textbooks, resulted in a reappraisal of the methods used for the detection of hemolysin in tube tests. The classic procedure is that of Greig and Pollitzer (98). Wabha and Takla (119) recommended the use of a 3% suspension of sheep red blood cells. The vibrios are grown on agar slants and washed off with saline. This suspension is standardized to contain $8 \times 10^8$ vibrios per ml. The sheep cell-vibrio mixture is incubated at 37 C for 2 to 4 hr, and then kept in a refrigerator for 4 to 6 hr. Feeley and Pittman (34) subjected the hemolysin test to a thorough study, and found that the composition as well as the pH of the medium influenced greatly the quantity of the hemolysin as well as the length of time during which it could be detected. Brain Heart Infusion broth (pH 7.4) proved to be the most feasible medium in their hands, and 24 hr at 35 C the most favorable incubation time. The test is carried out by incubating 0.5 ml of the growth with 0.5 ml of a 1% suspension of washed sheep red blood cells for 2 hr at 35 to 37 C and then overnight at 4 C.

Of the further differential tests, those of Tanamal, Gispen, and Meyer have been used most frequently (98). In the Tanamal soda-serum reaction (113), a high-titer cholera serum which does not contain a preservative is mixed with an equal part of a 0.3% aqueous solution of Na$_2$CO$_3$. A few drops of a thick emulsion of the organism to be tested are added, and the mixture is incubated at 37 C. True cholera vibrios lose their agglutinability, but the El Tor strains do not.

The Tanamal flocculation reaction (113) is carried out by mixing 0.5 ml of a 0.5% watery NaHCO$_3$ solution with a few drops of the vibrio suspension, then 0.5 ml of a 0.5% aqueous solution of HgCl$_2$ is added. True cholera vibrios show flocculation, but the El Tor organisms do not.

The heat-inactivation test of Gispen (see 98) is based on the observation that heating for 3 hr at 100 C destroyed the agglutinability of true cholera vibrios but not that of the El Tor strains. Meyer (see 98) found that El Tor vibrios, but not true cholera vibrios, remain agglutinable when suspended in 2% chloroform.

These tests were re-evaluated by Mukerjee and Guha Roy (85) and Mukerjee (81, 82) on true cholera vibrios from India and Thailand as well as on El Tor strains from Thailand and from the present El Tor epidemic. The Tanamal soda-serum agglutination test showed a deviation from the expected result in 3.5% of the true and in 2.6% of the El Tor vibrios. This is within the limits of statistical variation. The deviations of the other tests with true cholera and El Tor vibrios were: 10 and 21.8%, respectively, in the sublimate-flocculation test; 30.4 and 0% in the heat-inactivation test; and 36.8 and 0% in the chloroform test. Others also found discrepancies in these tests but to a lesser degree (2, 98).

Wabha and Takla (119) examined the action of metallic ions on vibrios and found that CuSO$_4$ is a useful reagent in the differentiation of these organisms. Their test is carried out by growing the vibrios on agar slants, washing them off with distilled water, and adjusting the resulting emulsion to contain $5 \times 10^8$ vibrios per ml. The suspension is boiled for 15 min, ten drops are added to 2 ml of a 0.25% watery solution of CuSO$_4$, and the mixture is shaken for 5 min. True cholera vibrios show flocculation, while El Tor organisms do not.

Gan and Tjia (55) noted two characteristics which may be useful in the differentiation of vibrios. The first is growth in trypsin broth. A 1:100 trypsin solution is prepared from trypsin (1:250, USP) in distilled water by shaking the mixture for 2 to 3 hr and filtering it through a Seitz filter after overnight standing in a refrig-
ator. This solution can be kept at -20°C for 1 week. Then 1 ml of the trypsin solution and 9 ml of nutrient broth are mixed. The final pH should be 7.0. In the experiments of Gan and Tjia, all of the 241 recently isolated El Tor cultures and 2 of 17 old El Tor cultures, but none of the 27 true cholera and NAG vibrios, formed pellicle and turbidity in this medium.

Gan and Tjia (55) also observed that El Tor and NAG vibrios were not susceptible to 50 μg of polymyxin B in discs on agar plates, whereas true cholera vibrios were inhibited.

Finkelstein and Mukerjee (45) reported that agglutination occurs when agar-grown El Tor vibrios are mixed on a slide with 0.85% saline to form a thick emulsion and with a 2.5% suspension of chicken red blood cells. True cholera vibrios did not give this reaction in the hands of the authors.

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**Table 1. Differential diagnosis of El Tor, “true” cholera, and NAG vibrio strains**

<table>
<thead>
<tr>
<th>Property or test</th>
<th>El Tor vibrios</th>
<th>“True” cholera vibrios</th>
<th>Nonagglutinable vibrios</th>
<th>Reference</th>
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<td>Celebes type</td>
<td>Zam-Zam type</td>
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<td>Variable</td>
</tr>
<tr>
<td>Voges-Proskauer reaction</td>
<td>+†</td>
<td>+†</td>
<td>-</td>
<td>Variable</td>
</tr>
<tr>
<td>Chicken red blood cell agglutination</td>
<td>+</td>
<td>+</td>
<td>0*</td>
<td>?</td>
</tr>
<tr>
<td>Tanamal soda-serum agglutination</td>
<td>+*</td>
<td>+*</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>Wabha and Takla copper sulfate flocculation</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Gan and Tjia trypsin test</td>
<td>+‡</td>
<td>+‡</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gan and Tjia polymyxin B test</td>
<td>Not sensitive</td>
<td>Not sensitive</td>
<td>Sensitive</td>
<td>Not sensitive</td>
</tr>
<tr>
<td>Susceptibility to phage type II of Mukerjee</td>
<td>-</td>
<td>-</td>
<td>Variable</td>
<td>-</td>
</tr>
<tr>
<td>Susceptibility to phage type IV of Mukerjee</td>
<td>-</td>
<td>-</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>Toxin production</td>
<td>Strong</td>
<td>Weak</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>Sayamov test in rabbit gall bladder</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Nonspecific inflammation*</td>
</tr>
<tr>
<td>Human pathogenicity</td>
<td>Cholera</td>
<td>None or very mild</td>
<td>Cholera</td>
<td>None or very mild</td>
</tr>
<tr>
<td>Resistance to environmental factors</td>
<td>Higher*</td>
<td>Higher*</td>
<td>Lower</td>
<td>Higher*</td>
</tr>
</tbody>
</table>

* Most often.
† Frequently.
‡ Recently isolated cultures are +; old stock strains are often -.
placed in bottles with 50 ml of alkaline peptone water and incubated at 35 to 37 C. Since not only true cholera and El Tor vibrios but also other organisms multiplied in this medium, selective plates were streaked from the surface growth of the peptone water bottles after 4, 8, and 24 hr.

Phage-typing has been strongly recommended as a routine measure in the diagnosis of vibrios (37, 82, 83). In small laboratories, the phages can be preserved by absorbing them on filter paper discs according to Watanabe (120). These discs may be kept in a refrigerator for a considerable length of time. (Phages as well as consultation on phage typing are available at the WHO-supported Cholera Phage Typing Centre, Department of Microbiology, Indian Institute for Biochemistry and Experimental Medicine, P27, Prinsep Street, Calcutta 13, India. The director of this activity is S. Mukerjee.) Table 1 shows the most helpful differential diagnostic features of El Tor, true cholera, and NAG vibrios.

IMMUNOLOGICAL TESTS WITH HUMAN SERA

Comments on the technique of agglutination reactions with human sera were published by De et al. (19, 24), Guha Roy and Mukerjee (60), and Vella and Fielding (117). Live organisms were considered generally the most effective agglutinogens. Mercuroic iodide-formaldehyde-treated and antibiotic-killed vibrios were also recommended (117).

Aragon and Famatiga (2) and Briones (see 37) reported that in the Philippine epidemic, which was caused by El Tor vibrios predominantly of the Ogawa subtype, the sera of the patients often agglutinated not only El Tor Ogawa but also true V. comma Ogawa organisms to high titers, while the sera of immunized individuals who received vaccines made from true V. comma Ogawa organisms developed lower titers against El Tor than against true cholera vibrios.

Significant antitoxin titers were found in convalences from true as well as from El Tor cholera (37, 40, 107). Felsenfeld (35) and Morgan et al. (79) developed a method for the determination of the lethal toxin neutralizing power ("antitoxin") of the serum. The supernatant fluid of the vibrios served as the antigen. It was titrated in developing chick embryos and standardized to contain 10 LD50 (for chick embryos) per ml. Doses of 0.1 ml of this antigen were matched against 0.1-ml amounts of the sera to be tested, by use of the Preer technique (99). The movements of the precipitation bands were determined after 3, 7, and 21 days. In sera from El Tor cholera convalescents in the Philippines, the antitoxin levels against El Tor vibrios were not higher than against true cholera vibrios. In the mild El Tor diarrhea in Ubol, fewer persons developed significant titers against the Philippine El Tor toxin than against the true cholera toxin and the Ubol El Tor vibrio toxin. More convalescents from true cholera infections in Thailand had high antitoxin levels in their sera against true cholera vibrio toxin than against El Tor toxin (37, 40). Vaccinated persons did not show antibodies against true cholera toxin in India (107).

Mallik et al. (69) were dubious about the significance of coproantibodies in cholera. Third (116) found that coproantibodies appeared in the absence of serum antibody, that these antibodies were present along the entire gut; and that they declined after injections of epinephrine or cortisone. Freter (46) introduced the Farr test in the titration of coproantibodies. This consists of labeling the antigen with I131 and, after agglutination, precipitating it with ammonium sulfate saturated to 37%. Freter (47) also recommended that epsom salt be given to the examined persons, since coproantibodies begin to decline soon after they are formed. These observations were made with true cholera vibrios, and are recorded here because of their applicability to the study of El Tor infections.

Vibriocidal antibodies and vibriocidal property-inhibiting antibodies were studied by Finkelstein (42). Complement was required for the titration of vibriocidal antibodies. The sera were serially diluted with 1:20 guinea pig complement, and to each tube 2 to 4,000 viable vibrio cells were added. After 1 hr of incubation at 37 C, the tubes were put into an ice bath and plated. The vibriocidal antibody was stable when heated at 56 C for 30 min. It was present also in the serum of about 50% of the nonexposed and not immunized individuals to titers not exceeding 1:100. The titers increased during exposure and after immunization. In the vibriocidal property-inhibiting test, "antigen" (cholera toxin or serum of a patient without vibriocidal property) was serially diluted, and, to each 0.25 ml, 0.25 ml of a serum
with constant (10 to 100 units) vibriocidal property was added. After incubation for 1 hr at 37 C, 0.25 ml of 1:20 complement and 0.25 ml of the vibrio suspension were pipetted into each tube and, after further incubation for 1 hr at 37 C, plates were streaked. The vibriocidal property-inhibiting test was specific for the causative organism but did not allow differentiation between AB (Ôgawa) and ABC (Hikojima) antigens. The authors considered it an indication of the nature of the circulating antibody.

**Vaccination**

Seal (106) concluded that both antitoxin and antibacterial immunity are necessary to prevent cholera. Burrows et al. (4, 5, 6) believed that immunity in cholera is due to several factors. The main protective antibody may be directed against the heat-stable antigen but is not necessarily identical with it. Bacteriolysin, immune opsonin, antibody against the heat-labile exotoxin, and antiendotoxin were listed as other factors of importance. Jenkins and Rowley (64) proved recently that an opsonin-like substance, which is important in the immunology of cholera, is present in the intestines. Thus, an ideal vaccine would be expected to stimulate antibody production against the organisms themselves and numerous other vibrio products.

Felsenfeld (36) carried out protection tests with different vaccines in mice, as well as with sera of vaccinated and convalescent persons. He expressed doubts as to whether a complete cross-immunity exists between true cholera and El Tor infections. Vella (116a) suggested the simultaneous use of vaccines prepared from both true cholera and El Tor vibrios. On the other hand, Pittman and Feeley (97) concluded from their mouse-protection experiments that vaccines prepared from true cholera vibrios protect also against El Tor infections. Watanabe and Verwey (124), however, found that the polysaccharide antigen isolated from El Tor supernatant fluids was highly protective against both El Tor and true cholera vibrios of the homologous subtype. The mouse-protective effect reached its maximum in 10 to 14 days, and persisted at a high level for at least 4 weeks. They recommended, therefore, El Tor vaccine against both true cholera and El Tor vibrio infections.

During the present El Tor epidemic, only one island, the Celebes, reported the use of an El Tor vaccine (37); in other areas, vaccines prepared from true cholera vibrios were employed in 1960 and 1961 (37). Morbidity rates are not available at this date except from the Philippines, where about 20% of the patients with El Tor cholera reportedly had been immunized with a vaccine prepared from true cholera vibrios prior to becoming ill with cholera (37). Reports from Formosa are expected to be published shortly, as well as a comparative study with El Tor and true cholera vibrio vaccines in the Philippines.

**Summary and Conclusions**

The taxonomic position of the El Tor vibrios has not been clarified as yet. Since hemolysis, a textbook characteristic of El Tor strains, may be slow or absent, phage susceptibility is recommended as a criterion in separating El Tor from true cholera vibrios. Additional tests are listed which may be of value in the differential diagnosis of these organisms. The subdivision of El Tor vibrios according to their pathogenicity is of great value for the clinician and the epidemiologist, but may be considered to be less important for the taxonomist. The culture of the El Tor organisms for diagnostic purposes in the laboratory does not present greater problems than that of true cholera vibrios. The trend to use simple and noninhibitory media whenever possible and plates which allow simultaneously the isolation of *Salmonella* and *Shigella* is noted. Much additional research is needed in the analysis of the toxic components of the El Tor vibrios and of their relationship to those present in, or produced by, other vibrios, as well as in the field of the immunological responses in immunized and infected persons. Vaccine field trials under well-controlled conditions should be carried out in the future.

**Literature Cited**


4. Burrows, W. 1957. Studies on immunity to Asiatic cholera. IX. Electrophoretic frac-
82

CHIBRIKOVA, 15.

CHUGH, 13.

DAVIES, J. M. 1962. The cholera outbreak in


