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Cholera

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INTRODUCTION

Cholera is a clinical-epidemiologic syndrome caused by *Vibrio cholerae*, usually of serogroup O1. In its severe form, cholera gravis, the clinical disease is characterized by the passage of voluminous stools of rice water character that rapidly lead to dehydration. Hypovolemic shock, acidosis, and death can ensue in adults, as well as in children, if prompt and appropriate treatment is not initiated. Two distinctive epidemiologic features of cholera are its tendency to appear in explosive outbreaks, often starting in several distinct foci simultaneously, and its propensity to cause true pandemics that progressively affect many countries in multiple continents over the course of many years. Since 1961, the world has been experiencing the seventh pandemic of cholera, the causative organism of which is *V. cholerae* O1 of the El Tor biotype. Beginning in late 1992 in India and then in Bangladesh, there appeared epidemic cholera due to a new serogroup, O139. Preliminary epidemiologic reports documenting the severe nature of disease caused by O139, its rapid spread to neighboring countries, and its ability to cause large explosive outbreaks (45, 53, 354, 357, 442) give testimony that this new serogroup may represent the etiologic agent of a new, eighth pandemic of cholera.

There is considerable debate over the origin of the term cholera and which ancient cultures were touched by the disease (350). One proposition is that the term cholera is derived from the Greek, meaning "bilious," whereas another possible deri-

vation is from the Hebrew, meaning "bad disease." These derivations suggest that cholera may have been known to ancient civilizations in the Mediterranean, in the Middle East, and in the subcontinent. Others contest this and contend that the clinico-epidemiologic syndrome of cholera that includes pandemic spread has been known only since the early 19th century (350).

The final decade of the 20th century has been a hallmark epoch for cholera. In 1991, the seventh pandemic reached the last developing nation populations previously untouched by El Tor cholera, as epidemic cholera appeared in South America (241, 331, 373, 440). This constitutes the return of cholera to this continent after a century of absence. It is ironic that this last extension of the El Tor pandemic resulted in the single largest and most rapidly spreading of all epidemics recorded during the 30-year history of the seventh pandemic. Abruptly and unexpectedly, 23 months later there appeared in the Indian subcontinent a new variant of *V. cholerae* that caused the typical clinical syndrome of cholera and that exhibited the characteristic fulminant epidemic behavior of cholera but that, surprisingly, was found to express surface antigens of a new O serogroup (53).

This review will focus on the clinical microbiology, epidemiology, pathogenesis, and clinical features of cholera. Special attention will be paid to the extraordinary advances that have been made in recent years in unravelling the molecular pathogenesis of this infection and in the development of new generations of vaccines to prevent it.

ISOLATION AND IDENTIFICATION

Nomenclature

The agent responsible for cholera was first described in 1854 in Italy by Pacini, who found a large number of curved bacteria in the intestinal contents of cholera victims which he called *Vibrio cholera* (350). This initial discovery was overshadowed by the work of Robert Koch, who studied cholera in Egypt and demonstrated in 1883 that cholera was caused by this comma-shaped organism (350). Koch named the organism *Kommabazillen* and the subsequent name *Vibrio comma* was used for several decades before the pioneering work of Pacini was recognized and the name was changed to *Vibrio cholerae*.

V. cholerae is a well-defined species on the basis of biochemical tests and DNA homology studies (20). However, this species is not homogeneous with regard to pathogenic potential. Specifically, important distinctions within the species are made on the basis of production of cholera enterotoxin (cholera toxin [CT]), serogroup, and potential for epidemic spread. Until recently, the public health distinction was simple; that is, *V. cholerae* strains of the O1 serogroup which produced CT were associated with epidemic cholera, and all other members of the species either were nonpathogenic or were only occasional pathogens. However, with the recent epidemic of cholera due to strains of the O139 serogroup (see below), such previous distinctions are no longer valid. There are two serogroups, O1 and O139, that have been associated with epidemic disease, but there are also strains of these serogroups which do not produce CT, do not produce cholera, and are not involved in epidemics. Conversely, there are occasional strains of serogroups other than O1 or O139 that are clearly pathogenic, either by the production of CT or other virulence factors (see below); however, none of these other serogroups have caused large epidemics or extensive pandemics. Therefore, in assessing the public health significance of an isolate of *V. cholerae*, there are two critical properties to be determined beyond the biochemical identification of the species *V. cholerae*. The first of these properties is production of CT, which is the toxin responsible for severe, choleralike disease in epidemic and sporadic forms. The second property is possession of the O1 or O139 antigen, which since the actual determinant of epidemic or pandemic potential is not known, is at least a marker of such potential. The major serogroups of *V. cholerae* are discussed below and summarized in Table 1.

TABLE 1. Serogroups, serotypes, biotypes, and CT production by the species *V. cholerae*

Serogroup	CT production (%)	Epidemic spread	Serological subtypes (no.)	Biotypes (no.)
O1	>95 (+)[a]	Yes	3 (Inaba, Ogawa, Hikojima)	2 (classical, El Tor)
O139	>95 (+)	Yes	None	1
O2-O138	>95 (−)	No	None	1

[a] +, positive; −, negative.

***V. cholerae* O1.** *V. cholerae* of the O1 serogroup that produces CT has long been associated with epidemic and pandemic cholera. Some isolates of *V. cholerae* O1 do not produce CT and do not possess the genes encoding CT (212). Environmental strains are usually CT negative and are considered to be nonpathogenic on the basis of volunteer studies (243) (see below). However, CT-negative *V. cholerae* O1 strains have been isolated from occasional cases of diarrhea (293) or extraintestinal infections. This serogroup can be further subdivided into serotypes of the O1 serogroup called Ogawa and Inaba; a third serotype which is rarely isolated is Hikojima (see below). *V. cholerae* O1 can also be divided into two biotypes, classical and El Tor, which are described below. All combinations can be found, i.e., classical strains which are Inaba or Ogawa and El Tor strains which are Inaba or Ogawa.

***V. cholerae* non-O1/non-O139.** In recent years, until the emergence of the O139 serogroup, all isolates that were identified as *V. cholerae* on the basis of biochemical tests but that were negative for the O1 serogroup were referred to as "non-O1 *V. cholerae*." In earlier years, the non-O1 *V. cholerae* were referred to as non-cholera vibrios or nonagglutinable vibrios. In view of the emergence of epidemic O139 disease, one might now refer to O2-O138 as nonepidemic *V. cholerae*. These non-O1/non-O139 strains have been divided into serogroups O2 through O138 on the basis of the lipopolysaccharide (LPS) somatic antigen. The great majority of these strains do not produce CT and are not associated with epidemic diarrhea (reviewed in reference 289). These strains are occasionally isolated from cases of diarrhea (usually associated with consumption of shellfish) and have been isolated from a variety of extraintestinal infections, including wounds, ear, sputum, urine, and cerebrospinal fluid (reviewed in references 181, 288, 289, and 296). They are regularly found in estuarine environments, and infections due to these strains are commonly of environmental origin (289). While the great majority of these strains do not produce CT, some strains may produce other toxins (see below); however, for many strains of *V. cholerae* non-O1/non-O139 isolated from cases of gastroenteritis, the pathogenic mechanisms are unknown.

TABLE 2. Differentiation of *V. cholerae* from related species

Test	Result for given species				
	V. cholerae	*A. hydrophila*	*Plesiomonas shigelloides*	Other *Vibrio* spp.[a]	*Enterobacteriaceae*
Oxidase	+	+	+	+[b]	−
String test	+	−/+[c]	−	+/−	−
Acid from mannitol	+	+	−	+/−	+/−
Acid from sucrose	+	+	−	+/−	+/−
Lysine decarboxylase	+	−	+	+/−	+/−
Arginine dihydrolase	−	+	+	+/−	+
Ornithine decarboxylase	+	−	+	+/−	+/−
Growth in 0% NaCl	+	−	−	−[d]	−
mol% G+C	47–49	58–62	51	38–51	38–60

[a] Vibrio spp. other than *V. cholerae.*
[b] Except for *V. metschnikovii.*
[c] +/−, positive or negative results depending on species or strain.
[d] Except for *V. mimicus.*

***V. cholerae* O139 Bengal.** The simple distinction between *V. cholerae* O1 and *V. cholerae* non-O1 was rendered obsolete in early 1993 when the first reports of a new epidemic of severe, choleralike disease emerging from eastern India and Bangladesh appeared (3). At first, the organism responsible for this outbreak was referred to as non-O1 *V. cholerae* because it did not agglutinate in O1 antisera. However, further investigations revealed that this organism did not belong to the O serogroups previously described for *V. cholerae* but to a new serogroup, which was given the designation O139 and a synonym "Bengal," in recognition of the origin of this strain (399).

The epidemiological features of the O139 serogroup are reviewed below. In general, this organism appears to be a hybrid of the O1 strains and the non-O1 strains. In important virulence characteristics, specifically, cholera enterotoxin and toxin-coregulated pilus (TCP), *V. cholerae* O139 is indistinguishable from typical El Tor *V. cholerae* O1 strains (152, 363). However, this organism does not produce the O1 LPS and lacks at least some of the genetic material necessary for production of the O1 antigen (269). Furthermore, like many strains of non-O1 *V. cholerae* and unlike *V. cholerae* O1, this organism produces a polysaccharide capsule (see below).

Isolation

Specimen collection. Although initiation of treatment for severe, dehydrating diarrhea need not and should not wait for identification of the etiologic agent, subsequent clinical and public health decisions can be greatly influenced by the identification of *V. cholerae* O1 (or O139) in a clinical specimen. Stool samples should be collected early in the illness, preferably before the initiation of antibiotic therapy. Samples can be collected satisfactorily from sterile bedpans or comparable containers, but in epidemic situations collection of specimens from bedpans may yield false-positives due to contamination by previous use or false-negatives due to the presence of residual disinfectants. Reasonable recovery rates are also possible with rectal swabs.

When processing of specimens is delayed, the stool sample or swabs can be placed in Cary-Blair transport medium; viability of *V. cholerae* for up to 4 weeks in this medium has been reported (218). Two broth media used for enrichment of *V. cholerae* can also be used for transport. Alkaline peptone water and tellurite-taurocholate-peptone broth have both been successfully employed for transport of specimens, although prolonged holding in alkaline peptone water at ambient temperatures may allow *Pseudomonas* spp. and non-O1 *V. cholerae* to overgrow *V. cholerae* O1 (330). The use of other transport media, such as buffered glycerol-saline, may lead to poor recovery of *V. cholerae* (218). Strips of filter paper soaked in liquid stool and sealed in airtight plastic bags have also been used to retain viable *V. cholerae* for up to 5 weeks (218). Vibrios are more susceptible to refrigeration than enteric bacilli, and so specimens in transport media should be shipped to the laboratory without refrigeration.

Culture media. In many specimens, the concentration of *V. cholerae* is so high (10^7 to 10^8 per ml of liquid feces) that enrichment is unnecessary. However, enrichment broth is commonly used to recover low levels of vibrios, particularly from formed stools. Alkaline peptone water is the most commonly used enrichment broth (330); the pH of this medium can range from 8.4 up to 9.2, thus taking advantage of the ability of *V. cholerae* to multiply at alkaline pH. Enrichment lasts for only 6 to 8 h, as incubation in alkaline peptone water for longer than 8 h may result in overgrowth of other organisms. Less commonly used enrichment media include alkaline peptone water with tellurite (330), Monsur's tellurite-taurocholate broth (287), and sodium-gelatin phosphate broth (362).

Examination of food and water samples for *V. cholerae* nearly always utilizes one if not two enrichment steps. When a second enrichment is used to avoid overgrowth of other organisms in the initial broth, alkaline peptone is used first for no more than 8 h followed by enrichment in a more selective broth such as Monsur's tellurite-taurocholate medium (386).

The most commonly used plating medium for *V. cholerae* is thiosulfate-citrate-bile salts-sucrose (TCBS) agar, which is available from several commercial sources. The sucrose-fermenting *V. cholerae* isolates are readily detected on this medium as large, yellow, smooth colonies. Additional selective media (reviewed in references 330 and 386) have been developed for isolation of *V. cholerae* and include Monsur's tellurite-taurocholate gelatin agar (287), vibrio agar (455), and polymyxin-mannose-tellurite agar (401), but these are not commercially available. Most selective media commonly used for isolation of members of the family *Enterobacteriaceae* are not suitable for isolation of *V. cholerae*, but some success has been reported with MacConkey agar (218). Nonselective media such as blood agar plates are excellent for the growth of *V. cholerae*, but with such media overgrowth by unwanted species is potentially a problem.

Identification

Description. *Vibrio* spp. are classified in the family *Vibrionaceae* along with *Aeromonas*, *Phobacterium*, and *Plesiomonas* spp. These genera can be distinguished by a variety of tests as shown in Table 2. Like other *Vibrio* species, *V. cholerae* is a facultatively anaerobic, asporogenous, gram-negative rod that is capable of respiratory and fermentative metabolism. *V. cholerae* is oxidase positive, reduces nitrate, and is motile by a single polar, sheathed flagellum. Growth of *V. cholerae* O1 is inhibited by the compound O/129 (2,4-diamino-6,7-diisopropylpteridine), but resistance to this compound is increasingly found, usually in association with resistance to trimethoprim-sulfamethoxazole (313, 359). *V. cholerae* O139 isolates are resistant to O/129 (3).

Like growth of other *Vibrio* species, that of *V. cholerae* is stimulated by the addition of 1% NaCl. However, an important distinction from other *Vibrio* species is the ability of *V. cholerae* to grow in nutrient broth without added NaCl. *V. mimicus* can also grow under these conditions but can be readily distinguished from *V. cholerae* by lack of sucrose fermentation. *V. mimicus* was formerly classified as *V. cholerae* Heiberg group V, a typing scheme for *V. cholerae* based on sugar fermentations pattern which is now infrequently used (161). Some strains of *V. mimicus* produce cholera enterotoxin and have been associated with sporadic cases of diarrhea (288).

Biochemical and serological identification. Suspected *V. cholerae* isolates can be transferred from primary isolation plates to a standard series of biochemical media used for identification of members of the *Enterobacteriaceae* and *Vibrionaceae* families. Both conventional tube tests and commercially available enteric identification systems are suitable for identifying this species. Several key characteristics for distinguishing *V. cholerae* from other species are given in Table 2, and a more complete summary of biochemical tests for *V. cholerae* is given in Table 3. A crucial test for distinguishing *V. cholerae* from members of the *Enterobacteriaceae* is the oxidase test; *V. cholerae* gives a positive test. The use of colonies obtained directly from TCBS agar may give erroneous oxidase results; therefore, yellow colonies from TCBS should be subcultured by heavy inoculation onto a nonselective medium such as blood agar, allowed to grow for 5 to 8 h, and then tested for oxidase (218).

The key confirmation for identification of *V. cholerae* O1 is agglutination in polyvalent antisera raised against the O1 antigen. As with the oxidase test, more satisfactory results for serological testing are found with colonies picked from nonselective media compared with those picked from TCBS (218). Polyvalent antiserum for *V. cholerae* O1 is commercially available and can be used in slide agglutination or coagglutination tests (355). A monoclonal antibody-based, coagglutination test suitable for testing isolated colonies or diarrheal stool samples has recently become available commercially (Cholera SMART; New Horizons Diagnostics Corp., Columbia, Md.) (see below). Oxidase-positive organisms that agglutinate in O1 antisera can be reported presumptively as *V. cholerae* O1 and then forwarded to a public health reference laboratory for confirmation (218).

Antiserum against O139 is now available in many reference laboratories and should soon be commercially available. Antisera against the other serogroups of *V. cholerae* are not commercially available as of this writing. The serological classification of *V. cholerae* is discussed in greater detail below.

In many developing countries, simpler identification schemes are employed for identification of *V. cholerae*. One such scheme involves inoculating suspected *V. cholerae* colonies from the isolation plate into Kligler iron agar (KIA) medium. Cultures yielding an alkaline slant over acid butt with no gas or H_2S are then tested for oxidase activity and reactivity with O1 or O139 antisera, using growth taken from the KIA slant.

TABLE 3. Biochemical and other characteristics of *V. cholerae*[a]

Test	% Positive
Indole production	99
Methyl red	99
Voges-Proskauer	75
Citrate, Simmons	97
H_2S on triple sugar iron	0
Urea hydrolysis	0
Phenylalanine deaminase	0
Arginine, Moeller	0
Lysine, Moeller	99
Ornithine, Moeller	99
Motility (36°C)	99
Gelatin hydrolysis (1% NaCl, 22°C)	90
KCN test (% that grow)	10
Malonate utilization	1
D-Glucose, acid production	100
D-Glucose, gas production	0
Acid production from:	
D-Adonitol	0
L-Arabinose	0
D-Arabitol	0
Cellobiose	8
Dulcitol	0
Erythritol	0
D-Galactose	90
Glycerol	30
myo-Inositol	0
Lactose	7
Maltose	99
D-Mannitol	99
D-Mannose	78
Melibiose	1
α-Methyl-D-glucoside	0
Raffinose	0
L-Rhamnose	0
Salicin	1
D-Sorbitol	1
Sucrose	100
Trehalose	99
D-Xylose	0
Mucate, acid production	1
Tartrate, Jordan	75
Esculin hydrolysis	0
Acetate utilization	92
Nitrate→nitrite	99
Oxidase	100
DNase	93
Lipase (corn oil)	92
ONGP test[b]	94
Tyrosine clearing	13
Growth in nutrient broth with:	
0% NaCl	100
1% NaCl	100
6% NaCl	53
8% NaCl	1
10% NaCl	00
String test	100
Polymyxin B, zone of inhibition	22

[a] Adapted from reference 218 with permission of the publisher.
[b] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

DNA probes and PCR techniques. Nucleic acid probes are not routinely employed for the identification of *V. cholerae*

because of the ease of identifying this species by conventional methods. DNA probes have been extremely useful in distinguishing those strains of *V. cholerae* that contain genes encoding CT (*ctx*) from those that do not contain these genes. This distinction is particularly important in examining environmental isolates of *V. cholerae* since the great majority of these strains lack *ctx* sequences. The first application of DNA probes to distinguish toxigenic from nontoxigenic strains utilized sequences encoding the closely related heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) (212). This first study in 1981 answered a crucial public health question about environmental strains isolated in non-cholera-endemic countries which did not express CT detectable by conventional assays. The public health concern was that these strains might contain minor mutations in genes encoding CT and therefore might possess the genetic potential to revert to toxigenicity. The DNA hybridization studies with the LT probe showed that strains did not contain toxin gene sequences and therefore could not revert to toxigenicity.

A number of DNA fragment probes and synthetic oligonucleotide probes have been developed to detect *ctx* sequences (reviewed in reference 353). Two synthetic oligonucleotide probes employing nonisotopic labels have been used to identify toxigenic *V. cholerae* O1 directly from primary isolation plates. An alkaline phosphatase-labeled *ctx* probe described by Wright et al. (479) was used to screen colonies grown on nonselective media inoculated with stool samples from volunteers experimentally infected with toxigenic *V. cholerae* O1. This probe successfully detected all stool samples with $>10^3$ *V. cholerae* organisms per g of stool. Another oligonucleotide probe labeled with alkaline phosphatase was used by Yoh et al. (490) to detect *ctx* genes in a stool sample from a patient recently returning from a cholera-endemic country. The time from picking the colonies from TCBS agar to final hybridization results was just 3 h.

The PCR technique has also been used to detect *ctx* sequences. The *ctx* genes are highly conserved among strains of *V. cholerae* (see below), and therefore sequence variation which might lead to poor annealing of the oligo primers does not seem to be a problem. Shirai et al. (403) reported a set of primers that amplified a 302-bp region and could detect as little as 1 pg of DNA or three viable cells from broth cultures after 40 cycles of amplification. These investigators also examined stool samples from patients with secretory diarrhea and detected *ctx* sequences by PCR in all samples that were positive for *V. cholerae* O1 by culture techniques. Stool samples may sometimes contain substances that can be inhibitory to the PCR assay, but the rice water stools from the cholera patients did not appear to inhibit this reaction (403). A recent study of 123 hospitalized patients in Calcutta compared *ctx*-PCR with CT-bead enzyme-linked immunosorbent assay (ELISA) and culture techniques (358). The PCR technique was more sensitive, yielding positive results with 94 specimens examined versus 81 samples that were positive with either culture or bead ELISA techniques. Other investigators have used PCR to detect *ctx* sequences in isolated strains from the Latin American cholera epidemic (105, 466).

The PCR technique has also been used to detect toxigenic *V. cholerae* O1 in food samples. Koch et al. (227) examined fruit, vegetables, and shellfish which had been seeded with *V. cholerae* O1. Amplification of the desired fragment was readily observed with fruit and vegetable samples, but seeded shellfish homogenates often inhibited the PCR assay. By diluting the shellfish homogenate to 1%, the desired fragment was readily amplified. PCR was used to investigate a small outbreak of imported cholera in New York in 1991 (353). Crabs brought in from Ecuador were incriminated in the outbreak but did not yield *V. cholerae* O1 after culture. However, the *ctx*-PCR technique detected *ctx* sequences in one of four crab samples examined.

Direct specimen examination. Although the usual method to diagnose *V. cholerae* O1 employs isolation on bacteriologic media, several techniques which detect the organism directly in stool samples have been reported. Detection of *V. cholerae* O1 in stool samples by DNA probes and PCR techniques is reviewed above. Among nongenetic approaches, examination of cholera stools by dark-field or phase-contrast microscopy often shows the highly motile vibrios darting through the field, particularly when the concentrations of vibrios are $>10^5$ per ml of stool. Addition of specific polyclonal (146) or monoclonal (151) antibodies directed against the O1 antigen can result in inhibition of motility, thereby identifying *V. cholerae* O1 in 2 to 5 min in about 50% of the cases (146). While this technique can be very valuable in areas of cholera endemicity, the sensitivity and specificity of the assay are highly dependent on the skill of the technician performing the test. A fluorescent-antibody technique using O1 antisera has also been used to identify *V. cholerae* O1 in stool specimens (111, 380) but is not widely used since it requires expensive equipment and large numbers of organisms (10^6 to 10^7/ml) (330). The fluorescent-antibody technique has also been used to identify *V. cholerae* O1 in water samples (33).

Rapid diagnosis of *V. cholerae* O1 has been achieved by coagglutination tests utilizing antibodies against the O1 CT B-subunit antigens (191, 355). A bead ELISA for diagnosis of CT has been shown to detect the toxin directly from stool specimens, yielding positive results in 85% of culture-positive specimens (356). A coagglutination test using monoclonal antibodies against the O1 antigen has recently become available commercially (CholeraScreen; New Horizons Diagnostics Corp.). This test takes only 5 min to complete and has been reported to be highly sensitive and specific in field trials in Guatemala and Bangladesh (64). In Guatemala, stool specimens from 17 patients were examined, and 10 samples were positive for *V. cholerae* O1 by both conventional culture and CholeraScreen. An additional four culture-negative specimens were positive with the coaglutination test, and patient sera available for two of these specimens showed immunologic evidence of infection with *V. cholerae* O1. In Bangladesh, 55 of 77 specimens were positive with CholeraScreen compared with 49 culture-positive specimens; the six culture-negative specimens that were positive with this test were all positive with a direct fluorescent-antibody test (64). This test was recently used to detect *V. cholerae* in stool samples from airline passengers on a flight from South America to Los Angeles (1). The authors found that the coagglutination test was nearly as sensitive as culture techniques (17 CholeraScreen-positive versus 19 culture-positive stool samples) and much more rapid. This kit has also been evaluated in volunteer studies in which culture techniques proved to be slightly more sensitive but not nearly as rapid as the coagglutination test. Evaluation of stool specimens from volunteers experimentally infected with *V. cholerae* O1 showed that the sensitivity of the coagglutination test was 82% for specimens containing less than 10^4 CFU of *V. cholerae* per gram and 89% for specimens with greater than 10^4 CFU/g (312).

A further modification of the CholeraScreen kit is the Cholera SMART kit (New Horizons Diagnostics Corp.), which is a colloidal gold-based colorimetric immunoassay (156). The O1 antigen present in a specimen is captured and concentrated on a solid-phase matrix and appears to the naked eye as a pink-to-red spot resulting from the deposition of colloidal

gold. Field trials of this kit in Mexico showed 100% agreement between these two kits (156). Similar kits to detect strains bearing the O139 antigen are now being developed (156).

Toxin Assays

A crucial distinction to be made in identifying *V. cholerae* is the determination of whether the strain produces CT. Initial nonanimal assays to detect CT involved cell culture assays employing Chinese hamster ovary (CHO) cells (150) or Y-1 adrenal cells (88). These assays, which detect a characteristic change in cell morphology induced by CT, are available only in reference and research laboratories. Various modifications of an ELISA using purified G_{M1} ganglioside receptor as the capture molecule (435) are now more commonly used to assay CT. A highly sensitive bead ELISA, which uses polystyrene beads coated with anti-CT antibody as the solid phase, can detect picogram quantities of CT in culture supernatants and can detect CT directly in stool specimens (356). A latex agglutination assay to detect CT was reported to be less complicated and less time-consuming than the ELISA, with excellent sensitivity and specificity (0.97 and 1.00, respectively) compared with an ELISA (7). Additional methods to detect CT have recently been reviewed (307).

In addition to the above assays for detecting the phenotypic expression of CT, a number of DNA probes and PCR methods have been developed to detect the *ctx* genes (see above). Such genetic methods are particularly suitable for screening large number of isolates and may ultimately replace the above phenotypic assays in all but the most specialized research laboratories.

Antibiotic Susceptibility

Although the most important treatment for cholera is prompt replacement of fluids and electrolytes, antibiotics can help shorten the course of disease and diminish markedly the volume of rehydration fluids that must be administered (see below). Since antibiotic resistance of *V. cholerae* is not uncommon in some geographic areas (e.g., East Africa) and some outbreaks elsewhere have yielded resistant strains, testing of clinical isolates is definitely indicated. Antibiotic susceptibility testing for *V. cholerae* isolates is performed with methods employed for other gram-negative enteric bacteria. Mueller-Hinton agar and broth support the growth of *V. cholerae*, and standardized disk diffusion or dilution susceptibility testing methods are suitable for this species.

Subtyping

Serotyping. The major surface antigen employed in characterization of *V. cholerae* is the O antigen. The O antigen is heat stable and is composed of a homopolymer containing the amino acid sugar D-perosamine (4-amino-4,6-dideoxy-D-mannose) in which the amino groups are acylated by 3-deoxy-L-glycero-tetronic acid (219, 269, 360). The different O groups are referred to as serogroups or serovars (386). The rough (R) antigen of *V. cholerae* is identical throughout the species (400). The R form is difficult to distinguish from the smooth (S) form by colony morphology alone, but it can be identified by using R antiserum (386). A flagellar (H) antigen is also present, but the value of this antigen for species identification is limited due to the presence of common H epitopes among all *Vibrio* species (402, 409). Unlike other enteric species for which the term serotype usually signifies an assortment of O and H antigens, for *V. cholerae* it usually refers to different antigenic forms of the O1 antigen (see below).

TABLE 4. Differentiation of classical and El Tor biotypes of *V. cholerae* O1

Test or property	Result for biotype:	
	Classical	El Tor
Hemolysis	−	(+)
Agglutination of chicken erythrocytes	−	+
Voges-Proskauer	−	+
Inhibition by polymyxin B (50-U disk)	+	−
Lysis by		
Classical IV bacteriophage (305)	+	−
FK bacteriophage (451)	+	−

There are multiple classification systems for the O antigen of *V. cholerae*. The most widely used system is the typing scheme of Sakazaki and Shimada (387), which uses sera raised against heat-killed organisms. This scheme is made up of 138 different O groups to which the newly described O139 group was recently added (399). Another system in use in the United States is that of Smith (414), employing sera raised against live organisms. Comparison of the two systems shows significant overlap, but many areas of disagreement are found (34, 406). A third system has been developed by Siebeling et al. (406), using methodology similar to that of Sakazaki and Shimada. A common feature of all of these systems is the use of the O1 serogroup to signify the serogroup of *V. cholerae* traditionally associated with epidemic cholera. Antisera for O groups other than O1 and O139 are available only from reference laboratories and have limited distribution.

The O1 serogroup is divided into three antigenic forms called Inaba, Ogawa, and Hikojima. These antigenic forms are referred to as serotypes or subtypes, depending on the reference. The O antigen of *V. cholerae* O1 consists of three factors designated A, B, and C; the A factor may be the D-perosamine homopolymer, but the nature of the B and C factors is unknown (428). The differences among the subtypes is largely quantitative; Ogawa strains produce the A and B antigens and a small amount of C, while Inaba strains produce only the A and C antigens (386). Specific Inaba and Ogawa sera are prepared by absorption with the other subtype. The Hikojima subtype contains all three factors, thereby reacting with both Inaba and Ogawa antisera. The Hikojima subtype is rare and unstable and is not recognized by some authorities who would report cultures as Inaba or Ogawa, depending on which serum causes the strongest reaction (218).

V. cholerae O1 strains have been shown to shift between Inaba and Ogawa (382). This interconversion is usually irreversible in the laboratory and occurs more frequently in the direction of Ogawa to Inaba (269). DNA sequence analysis of genes (*rfb*) encoding the O1 antigen reveals that the sequences for Inaba and Ogawa antigens are nearly identical (428). The shift from Inaba to Ogawa can result from a variety of changes, even from a single base change in the *rfbT* gene which creates a premature stop codon in this gene. Inaba strains are thus *rfbT* mutants, and the conversion from Ogawa to Inaba can be due to selective pressure from the anti-Ogawa immune response engendered during infection (428). Conversions from Ogawa to Inaba have also been shown to involve alterations to the *rfbT* gene (186). A recent study of the serotype shift indicated that one Ogawa strain may have arisen from two mutations: one deletion mutation producing the initial Ogawa to Inaba shift; and a second insertion mutation which restored the original *rfbT* reading frame, thereby restoring the Ogawa serotype (104).

Biotyping. *V. cholerae* O1 strains have historically been divided into two biotypes, classical and El Tor. Isolates from the sixth pandemic were nearly exclusively of the classical biotype. Until the seventh pandemic, isolates of the El Tor biotype (named after the El Tor quarantine station in the Sinai, where it was first isolated in 1905) were associated only with sporadic diarrhea (350). However, the vast majority of isolates of the seventh pandemic have been of the El Tor biotype. Indeed, in recent years, the only country where classical strains have been isolated has been Bangladesh (405).

The characteristics used to distinguish the classical and El Tor biotypes are given in Table 4. Hemolysis of sheep erythrocytes is of limited value since, except for isolates from the early 1960s, most isolates of *V. cholerae* El Tor are nonhemolytic (15) (see below). Notable exceptions to the recent trend of nonhemolytic El Tor strains are the U.S. Gulf Coast isolates, which are strongly hemolytic (15). Among other criteria, biotyping by bacteriophage susceptibility is limited to a very few reference centers, leaving the Voges-Proskauer reaction, polymyxin B susceptibility, and hemagglutination of chicken erythrocytes as the most useful characteristics for biotyping. A newly described technique for biotyping *V. cholerae* called the CAMP test (239) utilizes synergistic hemolysis in the presence of *Staphylococcus aureus* beta-hemolysin and an uncharacterized factor of *V. cholerae* which is apparently unrelated to the El Tor hemolysin/cytolysin. By stab inoculating *V. cholerae* and *S. aureus* ca. 8 mm apart on a blood agar plate, hemolytic zones of different sizes were observed, and the size of the zone correlated with the biotype of the *V. cholerae* isolate. In addition to phenotypic assays, genetic methods have also been used to distinguish classical and El Tor biotypes. One approach utilizes an oligonucleotide probe derived from sequences within the gene encoding the hemolysin to differentiate between the biotypes (5). A second approach to biotyping has recently been described; it utilizes differences in DNA sequence between genes encoding the TCP from classical and El Tor strains (217). By using multiplex PCR with primers derived from the *tcpA* gene sequence, classical strains gave amplicons of different sizes from those seen with El Tor isolates. Examination of restriction fragment length polymorphisms (RFLPs) in rRNA genes has also been reported to distinguish classical and El Tor strains (224, 352) (see below).

Phage typing. For many years, the primary technique used to differentiate *V. cholerae* O1 strains of the same biotype and serotype was bacteriophage typing. However, the use of this technique is limited by the availability of a set of well-characterized *Vibrio* phages, thus restricting it to a very small number of reference and research laboratories in the world. The use of bacteriophage for typing different isolates is further hampered by the lack of a consensus typing scheme, a situation that has recently been reviewed (379). The multiple typing schemes are also limited by the fact that two or three phage types account for up to 80% of all *V. cholerae* O1 strains examined (379). Recently, a new typing scheme was reported by Chattopadhyay et al. (51) which uses 10 typing phage. This new scheme separated 1,000 *V. cholerae* strains into 146 phage types.

As noted above, bacteriophage typing has been used to distinguish the classical and El Tor biotypes; the typing phage of Murkejee (305) and, recently, the FK phage of Takeya et al. (451) have been used primarily for this purpose. One interesting application of bacteriophage typing is the use of lysogenic phage as DNA hybridization probes to distinguish strains on the basis of chromosomal integration sites. This technique was used to confirm other studies showing that the U.S. Gulf Coast isolates are clonal and furthermore showed that some nontoxigenic strains from the Gulf Coast are highly related to the toxigenic strains isolated from patients in this area (144).

***ctx* gene variation.** Variations in genes encoding CT (*ctx*) and in flanking DNA sequences have yielded significant insights into the molecular epidemiology of *V. cholerae*. Kaper et al. (206, 213) examined RFLPs in the *ctx* locus to study the relationship among *V. cholerae* strains isolated in the U.S. Gulf Coast. Enterotoxigenic strains isolated from Texas in 1973 and Louisiana in 1978 to 1981 were shown to possess two small *Hin*dIII restriction fragments of 6 and 7 kb which contained *ctx* gene sequences. This pattern was not seen in any strains isolated from other countries and demonstrated the clonal nature of the U.S. Gulf Coast isolates. This approach subsequently showed that a *V. cholerae* O1 isolate from a cholera patient in Maryland was identical to isolates from Louisiana and Texas (257). These results concurred with epidemiological investigations showing that the crabs eaten by the Maryland patient were harvested along the Texas coast (257). In contrast to the highly conserved O1 isolates, toxigenic isolates of *V. cholerae* non-O1 and *V. mimicus* isolated from this area showed greater divergence (213). RFLPs of *ctx* have also been used to examine isolates of *V. cholerae* from the sixth pandemic (69), Australia (81), Hong Kong (481), Africa (106), and Latin America (468).

RFLP analysis using *ctx* gene probes primarily detects sequence divergence in genes flanking the *ctx* locus. Olsvik and colleagues (325) have used automated sequencing of PCR-generated amplicons to examine sequence divergence within the structural gene for the CT B subunit. The *ctxB* sequences were found to be highly conserved (99%) and could be divided into three classes or genotypes. Genotype 1 was found in strains of the classical biotype and El Tor strains from the U.S. Gulf Coast. Genotype 2 was found in El Tor strains from Australia, and genotype 3 was found in strains from the seventh pandemic and the recent Latin American outbreak.

MEE. Multilocus enzyme electrophoresis (MEE) analysis (or zymovar analysis) has been applied to many bacterial species to study divergence among bacterial isolates of the same species (394). Variations in electrophoretic mobility of several enzymes have been found for *V. cholerae* strains which can divide the species into multiple electrophoretic types (ETs) and distinguish classical and El Tor strains (286, 469). This technique was used to show that all strains of *V. cholerae* O1 isolated from humans in Australia belonged to the same ET, regardless of the ability to produce CT (80). In contrast, the environmental O1 isolates and the non-O1 strains belonged to a wide variety of ETs. In the most extensive study to date, Wachsmuth et al. (468) used 16 different enzymes and derived six different ETs among 197 El Tor isolates of *V. cholerae* O1. ET1 contained toxigenic isolates from Australia and was most closely related to ET2, which contained toxigenic isolates from the U.S. Gulf Coast and some nontoxigenic clinical and environmental isolates from the United States and Latin America. ET3 contained strains of the seventh pandemic from Asia and Africa and differed in only one locus from ET4, which contained isolates from the recent Latin America outbreak. ET5 and ET6 contained nontoxigenic isolates from Mexico and Brazil, respectively. Thus, while MEE may not be useful in distinguishing strains within a single outbreak, this technique appears to be quite useful in investigating the origin of new outbreaks of disease.

rRNA gene variation. RFLPs of genes encoding rRNA have also been employed to study divergence among *V. cholerae* isolates (224, 469). In the study by Wachsmuth et al. (468), rRNA RFLP analysis yielded greater diversity among the *V. cholerae* El Tor isolates studied than did the MEE technique.

The 197 isolates that gave six ETs gave 13 different patterns when analyzed for rRNA RFLPs. Both techniques showed that strains of the Latin America outbreak are clonal. However, whereas MEE analysis showed that strains of the seventh pandemic from Asia and Africa were clonal and belonged to a single ET, rRNA RFLP analysis showed that these strains were diverse and belonged to five different classes. These authors hypothesized that the observed differences were due to a higher mutation rate in the DNA sequences flanking rRNA genes than in genes encoding the "housekeeping" enzymes studied with MEE. Popovic and colleagues (352) have recently proposed a standardized ribotyping scheme for *V. cholerae*. In a study of 214 human and environmental isolates from 35 countries and 14 U.S. states collected over the past 60 years, the authors digested *V. cholerae* DNA with the restriction enzyme *Bgl*I and hybridized the digested DNA with *E. coli* 16S and 23S rRNA. The resulting rRNA RFLPs were grouped into seven different ribotypes among classical strains and 20 ribotypes and subtypes among El Tor strains. Ribotyping has also recently demonstrated the existence of multiple clones of classical *V. cholerae* in Bangladesh (96). The conclusion to be drawn from these studies is that when maximum divergence among *V. cholerae* isolates is sought, as in epidemiological investigations, rRNA RFLP analysis is preferable to other subtyping techniques that have been used for this species.

Serological Tests for Retrospective Diagnosis

Serological assays have been very useful in retrospectively diagnosing cholera infections. The various components of the immune response to infection with *V. cholerae* are discussed below, but for the purposes of retrospective diagnosis and seroepidemiology, there are two main serological assays, the vibriocidal antibody and the cholera antitoxin immune response. Older serological tests for retrospective diagnosis of cholera have been reviewed elsewhere (330). Due to high background levels of immunity in areas where cholera is endemic, the interpretation of antibody titers differs between cholera-endemic and non-cholera-endemic areas (138).

Studies of immune responses after experimental challenge of volunteers in a non-cholera-endemic area have given the clearest guidance for appropriate immune responses to use for retrospective diagnosis (61, 255, 256, 376). The great majority of individuals develop both vibriocidal and antitoxic immune responses after infection with *V. cholerae* O1, and nearly all seroconversions are seen by day 10 postchallenge (61). Seroconversions occurred less often in volunteers with inapparent infection than in individuals with diarrhea. For individuals in countries where cholera is not endemic, levels of vibriocidal antibodies return to baseline in 1 to 6 months after infection, while antitoxin titers diminish in 1 to 2 years but do not return to baseline (61, 255, 256, 417). Because of elevated vibriocidal antibody levels seen in some individuals even in non-cholera-endemic countries, the use of paired acute- and convalescent-phase sera is more helpful than a single serum sample (61). Even with a single serum sample from a patient in an area where cholera is not endemic, an antitoxin level by immunoglobulin G (IgG) ELISA of fourfold or greater than the negative control serum and a titer of vibriocidal antibody $\geq$1,280 are strong evidence for a recent infection with *V. cholerae* O1 (61). Initial volunteer studies with *V. cholerae* O139 indicate that this serogroup produces an antitoxic response but little or no homologous vibriocidal response (292).

TABLE 5. Clinical response of healthy North American volunteers to various doses of *V. cholerae* El Tor Inaba strain N16961[a]

Dose[b]	Clinical attack rate	Mean incubation (h)	Mean diarrheal stool vol per ill volunteer (range)	Mean no. of loose stools per ill volunteer (range)
10^6	9/10[c]	25.5	3.2 liters (0.4–13.1)	12.9 (2–39)
10^5	3/5	18	3.1 (0.4–3.7)	15 (9–21)
10^4	4/5	36.5	1.1 (0.6–1.5)	6.5 (4–10)
10^3	4/6	33.3	0.9 (0.4–1.9)	5.8

[a] From reference 245 with permission of the publisher.
[b] Volunteers ingested 2 g of sodium bicarbonate prior to ingesting inoculum.
[c] Number ill/number of volunteers challenged.

PATHOGENESIS AND VIRULENCE FACTORS

The major features of the pathogenesis of cholera are well established. Infection due to *V. cholerae* begins with the ingestion of contaminated food or water containing the organism. After passage through the acid barrier of the stomach, the vibrio colonizes the epithelium of the small intestine by means of the TCP and other factors. Cholera enterotoxin produced by the adherent vibrios (and possibly other toxins) disrupts ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes leads to the severe diarrhea characteristic of cholera.

Although the major features of the pathogenesis of *V. cholerae* are well established, there are still significant questions which are unanswered for several aspects of the disease process. The specific details of the major steps in pathogenesis as well as several unanswered questions are reviewed below.

Models of Pathogenesis

Volunteer studies. Volunteer challenge studies with *V. cholerae* have been extremely useful in studying many aspects of cholera. These studies, in which consenting informed adults are experimentally infected with *V. cholerae* under quarantine conditions, have yielded many insights into pathogenesis and host immune response. Early studies by Cash et al. (39) established that in fasting North American volunteers, approximately 10^{11} *V. cholerae* organisms were required to induce diarrhea unless $NaHCO_3$ was administered to neutralize gastric acid. When 2.0 g of $NaHCO_3$ is concomitantly administered, 10^6 vibrios can induce diarrhea in 90% of volunteers (39, 245). Further studies by Levine et al. (245) demonstrated that most volunteers who receive as few as 10^3 to 10^4 organisms with buffer develop diarrhea, although lower inocula correlated with a longer incubation period and decreased stool volumes (i.e., diminished severity) (Table 5). In addition to gastric acidity, other host factors, as yet poorly defined, play a role in disease susceptibility. The effect of other host factors is illustrated by a study in which the identical inoculum that caused 44 liters of diarrhea in one volunteer caused little or no illness in other individuals (240). One correlation with increased host susceptibility is blood type. Individuals of blood group O have more severe disease than those of other blood groups, but the mechanism responsible for this difference is not known (55, 136, 252, 448). The various factors affecting host susceptibility have recently been reviewed (370).

The crucial role of CT in disease was clearly shown by Levine et al. (248), who fed purified CT to volunteers. Ingestion of 25 μg of pure CT (administered with cimetidine and $NaHCO_3$ to diminish gastric acidity) caused over 20 liters of rice water stool, and ingestion of as little as 5 μg of pure CT

resulted in 1 to 6 liters of diarrhea in five of six volunteers. These studies suggested that the severe purging characteristic of cholera was due to a single toxin (248). However, later studies with genetically engineered *V. cholerae* strains specifically deleted for *ctx* genes encoding one or both subunits of CT demonstrated that milder diarrhea could result even in the absence of CT (250, 397) (see below). Thus, although CT is responsible for the profuse diarrhea of cholera, there are still additional secretogenic factors expressed by *V. cholerae*.

Other aspects of the pathogenesis of cholera have been answered by using the volunteer model. The essential roles of the TCP colonization factor and the ToxR regulatory system in virulence were demonstrated in volunteer studies conducted by Herrington et al. (164) (see below). Conversely, volunteer studies also demonstrated that *V. cholerae* O1 strains isolated from sewage water in Brazil which lacked genes encoding CT and the TCP colonization factor could not colonize the intestine well, cause disease, or elicit strong vibriocidal responses (243). Finally, volunteer studies have played a crucial role in evaluating host immune response and vaccine efficacy, as reviewed below.

Animal studies. A plethora of animal models has been developed to study cholera (reviewed in references 371 and 383). Despite the fundamental limitation that natural infection with *V. cholerae* O1 does not occur in animals, a few of these models have yielded useful information relevant to human disease.

A basic technique used to study the secretogenic response to CT is the rabbit ligated ileal loop, first developed by De and colleagues (75) in India. Another model with an occluded intestinal tract is the sealed adult mouse model of Richardson et al. (372), in which the ano-rectal canal is sealed with cyanoacrylate ester glue. Unoccluded infant mice have also been used to study diarrhea induced by *V. cholerae* strains or CT (18) and are particularly useful in studying the colonization potential of strains in competitive colonization assays (123). The infant mouse model, like the infant rabbit model of Dutta et al. (91), is limited by the fact that the animals are susceptible to infection only for a relatively short time after birth.

The most widely used adult intact animal model is the RITARD (removable intestinal tie-adult rabbit diarrhea) model of Spira et al. (423), which uses a temporary slip knot tie of the small bowel that is subsequently removed 2 h after inoculation of live *V. cholerae* O1 proximal to the tie. This model allows massive and often fatal diarrhea to occur within 1 to 5 days. The RITARD model is often used after oral immunization to test the protective immunogenicity of *V. cholerae* strains. Successful colonization and immunization of adult rabbits by oral inoculation (usually without diarrhea) can be accomplished with administration of tincture of opium to induce hypoperistalsis (71). Lethal watery diarrhea can also be induced in an adult dog model (381), although this model is infrequently used.

Toxins

V. cholerae produces a variety of extracellular products that have deleterious effects on eukaryotic cells. The massive, dehydrating diarrhea characteristic of cholera is induced by cholera enterotoxin, also referred to as CT or choleragen. The volunteer studies described above clearly show the pivotal role that CT plays in disease. However, *V. cholerae* strains deleted of genes encoding CT can still cause mild to moderate diarrhea in many individuals (see below), and this has prompted a search for additional toxins produced by *V. cholerae*. Recent reviews on toxins produced by this species have appeared, including two reviews on the history of the discovery of CT (108, 465), a review on the structure and function of CT (418), and a general review on all toxins produced by *V. cholerae* (207).

Cholera enterotoxin. The existence of a toxin responsible for the symptoms of cholera was first advanced by Robert Koch, who in 1884 proposed that the agent responsible for cholera produces "a special poison" which acted on the intestinal epithelium and that the symptoms of cholera could be "regarded essentially as a poisoning" (226). The existence of this hypothesized toxin was demonstrated in 1959 by two independent groups of investigators working in India. De et al. (75) and Dutta et al. (91) demonstrated that an outpouring of fluid resulted when culture filtrates or lysates of *V. cholerae* were introduced into the rabbit intestinal tract. Purification of the toxin 10 years later by Finkelstein and LoSpalluto (112) allowed numerous investigators to discover fundamental properties of the toxin such as structure, receptor binding, and mode of action. These discoveries have been extensively reviewed in an historical context (108, 465).

(i) Structure. The structure of CT is typical of the A-B subunit group of toxins in which each of the subunits has a specific function. The B subunit serves to bind the holotoxin to the eukaryotic cell receptor, and the A subunit possesses a specific enzymatic function that acts intracellularly. CT consists of five identical B subunits and a single A subunit, and neither of the subunits individually has significant secretogenic activity in animal or intact cell systems. The mature B subunit contains 103 amino acids with a subunit weight of 11.6 kDa. The mature A subunit has a mass of 27.2 kDa and is proteolytically cleaved to yield two polypeptide chains, a 195-residue A_1 peptide of 21.8 kDa and a 45-residue A_2 peptide of 5.4 kDa. After proteolytic cleavage, the A_1 and A_2 peptides are still linked by a disulfide bond before internalization (132). Formation of intrachain disulfide bonds within the A and B subunits is catalyzed by disulfide isomerase (491). The same enzyme is essential for assembly of the TCP of *V. cholerae*, which contains an intrachain disulfide bond (338). The other steps of CT assembly and secretion into the extracellular environment are not as well characterized as the disulfide bond formation (reviewed in reference 166).

The crystal structures for CT and the highly similar LT of ETEC have recently been determined (412). The B subunits form a pentamer via interactions between β sheets of adjacent monomers. A pore of 11 to 15 Å (1.5 to 1.5 nm) is formed in the center of the pentamer; the C-terminal end of the A_2 peptide sits in this pore and binds to the B pentamer by numerous interactions between polar and charged residues. The N-terminal half of the A_2 is a long α-helix which extends outside the B pentamer and interacts with the A_1 peptide. The A_1 peptide has structural homology with the catalytic region of *Pseudomonas aeruginosa* exotoxin A and diphtheria toxin (412).

(ii) Receptor binding. The receptor for CT is the ganglioside G_{M1} [Galβ1→3GalNAcβ1(NeuAcα2→3)→Glc-ceramide, where Gal is galactose, GalNAc is *N*-acetylgalactosamine, Glc is glucose, and NeuAc is *N*-acetylneuraminic acid (sialic acid)] (222, 418). The interaction between CT and the receptor occurs via the B subunit; addition of purified B subunit (also known as choleragenoid) or G_{M1} to rabbit ileal loops before addition of CT inhibits fluid secretion (222, 345). Antibodies directed against B, the immunodominant subunit, are much more efficient at neutralizing toxin activity than are antibodies directed against the A subunit (341, 433).

Binding of CT to epithelial cells is enhanced by a neuraminidase (EC 3.2.1.18; NANase) produced by *V. cholerae*. This

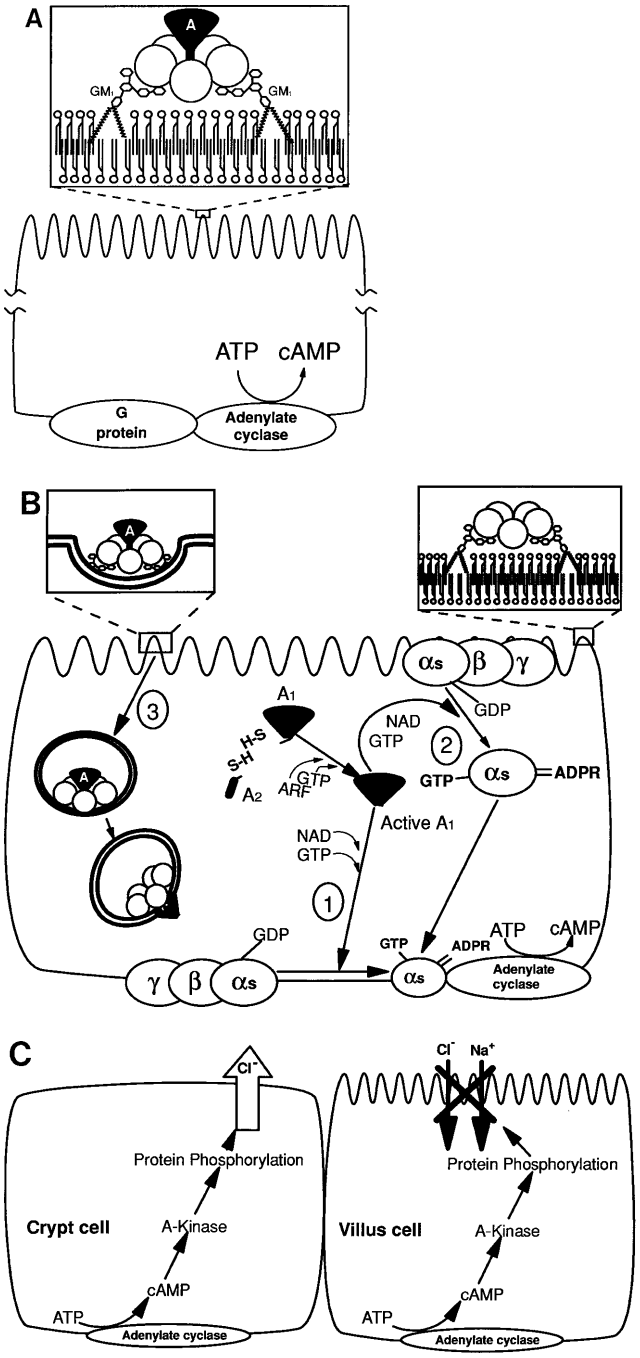

FIG. 1. Mode of action of CT. (A) Adenylate cyclase, located in the basolateral membrane of intestinal epithelial cells, is regulated by G proteins. CT binds via the B-subunit pentamer to the G_{M1} ganglioside receptor inserted into the lipid bilayer. (B) The A subunit enters the cell, probably via endosomes, and is proteolytically cleaved, with subsequent reduction of the disulfide bond to yield A_1 and A_2 peptides. The A_1 peptide is activated (at least in vitro) by ARFs and transfers an ADP-ribose moiety (ADPR) from NAD to the α subunit of the G_S protein. The ADP-ribosylated α subunit dissociates from the other subunits of $G_{S\alpha}$ and activates adenylate cyclase, thereby increasing the intracellular cAMP concentration. Three possible scenarios have been proposed to explain entry of the toxin and activation of adenylate cyclase. Possibility (1) proposes that the A_1 subunit translocates through the apical membrane, leaving the B pentamer on the apical membrane. The A_1 diffuses freely through the cytoplasm to the basolateral membrane, where it ADP-ribosylates $G_{s\alpha}$ (103). A second possibility (2) is that the A_1 peptide ADP-ribosylates an α subunit in the apical membrane. The ADP-ribosylated α subunit traverses the cell to attach to the adenylate cyclase located in the basolateral membrane (87). A third possibility (3) is that the entire toxin enters the cell via endosomes and the A subunit translocates through the endosomal membrane. The endosome travels through the cell with the A_1 peptide still associated with the endosomal membrane. The A_1 peptide ADP-ribosylates the $G_{S\alpha}$ located in the basolateral membrane, perhaps after an endosome-plasma membrane fusion (189). (C) Increased cAMP activates protein kinase A, leading to protein phosphorylation. In crypt cells, the protein phosphorylation leads to increased Cl^- secretion, and in villous cells, it leads to decreased NaCl-coupled absorption. From reference 207.

enzyme, with a predicted molecular mass of 83 kDa (127), enhances the effect of CT by catalyzing the conversion of higher-order gangliosides to GM_1 (170). It has been hypothesized that NANase may produce locally high concentrations of the GM_1 receptor for CT in vivo, thereby enhancing the binding of CT and leading to greater fluid secretion (170, 222). Experiments with purified NANase and ^{125}I-labeled CT showed that isolated rabbit small-intestine brush borders or cultured mouse neuroblastoma cells exposed to NANase bound two to seven times more CT than did untreated cells (148, 284). In ligated canine ileal loops perfused with CT, secretion increased fourfold in loops pretreated with NANase compared with untreated control loops (424).

Using isogenic *V. cholerae* strains specifically mutagenized in the gene encoding NANase (*nanH*) and flow cytometry, Galen et al. (127) showed that fluorescence due to binding of fluorescein-conjugated CT to mouse fibroblasts exposed to NANase-positive culture filtrates increased five- to eightfold relative to binding to cells exposed to NANase-negative filtrates. Furthermore, the short circuit current measured in Ussing chambers increased 65% with NANase-positive filtrates compared with NANase-negative filtrates. These results indicated that NANase plays a subtle but significant role in binding and uptake of CT, although this enzyme is not a primary virulence factor of *V. cholerae*.

(iii) Enzymatic activity. The intracellular target of CT is adenylate cyclase, one of the most important regulatory systems of the eukaryotic cell. This enzyme mediates the transformation of ATP to cyclic AMP (cAMP), a crucial intracellular messenger for a variety of cellular pathways. Normally, adenylate cyclase is activated or inactivated in response to a variety of stimuli. Regulation of adenylate cyclase is mediated by G proteins, which serve to link many cell surface receptors to effector proteins at the plasma membrane. G proteins are heterotrimers composed of three distinct subunits: α (M_r = 39 to 46 kDa), β (M_r = 37 kDa), and γ (M_r = 8 kDa) (163). The specific G protein involved is the G_s protein, activation of which leads to increased adenylate cyclase activity. CT catalyzes the transfer of the ADP-ribose moiety of NAD to a specific arginine residue in the $G_{s\alpha}$ protein, resulting in the activation of adenylate cyclase and subsequent increases in intracellular levels of cAMP. cAMP activates a cAMP-dependent protein kinase, leading to protein phosphorylation, alteration of ion transport, and ultimately to diarrhea (Fig. 1).

After CT binds to intact cells, there is a lag of 15 to 60 min before adenylate cyclase is activated (131). The lag time is necessary to allow the A_1 peptide to translocate through the membrane and to come into contact with the G proteins. The adenylate cyclase is located on the basolateral membrane of polarized intestinal epithelial cells, and a variety of mechanisms have been proposed to explain how CT bound to the brush border (apical) membrane reaches the basolateral adenylate cyclase (Fig. 1B). The most likely explanation is that whole CT enters the cells by endocytosis and the low pH of the endosomal compartment facilitates the translocation of the A subunit across the endosomal membrane. The endosome then travels through the cell with the A_1 peptide still associated with

the endosomal membrane. Ultimately, the A_1 peptide ADP-ribosylates $G_{s\alpha}$ located in the basolateral membrane. Several investigators have recently reported that the action of CT is completely inhibited by addition of brefeldin A, a fungal metabolite known to interfere with vesicular transport in endosomal and transcytotic pathways of many eukaryotic cells (89, 237, 238, 311). These results show that an intact Golgi region is required for intracellular trafficking of CT and implicate toxin endocytosis in the entry of CT into cells.

The A_1 peptide catalyzes the transfer of ADP-ribose from NAD to an arginine residue on $G_{s\alpha}$ in the following reaction: NAD + $G_{s\alpha}$ → [ADP-ribosyl $G_{s\alpha}$] + nicotinamide + H^+ (301). The α subunit of G_s contains a GTP binding site and an intrinsic GTPase activity (reviewed in reference 163). Binding of GTP to the α subunit leads to dissociation of the α and the β-γ subunits and subsequent increased affinity of α for adenylate cyclase. The resulting activation of adenylate cyclase continues until the intrinsic GTPase activity hydrolyzes GTP to GDP, thereby inactivating the G protein and adenylate cyclase. ADP-ribosylation of the α subunit by the A_1 peptide of CT inhibits the hydrolysis of GTP to GDP, thus leaving adenylate cyclase constitutively activated, probably for the life of the cell (40, 203).

The ADP-ribosylation activity of A_1 is stimulated in vitro by a family of proteins termed ADP-ribosylation factors (ARFs; reviewed in reference 302). The ARF proteins are ca. 20-kDa GTP binding proteins and constitute a distinct family within the larger group of ca. 20-kDa guanine nucleotide-binding proteins which include the *ras* oncogene protein. In the presence of GTP, ARF appears to act directly on the A_1 peptide, independently of B subunits or $G_{s\alpha}$. At least in vitro, ARFs serve as allosteric activators of A_1 and increase the ADP-ribosyltransferase activity of the proteins.

(iv) Cellular response. The increased intracellular cAMP concentrations resulting from the activation of adenylate cyclase by CT lead to increased Cl^- secretion by intestinal crypt cells and decreased NaCl-coupled absorption by villus cells (100) (Fig. 1C). The net movement of electrolytes into the lumen results in a transepithelial osmotic gradient which causes water flow into the lumen. The massive volume of water overwhelms the absorptive capacity of the intestine, resulting in diarrhea. The steps between increased levels of cAMP and secretory diarrhea are not known in their entirety. Pioneering work in this area by Field et al. (99, 102) and subsequent investigators (reviewed in reference 108) demonstrated that CT could concomitantly increase cAMP formation and ion transport in isolated intestinal epithelium mounted in Ussing chambers. One crucial step resulting from increased cAMP levels is activation of protein kinase A, which subsequently phosphorylates numerous substrates in the cell (50).

Although it is clear that chloride channels can be regulated by cAMP-dependent protein kinases, the actual ion channel or channels affected by the CT are not known with certainty. There are multiple types of Cl^- channels in apical membranes with different modes of activation and subcellular distribution (77). It is also not certain if protein kinase A directly phosphorylates the ion channel or if it phosphorylates an intermediate protein which then phosphorylates other proteins in a cascade. One attractive candidate for a relevant target protein is the cystic fibrosis (CF) gene product, CFTR. The CFTR protein is a Cl^- channel (21) and has multiple potential substrate sequences for kinase A. Unlike normal intestinal tissue, tissues obtained from patients with CF (CF homozygotes) do not respond to either cAMP- or Ca-mediated secretagogues (25). Interestingly, it has been hypothesized that individuals who are CF heterozygotes may have a selective advantage over "normal" homozygotes in surviving cholera (377). Heterozygotes presumably have only half of the normal number of chloride channels responsive to kinase. After infection with *V. cholerae*, the CF heterozygote may have less intestinal chloride secretion, and therefore less diarrhea, due to the smaller number of chloride channels.

(v) Alternate mechanisms of action. The activation of adenylate cyclase leading to increased cAMP and subsequent altered ion transport is the "classic" mode of action of CT. However, data from several groups suggest that the increased levels of cAMP and subsequent protein kinase A activation may not explain all of the secretory effects of CT. There is compelling evidence that prostaglandins and the enteric nervous system (ENS) are involved in the response to CT in addition to the mechanism outlined above. The majority of the studies presenting these alternative mechanisms do not conclude that the above scenario is wrong; rather, they suggest that this scenario simply does not explain all of the secretion due to CT.

(a) Prostaglandins. Several reports have implicated prostaglandins in the pathogenesis of cholera (23, 27, 342). Cholera patients in the active secretory disease stage have elevated jejunal concentrations of prostaglandin E_2 (PGE_2) compared with patients in the convalescent stage (419), and prostaglandins PGE_1 and PGE_2 have been reported to stimulate adenylate cyclase and increase short circuit current (Isc) in Ussing chambers (221). The role of prostaglandins, leukotrienes, and other metabolites of arachidonic acid in causing intestinal secretion have been well documented (126), although the exact mechanisms are unclear. Peterson and Ochoa (342) have reported that addition of cAMP induced only a small, transient fluid accumulation in rabbit intestinal loops whereas addition of PGE_2 caused a much stronger fluid accumulation in rabbit loops. Addition of CT led to increases in both cAMP and PGE in rabbit loops and in CHO cells, resulting in the release of arachidonic acid from membrane phospholipids (361). A model has been suggested in which cAMP levels increased by CT serve not only to activate protein kinase A but also to regulate transcription of a phospholipase or a phospholipase-activating protein. The activated phospholipase could act on membrane phospholipids to produce arachidonic acid, a precursor of prostaglandins and leukotrienes (343). Consistent with this model, de Jonge and colleagues (77), using the human intestinal cell line HT29.c1.19A mounted in Ussing chambers, report that 40 to 60% of the Isc response to CT is inhibited by relatively low concentrations of the phospholipase A_2 inhibitor mepacrine. The effect of CT on arachidonic acid metabolism might occur through ADP-ribosylation of G_s in the apical membrane, which would then directly activate a phospholipase, or the effect may be mediated through protein kinase A, which would activate the phospholipase.

(b) ENS. The ENS, as part of the autonomic nervous system, plays an important role in intestinal secretion and absorption (70). The intestine also contains a variety of cells that can produce hormones and neuropeptides, products that can affect secretion. Two such substances with well-established roles in causing secretion are vasoactive intestinal peptide (VIP) and serotonin (5-hydroxytryptamine) (70). Lungren (261) has reported several studies that support the concept that the ENS plays a crucial role in secretion due to CT. The overall hypothesis proposed by these investigators is that CT binds to "receptor cells," namely, enterochromaffin cells, which release a substance such as serotonin which activates dendritelike structures located beneath the intestinal epithelium. This leads to the release of VIP, resulting in electrolyte and fluid secretion.

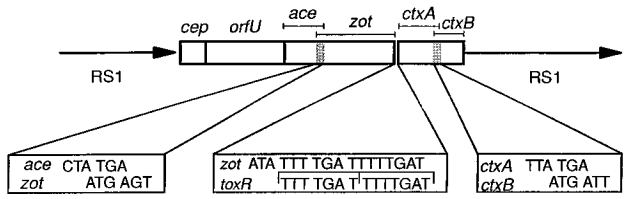

FIG. 2. Arrangement of *ctxAB*, *zot*, and *ace* genes in the *V. cholerae* core region. RS1 elements are shown on both sides of the core region, a common arrangement in El Tor but not classical strains. The enlarged regions depict the overlapping open reading frames of *ctxA* and *ctxB* and of *ace* and *zot*. In addition, the region where the stop codon of *zot* overlaps with the first ToxR binding repeat upstream of the *ctx* operon is also enlarged. Also shown are *orfU*, an open reading frame of unknown function, and *cep* (core-encoded pilus), a recently described colonization factor (337). From reference 207.

This model is supported by a variety of evidence: (i) CT-induced secretion is inhibited by a variety of ganglionic or neurotransmitter blockers (41, 42, 44), (ii) CT stimulates release of serotonin (5-hydroxytryptamine) from enterochromaffin cells into the intestinal lumen (314) and fluid secretion induced by CT is markedly diminished by 5-hydroxytryptamine receptor antagonists (413), and (iii) CT-induced secretion is accompanied by an increased release of VIP from the small bowel and the increased release of VIP is blocked by tetrodotoxin (314). In addition, cholera patients also show increased levels of VIP in blood (169).

Cassuto et al. (43) estimate that ca. 60% of the effect of CT on intestinal fluid transport could be attributed to nervous mechanisms. The release of serotonin from enterochromaffin cells in response to binding of CT may cause secretion by two pathways: (i) the 5-hydroxytryptamine could directly stimulate the ENS, leading to release of VIP; or (ii) the 5-hydroxytryptamine releases prostaglandins that could alter transport function directly (see above) or could activate the ENS. Besides the direct secretory effect, there is also evidence that CT increases intestinal motility and could thereby contribute to diarrhea (271).

(vi) Immunomodulation. CT is one of the most potent oral immunogens ever studied (262). Vigorous immune responses are engendered not only against CT delivered orally by itself but also against unrelated antigens delivered orally with CT (reviewed in reference 171). CT can stimulate immune responses to protein antigens such as keyhole limpet hemocyanin up to 50-fold greater than those observed when antigen is delivered alone (266). Chemical conjugation or genetic fusion of antigens to the B subunit of CT or LT has yielded enhanced responses to streptococcal antigens (74, 79), hepatitis B antigen (390), and ETEC heat-stable enterotoxin (ST) (388).

While the B subunit by itself has been reported to have an adjuvant effect, probably due to the avid binding of B subunit to G_{M1} (74, 116), the most potent adjuvant effect of CT and LT is due to the ADP-ribosyltransferase activity of the A subunit. Lycke et al. (265) have shown that alteration of a single residue in the active site of the A subunit greatly diminishes the adjuvant effect of LT. CT has a variety of effects on cells of the immune system including stimulating interleukin-1 proliferation and enhancing antigen presentation by macrophages (35), promoting B-cell isotype differentiation (264), and inhibiting Th1 cells (306). It has also been suggested that the adjuvant action of CT is due to increased intestinal permeability in response to CT, perhaps providing increased access of antigens to the gut mucosal immune system (263).

(vii) Genetics. The genes encoding CT (*ctxAB*) were initially cloned by exploiting their homology to the genes encoding LT of ETEC (130, 208, 336). DNA sequence analysis shows 78% overall nucleotide homology and 80% predicted protein homology between the two genes (258, 259, 276, 485, 486). The A and B subunits are encoded on two separate but overlapping open reading frames; the first two bases of the *ctxA* translation termination signal (TGA) are the last two of the *ctxB* translation initiation codon (ATG) (Fig. 2) (258, 276). The A and B cistrons possess ribosomal binding sites immediately upstream of their start codons, with the site for *ctxB* being located in the 3′ end of the *ctxA* sequence. The higher expression of the B subunit, leading to the 5:1 ratio of B-A subunits in the holotoxin, results from more efficient translation due to a stronger ribosomal binding site for the B subunit (276). Transcription of the *ctx* operon is regulated by the ToxR regulatory system (see below).

Many strains of *V. cholerae* O1 contain multiple copies of the *ctx* operon. Classical strains contain two copies which are separated on the *V. cholerae* O1 chromosome by an unknown distance (274, 297). Most El Tor strains contain only a single copy of the *ctx* operon, but about 30% of El Tor strains contain two or more adjacent gene copies (274, 275). The *ctx* operon, along with genes encoding the zonula occludens toxin (Zot) and accessory cholera enterotoxin (Ace) (see below) are located on a 4.5-kb region called the core region (140) (Fig. 2). Flanking the 4.5-kb region are one or more copies of a 2.7-kb sequence called RS1. Recombination between RS1 sequences can lead to tandem duplication and amplification of the core region as well as deletion of the core region (140). Serial passage of an El Tor strain through rabbit ligated intestinal loops led to in vivo amplification, resulting in strains expressing higher levels of CT and containing more *ctx* copies than the strain injected into the first loop (274). Although CT by itself can aid colonization (347), the in vivo amplification of the entire 4.5-kb core region may be due to the presence in this region of a gene encoding an intestinal colonization factor (337) (see below). Classical strains and some El Tor strains possess RS1 sequences only on one side of the core region, and therefore this gene duplication and amplification are not seen (274, 275). The RS1 sequence encodes a site-specific transposable element which can insert into a specific 18-bp sequence called attRS1 (337). Naturally occurring isolates of *V. cholerae* which do not produce CT lack sequences homologous to *ctx* and the rest of the core region (212, 281) but do contain attRS1 sequences (337). Pearson et al. (337) recently demonstrated that when core region and RS1 sequences are cloned into a suicide plasmid incapable of replicating in *V. cholerae* and transferred from a conjugation-proficient *E. coli* strain, the RS1 and *ctx* sequences can insert into the attRS1 site in a *recA*-independent manner.

Other toxins produced by *V. cholerae*. (i) Zot. Zot was described by Fasano et al. (97), who reported that *V. cholerae* produced a toxin that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens. This activity was discovered by testing culture supernatants of *V. cholerae*, both wild type and Δ*ctx*, in Ussing chambers, a classic technique for measuring transepithelial transport of electrolytes across intestinal tissue. When culture supernatants of *V. cholerae* CVD101 (Δ*ctxA*) were added to rabbit ileal tissue mounted in Ussing chambers, an immediate increase in tissue conductivity (i.e., a decrease in tissue resistance) was observed (97). Unlike the increase in potential difference observed in response to CT, which reflects ion transport across the membrane (101), i.e., the transcellular pathway, variation in transepithelial conductance reflects primarily, although not exclusively, modification of tissue permeability through the intercellular space, i.e., the

paracellular pathway (82). Examination by electron microscopy of the epithelial tight junctions, the major barrier in this paracellular pathway (82, 267), revealed that exposure of ileal tissue to culture supernatants of CVD101 resulted in the "loosening" of the tight junction so that an electron-dense marker could permeate the paracellular space (97). In contrast, tissue treated with uninoculated broth control was not permeable to this marker. Freeze-fracture electron microscopy showed that the anastomosing network of strands made up of tight junctions suffered a striking decrease in strand complexity in tissue treated with supernatants (97). The toxin responsible for this striking effect on zonula occludens was named Zot, for zonula occludens toxin. Fasano and colleagues (97) speculate that, by increasing intestinal permeability, Zot might cause diarrhea by leakage of water and electrolytes into the lumen under the force of hydrostatic pressure.

The gene encoding Zot was cloned and found to be located immediately upstream of the *ctx* locus (19). *zot* gene sequences are present in both *V. cholerae* O1 and non-O1 strains, and strains that contain *ctx* sequences almost always contain *zot* sequences and vice versa (95, 193, 215). The *zot* gene consists of a 1.3-kb open reading frame which could potentially encode a 44.8-kDa polypeptide. The predicted amino acid sequence of the Zot protein shows no homology to any other bacterial toxin including the toxin A of *Clostridium difficile*, a large 300-kDa protein which also alters tight junctions (159). Some homology to ATPase protein sequences has been reported, but the significance of this observation is unknown (229).

(ii) Ace. Ace of *V. cholerae* was recently identified by Trucksis et al. (463). These investigators demonstrated that the gene product of an open reading frame located immediately upstream of *zot* can increase short circuit current in Ussing chambers. Like CT, and in contrast to Zot, this new toxin increases potential difference rather than tissue conductivity. Strains containing the cloned *ace* gene caused significant fluid accumulation in rabbit ligated ileal loops. The *ace* gene could potentially encode a 96-residue peptide with a predicted M_r of 11,300 (463). The predicted amino acid sequence of Ace shows a striking similarity to that of a family of eukaryotic ion transporting ATPases, including the human plasma membrane calcium pump, the calcium-transporting ATPase from rat brain, and the product of the CF gene, the CF transmembrane regulator. The first two proteins are involved in transport of calcium ions across the membrane, and CFTR functions as a chloride ion channel (21). In addition, Ace also shows sequence similarity with a virulence protein of *Salmonella dublin*, SpvB, which is essential for virulence in mice (231).

The predicted structure of the Ace protein suggests a model in which multimers of Ace insert into the eukaryotic membrane with hydrophobic surfaces facing the lipid bilayer and the hydrophilic sides facing the interior of a transmembrane pore. Such a structure is found for the 26-residue δ toxin of *S. aureus* (120) which has a variety of effects on eukaryotic tissue, including increasing vascular permeability in guinea pig skin and inhibiting water absorption and increasing cAMP concentration in the ileum (214, 319). The C-terminal region of Ace shows 47% amino acid similarity with residues 2 to 20 of δ toxin (463), thus lending some support to a model in which Ace acts by aggregating and inserting into the eukaryotic membrane to form an ion channel.

(iii) Hemolysin/cytolysin. As noted above, hemolysis of sheep erythrocytes was traditionally used to distinguish between the El Tor and classical biotypes of *V. cholerae*, although more recent El Tor isolates are only poorly hemolytic on sheep erythrocytes (15, 128). The hemolysin was initially purified by Honda and Finkelstein (175) and shown to be cytolytic for a variety of erythrocytes and mammalian cells in culture and rapidly lethal for mice. The hemolysin/cytolysin is initially made as an 82-kDa protein and processed in two steps to a 65-kDa active cytolysin (483). An identical hemolysin, indistinguishable biologically, physicochemically, and antigenically from hemolysin produced by El Tor strains, is produced by *V. cholerae* non-O1 (482). Genes encoding this hemolysin, *hlyA*, are present in classical, El Tor, and non-O1 strains of *V. cholerae* (36). Although classical strains are negative for lysis of sheep erythrocytes, *E. coli* containing cloned *hlyA* genes from classical strain 395 are capable of lysing chicken and rabbit, but not sheep, erythrocytes (367). The molecular basis for differences in hemolytic activity among classical strains, old El Tor isolates, and recent El Tor isolates is not completely understood, but in one nonhemolytic classical strain, 569B, an 11-bp deletion which would produce a truncated *hlyA* gene product of 27 kDa was found (6). An oligonucleotide probe spanning this 11-bp region hybridized to all El Tor and non-O1 strains tested but did not hybridize to any classical strains tested (5). Genes encoding a second hemolysin, genetically distinct from the El Tor hemolysin/cytolysin, have been cloned from classical strain 395 (367), but no further characterization of this hemolysin is available.

The purified hemolysin is capable of causing fluid accumulation in ligated rabbit ileal loops (183). In contrast to the watery fluid produced in response to CT, the accumulated fluid produced in response to hemolysin was invariably bloody with mucous (183). McCardell et al. (272) and Spira et al. (422) have described a cytolysin which is cytotoxic for Y-1 adrenal and CHO cells and causes fluid accumulation in rabbit ileal loops. This cytolysin was proposed to be identical to the El Tor hemolysin. To test the hypothesis that this hemolysin was responsible for the diarrhea seen with Δ*ctx* strains of *V. cholerae*, Kaper et al. (211) constructed derivatives of such strains which were mutated in the *hlyA* gene by deletion of an internal 400-bp *Hpa*I fragment. When tested in volunteers, the Δ*hlyA* strains CVD104 and CVD105 still caused diarrhea in 33% of the subjects (250), thus indicating that the hemolysin/cytolysin is probably not the cause of diarrhea seen in recipients of Δ*ctx V. cholerae* strains.

(iv) Miscellaneous toxins. In addition to CT, Zot, Ace, and hemolysin/cytolysin, which are widely distributed in *V. cholerae* and for which genes have been cloned and sequenced, a number of other toxins have been reported for this species.

(a) Shiga-like toxin. A Shiga-like toxin, identified on the basis of cytotoxicity in Hela cells neutralizable by antibody raised against Shiga toxin purified from *Shigella dysenteriae* 1, has been reported for *V. cholerae* O1 (318). This toxin was hypothesized to be responsible for diarrhea in volunteers who ingested genetically engineered CT^- strains. Consistent with this hypothesis, *V. cholerae* CVD103-HgR (Δ*ctx*), which does not produce detectable Shiga-like activity, causes little or no reactogenicity in volunteers (249). Genes encoding a Shiga-like toxin activity have not been cloned from *V. cholerae*, despite repeated efforts (278, 335).

(b) ST. Some strains of *V. cholerae* non-O1 produce a toxin that shares 50% protein sequence homology to the ST of ETEC (320). Morris et al. showed that a non-O1 strain of *V. cholerae* which produced this toxin (also called NAG-ST) could cause diarrhea in volunteers (294). Sequences encoding the NAG-ST were found in 6.8% of clinical *V. cholerae* non-O1 isolates from Thailand and none of the 78 isolates from Mexico and the United States (167). A survey of environmental isolates of *V. cholerae* non-O1 from Calcutta, India, found the ST sequences in 2.3% of the 521 strains examined (329). Recently, an epidemic of diarrhea caused by *V. cholerae*

non-O1 that produced ST was reported in a refugee camp in Thailand (13). This toxin is only rarely found in *V. cholerae* O1, but one study did identify this toxin in 1 of 197 isolates of *V. cholerae* O1 (449). The genes encoding the ST enterotoxin in *V. cholerae* O1 and non-O1 are flanked by 123-bp direct repeats (321), thus suggesting that this toxin has been introduced into *V. cholerae* via a transposon.

(c) New cholera toxin. In 1983, Sanyal and colleagues reported that environmental strains of *V. cholerae* O1 which lack genes encoding CT could cause fluid accumulation in ligated rabbit ileal loops (389). Fluid accumulation was observed when either whole cells or culture filtrates were used. The filtrates also increased the capillary permeability of rabbit skin but, unlike CT, also caused blanching or necrosis along with the bluing reaction (389). The enterotoxic activity was subsequently found in CT^+ strains such as classical Inaba 569B (384). The toxin was termed new cholera toxin and proposed to be the cause of diarrhea in volunteers fed genetically engineered CT^- *V. cholerae* strains (385). This toxin has been found in both clinical and environmental isolates of *V. cholerae* and was also found in strains that naturally lack *ctx*, *zot*, and *ace* sequences (19). No additional biochemical or genetic characterization of this toxin has been reported.

(d) Sodium channel inhibitor. Tamplin et al. (453) reported that strains of *V. cholerae* O1, including CT^- CVD101, produced a factor that inhibited sodium channels. The initial characterization of this factor suggested that the active compound is tetrodotoxin or a related toxin, but no further characterization of this sodium channel inhibitor in *V. cholerae* O1 has been reported.

(e) Thermostable direct hemolysin. The thermostable direct hemolysin (Kanagawa Phenomenon hemolysin) of *V. parahaemolyticus* is responsible for the enterotoxic activity of this species (315). Genes encoding a thermostable direct hemolysin-like toxin have been found on a plasmid in some strains of *V. cholerae* non-O1 (176), but homologous sequences have not yet been found in *V. cholerae* O1 (461).

(v) Role of additional toxins in disease. The role of toxins other than CT in the pathogenesis of disease due to *V. cholerae* is unknown. These toxins clearly cannot cause cholera gravis because the diarrhea seen with Δ*ctx* strains presumably still producing these toxins is not the severe purging seen with wild-type *V. cholerae* strains (250). However, toxins other than CT may contribute in part to the diarrhea and other symptoms seen with wild-type *V. cholerae* strains and may be responsible for the symptoms seen with CT-negative strains. Such toxins may serve as a secondary secretogenic mechanism when conditions for producing CT are not optimal. The five miscellaneous toxins reviewed above, Shiga-like toxin, ST, thermostable direct hemolysin, new cholera toxin, and the sodium channel inhibitor, either are found only in a very small percentage of strains or are insufficiently characterized to assess the potential role in disease.

The three non-CT *V. cholerae* toxins that are the best characterized are the hemolysin/cytolysin, Zot, and Ace. Genes encoding the hemolysin/cytolysin are found in nearly all pathogenic and nonpathogenic strains of *V. cholerae* O1 and non-O1, with no correlation seen with the presence of *ctx* sequences. As noted above, a CT-negative strain specifically mutated in *hlyA* still caused diarrhea in volunteers. The *ace* and *zot* genes are almost always found in strains containing *ctx* but are rarely found in strains lacking *ctx*. Thus, there is a strong epidemiological correlation of the presence of *zot* and *ace* with disease. It is intriguing that genes encoding three distinct toxins acting by different mechanisms are located in tandem on a dynamic region of the *V. cholerae* chromosome. The arrangement of the 4.5-kb core region flanked by RS1 elements can, in some strains, lead to amplification or deletion of all three toxin genes as a unit (140). This region was recently shown to also contain a gene encoding an intestinal colonization factor, *cep* (core-encoded pilin) (337). Thus, this region may be perceived as a "virulence cassette" of *V. cholerae* (463).

A recent volunteer study tested a *V. cholerae* strain specifically deleted of sequences encoding Zot, Ace, and hemolysin/cytolysin as well as the CT A subunit (446). Interestingly, this strain, *V. cholerae* CVD110 (277), still caused mild to moderate diarrhea in 7 of 10 volunteers as well as fever and abdominal cramps. While these volunteer studies do not exclude a role for Ace, Zot, and hemolysin/cytolysin in the pathogenesis of cholera, they clearly indicate that there are additional features of *V. cholerae* that result in diarrhea.

Colonization Factors

Although the toxin responsible for the severe symptoms of cholera was discovered many years ago, it was only recently that the mechanisms by which *V. cholerae* adheres to the small bowel epithelium have been elucidated. For many years, work focused on agglutination of erythrocytes as a surrogate for intestinal colonization factors. *V. cholerae* produces at least four hemagglutinins (HA) (155): (i) a D-mannose-, D-fructose-sensitive HA expressed by El Tor strains; (ii) an L-fucose-sensitive HA expressed by classical strains; (iii) a D-mannose-, L-fucose-resistant HA (MFRHA) expressed by both biotypes; and (iv) a soluble HA produced by both biotypes which also has protease activity. These HAs may still play a role in intestinal colonization, but it was not until the discovery of the TCP that an intestinal colonization factor critical for disease was demonstrated (164). Although TCP is critical for adherence to epithelial cells, intestinal colonization by *V. cholerae* is probably multifactorial.

The identity of the intestinal cell receptor(s) which is the target of *V. cholerae* adherence is not known. Jones and Freter (197) found that L-fucose partially inhibited the attachment of *V. cholerae* to brush border membranes and proposed that this sugar formed part of the intestinal receptor. Yamamoto and colleagues (484) used isolated human small intestinal cells to study attachment and found that L-fucose had the most striking inhibitory effect on *V. cholerae* binding to human intestinal cells. These investigators found that the primary targets for *V. cholerae* adhesion in human small intestinal cells were the following (in order of adherence efficiency): (i) mucus, (ii) the surface of the epithelial cells of ileal lymphoid follicles, and (iii) the surface of absorptive cells of jejunal or ileal villi (484, 487). Within the lymphoid follicles, the M cells were better adherence targets than other epithelial cells of the follicles (487). The specificity of the targets could vary according to the conditions used to grow the culture (484), thus indicating that expression of *V. cholerae* adherence factors is highly regulated.

Earlier work on *V. cholerae* adhesion usually employed chemically mutagenized *V. cholerae* strains to study the role of various factors in colonization. The lack of specific, well-characterized mutations complicates the interpretation of these earlier studies, which have been previously reviewed (248). Recent information on several potential colonization factors, most of which have been studied using recombinant DNA techniques, is reviewed below.

TCP. In 1987, Taylor et al. (459) reported the discovery of a pilus colonization factor in *V. cholerae* O1 which was composed of long filaments 7 nm in diameter which were laterally associated in bundles. This pilus was discovered by using Tn*phoA*, a transposon which has been used to identify many

bacterial virulence factors. When Tn*phoA* inserts in-frame into a gene encoding a secreted or membrane-spanning protein, a fusion protein which expresses alkaline phosphatase activity is formed (270), thus allowing easy differentiation from fusion proteins which are targeted for the cytoplasm where the alkaline phosphatase enzyme is inactive. Taylor and colleagues screened colonies expressing alkaline phosphatase activity and found one Tn*phoA* mutant that was greatly decreased in colonization ability when tested in an infant mouse model. This mutant was defective in production of a pilus which was associated with hemagglutination in the presence of fucose. The pilus was named TCP because expression of the pilus was correlated with expression of CT.

TCP is the only colonization factor of *V. cholerae* whose importance in human disease has been proven. Herrington et al. (164) conducted volunteer studies showing that intestinal colonization by *V. cholerae* was abolished when the gene encoding the TCP subunit (*tcpA*) was inactivated in a derivative of the classical Ogawa strain 395. Volunteers ingesting the mutant strain suffered no diarrhea, and no vibrios were recovered from the stools of the volunteers. Furthermore, no significant immune response to *V. cholerae* was seen after ingestion of the *tcpA* mutant. Curiously, despite the absolute requirement of a functional TCP for an immune response, volunteers ingesting wild-type *V. cholerae* do not exhibit antibodies against TCP even in the presence of a strong vibriocidal and antitoxin response (153). Although antibodies directed against TCP can protect mice from disease in the infant mouse model (431, 432), solid long-term protection can be engendered in humans in the absence of a detectable anti-TCP immune response.

The predicted amino acid sequence of the 20.5-kDa TcpA subunit shows significant homology to the type IV pili of *P. aeruginosa*, *Neisseria gonorrhoeae*, *Moraxella bovis*, and *Bacteroides nodosus* (398). TCP is found in both El Tor and classical strains, and the predicted protein sequences share 82 to 83% homology between the biotypes (184, 363). Monoclonal antibodies against TCP show epitope differences in pili produced by classical and El Tor strains (199, 431). El Tor strains produce less TCP than classical strains, and culture conditions for optimal TCP expression differ between biotypes (200, 432, 467). Sequences encoding TCP were not found in a collection of *V. cholerae* non-O1 strains nor in environmental isolates of *V. cholerae* O1 which do not produce CT (458). Synthesis of TCP is complex and incompletely understood; up to 15 open reading frames are found in the *tcp* gene cluster (216, 323, 324), and at least one other unlinked gene, *tcpG* (*dsbA*), is also required for expression of a functional pilus (338). Regulation of *tcp* gene expression is similarly complex and is under control of the ToxR system (216, 282, 324, 459) (see below). There is also evidence that expression of TCP is decreased in the presence of the conjugative P plasmid of *V. cholerae* (17), thus suggesting that additional levels of regulation may also be involved in the biosynthesis of TCP.

TCP has been studied almost exclusively in *V. cholerae* O1, and production of TCP or possession of *tcp* genes by *V. cholerae* non-O1 strains has not been reported. However, TCP is expressed by *V. cholerae* O139 when strains are grown under conditions that favor expression of TCP in El Tor *V. cholerae* O1 (152) and the *tcpA* gene sequence of *V. cholerae* O139 is identical to that of an El Tor strain (363).

Accessory colonization factor. Tn*phoA* mutagenesis was also used to discover another potential colonization factor of *V. cholerae*. Peterson and Mekalanos (344) isolated Tn*phoA* mutants which were regulated by ToxR (see below) and found four mutants whose colonization ability was diminished in infant mice. The reduction of colonization observed with insertions in the *acf* (for accessory colonization factor) locus, i.e., ca. 10- to 50-fold compared with the wild-type strain, was not as severe as that observed with the TCP mutant (ca. 1,000-fold [459]). The exact nature of the accessory colonization factor has not been reported, but one of the four open reading frames in this locus (*acfD*) encodes a lipoprotein (333). The nucleotide sequence of the *acf* locus has been determined (94) and found to be located immediately adjacent to the *tcp* and *toxT* loci (see below). Thus, there appears to be a second region of the *V. cholerae* chromosome, in addition to the region containing *ctx*, *zot*, and *ace*, in which genes encoding virulence factors are clustered.

MFRHA. A cell-associated HA which is not inhibited by mannose, fucose, or other sugars is produced by both biotypes of *V. cholerae*. The gene encoding this HA, termed MFRHA, was cloned by Franzon and Manning (119). The nucleotide sequence of a 693-bp open reading frame predicts a 26.9-kDa protein associated with this activity (118). An isogenic strain mutated in this gene was tested for virulence in infant mice and found to be markedly attenuated; whereas the parent strain had a 50% lethal dose (LD_{50}) of 9×10^5, the LD_{50} of the mutant was $>10^8$ (118). Competitive colonization experiments in infant mice showed that in vivo colonization of the mutant was reduced 500- to 1,300-fold relative to the parent strain. The exact nature of the MFRHA is not known, but it was suggested that the MFRHA is a cationic outer membrane protein (OMP) which is held on the cell surface primarily by charge interactions with the LPS (268).

MSHA. The mannose-sensitive HA (MSHA) of *V. cholerae* is expressed by strains of the El Tor biotype but is only rarely expressed by strains of the classical biotype (198). Jonson et al. (198) have identified this factor as a thin, flexible pilus composed of subunits with a molecular mass of 17 kDa. The protein sequence predicted from the *mshA* gene sequence shows that the MSHA is a type IV pilus (200a). A monoclonal antibody capable of inhibiting El Tor vibrio-mediated agglutination of chicken erythrocytes bound specifically to the length of the pilus, suggesting that the receptor binding portion may reside in the actual structural subunit rather than a minor pilus protein (198). The same monoclonal antibody protects against experimental cholera caused by El Tor vibrios in the infant mouse and rabbit intestinal loop models (326). The monoclonal antibody did not protect against challenge by *V. cholerae* O1 of the classical biotype, thus suggesting that there is an El Tor-specific protective antigen. Previous work by Finn et al. (113) characterized Tn*5* mutants of an El Tor strain which lacked MSHA. These mutants were defective in colonization of rabbit ileal tissue by a factor of 4 logs relative to the parent strain. Initial nucleotide sequence analysis of the genes inactivated by the Tn*5* inserts suggests that the transposons are not inserted into the structural gene of the MSHA pilus but rather into a gene required for secretion and/or assembly of the pilus (157).

Core-encoded pilus. In addition to containing the toxin genes *ctx*, *zot*, and *ace*, the 4.5-kb core element contains a gene encoding a factor that enhances colonization in infant mice. Pearson et al. (337) showed that an 82-amino-acid protein is encoded by the *cep* locus (core-encoded pilus) (Fig. 2). The predicted amino acid sequence of this protein shows homology to a flexible pilus of *Aeromonas hydrophila*, although a pilus structure has not yet been identified with the *cep* gene product. Deletion of the *cep* locus reduced colonization in infant mice 13- to 21-fold (337). Previous experiments showing that the 4.5-kb core region can be amplified in vivo (274) may be explained by the selective colonization advantage conferred by

the *cep* locus. The available evidence suggests that *cep* contributes little, if any, to intestinal colonization of humans. Tacket et al. (446) fed a derivative of El Tor strain E7946 which was deleted of the 4.5-kb core region and the *cep* genes. The mutant strain was shed in stools and engendered immune responses indistinguishable from those observed with the wild-type strain containing *cep*.

Soluble HA/protease. The *V. cholerae* soluble HA/protease does not appear to be a colonization factor but is a zinc metalloenzyme that nicks cholera enterotoxin and also cleaves fibronectin, mucin, and lactoferrin (110). This enzyme is produce by strains of both biotypes, but El Tor strains produce higher levels of HA/protease than do classical strains (90, 438). The gene encoding the HA/protease (*hap*) has been cloned (158), and the predicted amino acid sequence of the 46.7-kDa protein shows 61.5% identity with the *P. aeruginosa* elastase. In the absence of protein inhibitors, the 46.7-kDa protein is processed to a 32-kDa protein, which is the form usually isolated (158). Although the HA/protease is a very attractive potential virulence factor, an isogenic strain of *V. cholerae* specifically mutated in the gene encoding this protein was no less virulent in infant rabbits than the parent strain (109). Interestingly, the HA/protease has a "detachase" activity which allows the vibrios to detach from cultured human intestinal epithelial cells (109). The significance of this activity is not known, but it could conceivably aid the spread of the organism through the environment (109).

Other pili. There have been additional fimbriae reported for *V. cholerae* O1, but the relationship of these structures to the potential colonization factors described above is not clear. Hall et al. (154) described at least two other fimbrial types besides TCP and reported that multiple fimbrial types can be expressed by the same strain. Ehara and colleagues (92) purified pili of *V. cholerae* O1 which had a molecular mass of 16 kDa. This size is similar to the 17-kDa size reported for the MSHA, but the pili of Ehara et al. had hemagglutination activity which was both mannose and fructose sensitive. Nakasone and Iwanaga (308) purified pili from a non-O1 strain of *V. cholerae* with a subunit size of 16 kDa. Antibody directed against this pilus reacted with 12 of 49 *V. cholerae* non-O1 strains and 25 of 99 *V. cholerae* O1 strains, thus indicating a pilus that is common to many *V. cholerae* strains of different serovars. Further studies using isogenic strains specifically mutagenized in genes encoding these pili will be necessary to determine the relationships among the various pili and HAs.

OMPs. Numerous OMPs of *V. cholerae* have been identified, and the genes encoding many of these proteins have been cloned. Antibodies to purified OMPs have been shown to inhibit intestinal colonization in an infant mouse model (395). One OMP which has been shown to be important for virulence in infant mice is IrgA, a 77-kDa OMP whose expression is regulated by iron. A *V. cholerae* O1 strain specifically mutated in the *irgA* gene showed a ca. 100-fold increase in LD_{50} compared with the parent strain (143). The competitive colonization ability of the *irgA* mutant was reduced about 10-fold (143), suggesting a colonization defect in this mutant. An OMP which is necessary for biogenesis of the TCP pilus is TcpC, which has a molecular mass of 55 kDa as predicted from the sequence of the *tcpC* gene (323). TcpC is a lipoprotein which is involved in serum resistance of *V. cholerae* (333).

The role of other OMPs in virulence has not been established in either animal or human studies. OmpV is a 25-kDa OMP, the gene for which has been cloned and sequenced (349). Despite the fact that this is a major immunogenic component of the *V. cholerae* surface, a Tn*phoA* insertion into this gene did not affect LD_{50} or competitive colonization in infant mice (459). Another immunogenic OMP is a 22-kDa protein called OmpW (188), for which no role in virulence has been shown. OmpS is a 44-kDa protein which is induced by maltose and shares homology with the bacteriophage λ receptor LamB of *E. coli* (232, 233); in *E. coli*, the LamB protein functions as a pore, and a similar role has been proposed for the OmpS protein of *V. cholerae* (232). *V. cholerae* also produces a 35-kDa OMP which cross-reacts with antisera against the *E. coli* OmpA protein and a *Serratia marcescens ompA* gene probe hybridized weakly to *V. cholerae* (4). Two additional OMPs, OmpT (40 kDa) and OmpU (38 kDa), are under the control of ToxR (see below) (282). OmpU has recently been shown to mediate adherence to human epithelial cells in vitro and to engender serum antibodies in volunteers experimentally infected with *V. cholerae* O1 (419a).

Motility and flagella. *V. cholerae* are motile by means of a single, polar, sheathed flagellum. The flagellar core consists of two proteins of 47 and 49 kDa (368). Antibodies against these proteins do not agglutinate or inhibit the motility of *V. cholerae* (368). A membrane sheath covers the flagellar core and appears to be contiguous with the outer membrane (114). The proteins associated with the flagellar sheath have been variously reported to be three polypeptides of 56, 60, and 61.5 kDa (179) or two proteins of 38 and 40 kDa (410). Antibodies against the 38- and 40-kDa proteins inhibited motility of *V. cholerae*, but these proteins did not induce protective immunity against disease in the rabbit (368). Antibodies against the *V. cholerae* O1 LPS labeled the surface of the sheathed flagella, indicating that the flagellum and cell surface share a common set of LPS antigens (125).

The majority of studies have shown that motility is an important virulence property. Freter and colleagues (121, 123, 124) have shown that motile *V. cholerae* organisms direct themselves to the mucosal surface in response to chemotaxins. In several animal and in vitro models, it has been shown that motile *V. cholerae* cells rapidly enter the mucus gel and can be found in intervillous spaces within minutes to a few hours (123, 149, 197, 391). A variety of workers have shown that nonmotile, fully enterotoxinogenic mutants are naturally diminished in virulence (10, 149, 365, 391). In addition to its role in motility, it has also been suggested that the flagellum serves as an adhesin (10, 93, 179). One relevant issue concerns whether it is the mere presence of a flagellum that is sufficient for colonization or whether it is the actual property of motility that is important. Many of the studies in the literature use nonmotile mutants that have not been characterized as to whether the strains are nonmotile due to a complete lack of a flagellar structure or to a paralyzed flagellum. Furthermore, many studies used nonisogenic strains for the comparison of motile and nonmotile strains or employed a closed, static animal model such as the ligated rabbit ileal loop assay that could minimize the importance of motility (460). A study by Richardson (365) utilized isogenic strains derived spontaneously or by transposon mutagenesis in three different animal models. (Classical *V. cholerae* strains undergo a high frequency [ca. 10^{-4}] of spontaneous mutation to a nonmotile form [303].) The results varied between biotypes and among animal models, but in general it was concluded that motility is clearly a major contributing factor to *V. cholerae* pathogenicity and colonization while the flagellar structure alone seemed to be less important (365).

LPS. There is evidence to suggest that LPS is involved in adherence of *V. cholerae* O1 to the intestinal mucosa. In one study, purified Inaba LPS significantly inhibited attachment of *V. cholerae* Inaba to rabbit mucosa (52). In other in vitro and animal studies, antibodies against Ogawa or Inaba LPS were

shown to prevent adhesion of *V. cholerae* to intestinal mucosa (52, 122).

Polysaccharide capsule. Although *V. cholerae* O1 is unencapsulated, strains of *V. cholerae* non-O1 have been shown to produce a polysaccharide capsule (194). Such a capsule could facilitate septicemia in susceptible hosts, an outcome which is not uncommon with non-O1 strains (see above). Johnson et al. (194) found that an unencapsulated mutant derived by transposon mutagenesis was less virulent in a mouse model than the encapsulated parent. The encapsulated parent strain was protected from serum bactericidal activity, while the unencapsulated mutant was readily killed. There is also evidence that the capsular polysaccharide mediates adherence to epithelial cells. Using the intestinal epithelial cell line Caco-2, these investigators found a clear correlation between the amount of capsular material expressed and the avidity of binding to Caco-2 cells (192). Preincubation of Caco-2 cells with purified capsular polysaccharide from encapsulated strain NRT-36S inhibited binding of this strain by at least 50%, while capsular polysaccharide from a different encapsulated strain was not found to inhibit binding. Interestingly, strains of *V. cholerae* O139 also produce a polysaccharide capsule (195) which could possibly contribute to a case of septicemia reported with this serogroup (190).

Other factors. A slime agglutinin present on the flagellum has been reported to play a role in nonspecific in vitro adherence (11), although the exact nature of this factor is not clear. A 33-kDa surface protein of *V. cholerae* El Tor was recently reported to engender antibodies that blocked adherence of *V. cholerae* to mouse and rabbit mucosa (187).

Regulation

There are multiple systems involved in the regulation of virulence in *V. cholerae*. The ToxR regulon controls expression of several critical virulence factors and has been the most extensively characterized. Regulation in response to iron concentration is a distinct regulatory system that controls additional putative virulence factors. Other putative virulence factors such as NANase and various HAs are apparently not controlled by either regulatory system. There is also a set of genes that are expressed only in vivo and do not belong to the ToxR or iron regulatory systems. These different regulatory systems no doubt allow *V. cholerae* to vary expression of its genes to optimize survival in a variety of environments, from the human intestine to the estuarine environment.

ToxR regulon. As shown by Miller and Mekalanos (281, 282), expression of several virulence genes in *V. cholerae* O1 is coordinately regulated so that multiple genes respond in a similar fashion to environmental conditions. This theme of coordinate regulation and sensory transduction in the control of virulence factors has been found for numerous bacterial pathogens (280). For *V. cholerae* O1, the "master switch" for control of these factors is ToxR, a 32-kDa transmembrane protein (283). ToxR binds to a tandemly repeated 7-bp DNA sequence found upstream of the *ctxAB* structural genes and increases transcription of *ctxAB*, resulting in higher levels of CT expression (281, 283). The activity of ToxR is enhanced by the ToxS protein, a 19-kDa transmembrane protein that interacts with ToxR (84). A model in which ToxR dimers are active for DNA binding and transcriptional enhancement while ToxS serves to assemble or stabilize ToxR monomers into the dimeric form has been proposed (84).

In addition to cholera enterotoxin, ToxR also controls expression of the TCP colonization factor (459), the accessory colonization factor (344), the outer membrane proteins OmpT and OmpU (282), and three lipoproteins (333). The effect of ToxR on expression of most of these factors is to increase expression, but expression of OmpT is decreased in the presence of ToxR (282). A survey of genes whose expression is controlled by ToxR was undertaken by Peterson and Mekalanos (344), who found at least 17 distinct genes that are regulated by ToxR. These genes make up what is termed the ToxR regulon. The importance of ToxR in human disease was demonstrated by Herrington et al. (164), who fed volunteers a derivative of classical *V. cholerae* 395 in which the *toxR* gene was mutated. None of the volunteers ingesting the ToxR mutant suffered any diarrheal symptoms, and the challenge organism could not be recovered from stool samples.

Binding of ToxR directly to the *ctx* operon appears to be the exception rather than the rule for the ToxR regulon. The repeated 7-bp target sequence for binding of ToxR is not present upstream of any other genes regulated by ToxR (83). Expression of at least some of the genes of the ToxR regulon is controlled by another regulatory factor, ToxT (85). ToxT is a 32-kDa protein that shares significant sequence homology with the AraC family of transcriptional activators (165, 322). The studies of DiRita et al. (85) indicate that ToxR controls transcription of the *toxT* gene. The resulting increased expression of the ToxT protein then leads to activation of other genes in the ToxR regulon. Thus, a regulatory cascade controls expression of important virulence factors in *V. cholerae* where ToxR is at the top of the hierarchy, ToxT is at the next level, and a number of virulence genes controlled by ToxT are at the lowest level (85). This regulatory cascade has recently been reviewed by DiRita (83).

The influence of environmental conditions on expression of CT has been known for many years (369). Expression of other virulence factors such as TCP responds in similar fashion, and many of these responses are mediated by the ToxR regulon. It has been proposed that the ToxR protein senses environmental conditions and transmits this information to other genes in the ToxR regulon by signal transduction (83). For classical strains, increased expression of the ToxR regulon is found at pH 6.5 versus 8.5, 30 versus 37°C, and 66 mM NaCl versus lower or higher concentrations (282). These conditions apply only to strains of the classical biotype, in which the ToxR regulon has primarily been characterized. Expression of CT, TCP, and other potential virulence factors differs between the classical and El Tor biotypes (202), and control of the ToxR regulon in El Tor strains has not been thoroughly characterized. The decreased in vitro expression at 37°C of virulence factors known to be expressed at 37°C in the intestine is paradoxical. Perhaps this in vitro temperature effect may be due to the lack of other signals in vitro that are present at 37°C in vivo (83). Additional regulatory factors may play a role in regulation of the ToxR regulon. Immediately upstream of the gene encoding ToxR is a gene for a heat shock protein, *htpG* (332). The *htpG* and *toxR* genes are transcribed in opposite directions, and the promoters for the two genes are so close that only one RNA polymerase can bind in the intergenic region. In the model proposed by Parsot and Mekalanos (332), the normal sigma-70 RNA polymerase can bind to the *toxR* promoter and transcribe the *toxR* gene only at low temperatures. At elevated temperatures (>37°C), the RNA polymerase sigma subunit involved in transcription of heat shock genes, sigma-32, binds to the *htpG* promoter, thus repressing the *toxR* promoter. It has been suggested that upon entry into the body and passage through the stomach the heat shock response is first induced, thereby decreasing expression of the ToxR regulon (332). Once the organism is in the small bowel,

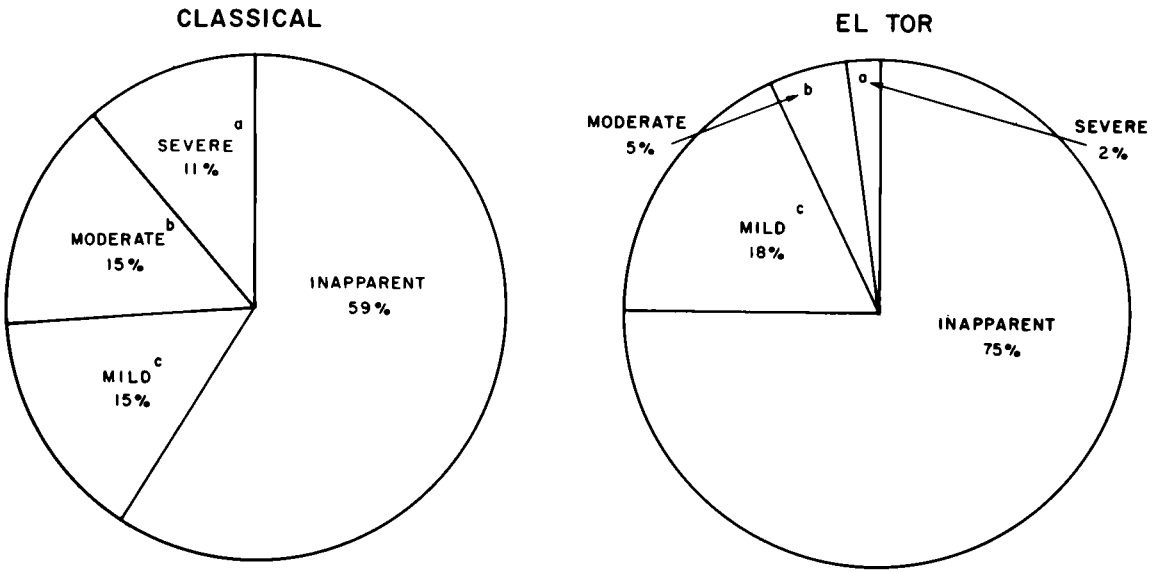

FIG. 3. Spectrum of illness showing percent distribution of individuals infected with the classical biotype of *V. cholerae* compared with those infected with the El Tor biotype. [a]Severe includes hospitalized cases of cholera gravis; [b]moderate includes cases detected in outpatient clinics; and [c]mild includes cases detected in bacteriological surveys. From reference 129 with permission of the publisher.

the heat shock system would no longer be expressed and the ToxR regulon would then be expressed.

Iron regulation. Growth of *V. cholerae* under low-iron conditions induces the expression of several new OMPs that are not seen with cells grown in iron-rich media (407). Many of these proteins are similar to proteins induced by in vivo growth of *V. cholerae*, indicating that the intestinal site of *V. cholerae* is a low-iron environment (392). In addition, expression of some OMPs decreases under iron-limiting conditions (392, 407). Several proteins whose expression are increased under low-iron conditions have been identified, including the hemolysin/cytolysin, vibriobactin, and IrgA (see above) (143, 427).

V. cholerae has at least two high-affinity systems for acquiring iron. The first system involves a phenolatelike siderophore, vibriobactin, which is produced under low-iron conditions (147, 334). Vibriobactin binds iron extracellularly and transports it into the cell through a specific receptor (426). The vibriobactin receptor is a 74-kDa OMP encoded by the *viuA* gene (37). Mutation of *viuA* prevents growth in low-iron media but does not affect synthesis of vibriobactin or transport of other iron complexes including ferrichrome, hemin, and ferric citrate (426). Vibriobactin production is not essential for virulence (408), thus suggesting the existence of other iron transport systems. A second system for acquiring iron utilizes heme and hemoglobin (427). Henderson and Payne (162) have recently cloned genes encoding a 26-kDa inner membrane protein and a 77-kDa OMP (distinct from IrgA) which allow transport of heme into the cell.

Iron regulation of gene expression in *V. cholerae* involves a protein called Fur which shares 76% homology with the *E. coli* Fur protein (37). Regulation by Fur involves binding of the Fur protein in the presence of iron to a 21-bp operator sequence found in the promoter of iron-regulated genes, thereby repressing transcription (38). In *V. cholerae*, Fur acts as a repressor for the *irgA* and *viuA* genes (37, 141). Regulation of *irgA* also requires a second protein, IrgB, which acts as a positive transcriptional activator (142). The *irgB* gene is located immediately upstream of *irgA*, and transcription of *irgB* itself is repressed by Fur in the presence of iron.

In vivo regulation. In addition to the genes whose expression is regulated by ToxR or iron, there are additional genes that are expressed in vivo but not in vitro. Sciortino and Finkelstein (392) first demonstrated that *V. cholerae* grown in ligated rabbit ileal loops expressed a protein profile different from that expressed by *V. cholerae* grown in vitro. Many, but not all, of these proteins also appeared to be induced under iron-limiting conditions. Subsequent studies by Richardson et al. (366) and Jonson et al. (201) also reported novel proteins expressed in vivo. Additional antigens that were expressed in vitro but were down-regulated during in vivo growth were also noted. Jonson and colleagues reported in vivo proteins ranging from 29 to 200 kDa, and most were not induced during culture in iron-depleted media (201). These investigators did not observe any increase in expression of ToxR-regulated proteins such as OmpU and TCP in the bacteria grown in vivo. The human immune response to proteins expressed in vivo was examined by Richardson and colleagues (366), using serum IgG and jejunal fluid IgA from convalescent volunteers experimentally infected with *V. cholerae*. In addition to containing antibodies reacting to antigens expressed in vitro, both serum and jejunal fluid samples reacted to several antigens found only in cells grown in vivo.

CLINICAL FEATURES

Symptoms

Only a minority of persons infected with CT-producing *V. cholerae* develop the most severe manifestations of the disease, termed cholera gravis (129) (Fig. 3). As shown in Fig. 3, infections with classical strains are generally more severe than those with El Tor strains. It has been estimated that 11% of patients with classical infections develop severe disease compared with 2% of those with El Tor infections. An additional 5% of El Tor infections and 15% of classical infections result in moderate illness (defined as cases detected and managed in outpatient clinics). While cholera gravis is a striking clinical entity, milder illnesses are not readily differentiated from other

causes of gastroenteritis in cholera-endemic areas. Symptoms of persons infected with *V. cholerae* O139 Bengal appear to be virtually identical to those of persons infected with O1 strains (28, 53).

The incubation period of cholera can range from several hours to 5 days and is dependent in part on inoculum size (245). Onset of illness may be sudden, with profuse, watery diarrhea, or there can be premonitory symptoms such as anorexia, abdominal discomfort, and simple diarrhea. Initially the stool is brown with fecal matter, but soon the diarrhea assumes a pale gray color with an inoffensive, slightly fishy odor. Mucus in the stool imparts the characteristic rice water appearance. Tenesmus is absent; instead, there is often a feeling of relief as enormous amounts of fluid are passed effortlessly. Vomiting is often present, occurring a few hours after the onset of diarrhea (39, 310).

In cholera gravis, the rate of diarrhea may quickly reach 500 to 1,000 ml/h, leading rapidly to tachycardia, hypotension, and vascular collapse due to dehydration (288, 290, 348). Peripheral pulses may be absent, and blood pressure may be unobtainable. Skin turgor is poor, giving the skin a doughy consistency; the eyes are sunken; and hands and feet become wrinkled, as after long immersion ("washerwoman's hands"). Phonation is impaired, and the patient speaks in a whisper. Patients are restless and extremely thirsty. Major alterations in mental status are uncommon in adults; the patient usually remains well oriented but apathetic, even in the face of severe hypovolemic shock. Painful muscle cramps may occur, probably resulting from hypokalemia. Body temperature usually is normal or subnormal, although low-grade fever in up to 20% of individuals can occur. Oliguria may be present until dehydration and electrolyte deficiencies are corrected.

Dehydration is reflected in a higher plasma protein concentration, hematocrit, serum creatinine, urea nitrogen, and plasma specific gravity (470, 472). Stool bicarbonate losses and lactic acidosis associated with dehydration can result in severe acidosis manifested by depression of blood pH and plasma bicarbonate and an increased serum anion gap (mean of 20.2 mmol/liter in one study [470]). The isotonic dehydration results in serum sodium and chloride concentrations which are usually within the normal range. Despite profound potassium loss, uncorrected acidosis may result in a normal or high serum potassium level. However, loss of cellular potassium can lead to hypokalemic nephropathy and focal myocardial necrosis. Ischemic renal tubular necrosis due to prolonged circulatory collapse may be seen in patients in whom treatment is long delayed or inadequate. Hypoglycemia with coma and convulsions may occur in children.

Differential Diagnosis

Cholera gravis is by far the main illness that can consistently cause dehydrating diarrhea in an adult. In a disease-endemic or epidemic setting, treatment should never be withheld pending laboratory confirmation; laboratory diagnosis is important primarily for epidemiologic and control purposes.

As outlined above, a definitive diagnosis is based on isolation of *V. cholerae* from a patient's stool. In the midst of a cholera epidemic, further characterization may not be necessary. In other situations, isolates should be screened for agglutination with O1 and O139 antiserum and for production of CT or the presence of the *ctx* genes. *V. cholerae* strains outside of the O1 and O139 groups and/or strains that do not produce CT can cause diarrhea and asymptomatic intestinal colonization (293, 294, 296). However, their identification does not carry the same epidemiologic and public health implications as isolation of CT-producing O1 and O139 strains. As outlined above, serologic assays may also be useful in making a retrospective diagnosis of cholera.

TABLE 6. Electrolyte concentration of cholera stool and of solutions used for intravenous and oral rehydration[a]

Stool or solution	Electrolyte concn (mmol/liter)				
	Sodium	Potassium	Chlorine	Base	Glucose
Cholera stool,[b] child	105	25	90	30	
Cholera stool,[b] adult	135	15	100	45	
Lactated Ringer's solution	130	4	109	28	
Isotonic saline (0.9%)	154		154		
Oral glucose-electrolyte solution	90	20	80	30	111

[a] Modified from reference 30 with permission of the publisher.
[b] When rate of stool output is 50 ml/kg per 24 h or more.

Therapy

The key to therapy is provision of adequate rehydration until the disease has run its course (usually 1 to 5 days in the absence of antimicrobial therapy). Rehydration can be accomplished by intravenous infusion of fluid (in severe cases) or by oral rehydration with an oral rehydration solution (ORS) (30, 290, 441). Antimicrobial agents play a secondary but valuable role in therapy by decreasing the severity of illness and the duration of excretion of the organism. The hypoglycemia that is sometimes seen in pediatric patients should be treated with 25 or 50% glucose given intravenously.

Intravenous rehydration. For adults, the intravenous replacement solution should be infused as rapidly as possible so that about 2 liters is given in the first 30 min. If at this point the patient's clinical condition improves, the infusion can be slowed to deliver approximately 100 ml/kg of body weight within the first 4 h of therapy. Children in shock should receive 30 ml of intravenous fluid per kg of body weight in the first hour and an additional 40 ml/kg in the next 2 h. In both adults and children, ORS (with its glucose and potassium) should be administered as soon as possible in the course of illness. The precise rates of fluid administration should be adjusted according to monitoring of the patient's state of hydration and continuing stool losses.

The intravenous fluid chosen for rehydration should be adequate to replace the isotonic fluid and electrolyte losses of cholera (Table 6). The World Health Organization (WHO) recommends Ringer's lactate as the best commercial solution. Since this solution does not have sufficient potassium, potassium chloride may be added to the bottle (10 meq/liter) or given orally. Isotonic saline corrects hypovolemia, but potassium, base, and glucose must be supplemented.

Oral rehydration. Patients with mild or moderate dehydration can receive initial fluid replacement to repair water and electrolyte deficits exclusively by the oral route (30). For mild dehydration, the WHO recommends ORS be given in a volume of 50 ml/kg within the first 4 h. For moderate dehydration, twice this volume (100 ml/kg) should be given in the same time period. Vomiting rarely prevents successful use of ORS and is not a contraindication to its use.

Stool output must be carefully monitored; in epidemic areas, this is often done with a "cholera cot," which contains a hole strategically cut to permit collection of diarrheal stool in a container placed under the cot. After initial stabilization by either intravenous or oral fluids, ongoing stool losses in adults should be replaced with ORS at a ratio of 1.5:1 (i.e., 150 ml of

TABLE 7. Antibiotics for treatment of cholera[a]

Antibiotic(s)	Administration	Dose	
		Children (mg/kg)	Adults (mg)
First choices			
Tetracycline	Four times per day for 3 days	12.5	500
Doxycycline	One single dose	6	300
Alternatives (tetracycline-resistant strains)			
Erythromycin	Four times per day for 3 days (adults) Three times per day for 3 days (children)	10	250
TMP-SMX[b]	Two times per day for 3 days	5 (TMP), 25 (SMX)	160 (TMP), 800 (SMX)
Furazolidone	Four times per day for 3 days	1.25	100

[a] From reference 441 with permission of the publisher.
[b] TMP, trimethoprim; SMX, sulfamethoxazole.

ORS for every 100 ml of diarrheal stool passed). For children, who tend to have lower concentrations of sodium in their stool, fluid losses should be replaced 1:1 with ORS. In practical terms, for adult patients with cholera, the upper limit of ongoing replacement of losses that can be replenished is approximately 750 ml/h by mouth or via orogastric tube. Thus, a cholera purge of approximately 500 ml/h (12 liters/day) is the maximum severity that can be handled with oral hydration alone. More voluminous purges will require that intravenous fluids be used in addition to oral fluids.

The oral solution used should be the one recommended by WHO (Table 6). This solution provides adequate quantities of electrolytes to correct the deficits associated with diarrheal dehydration and an optimal amount of glucose to facilitate the absorption of sodium and water. In developing countries there has been some success in the use of common, locally available sugar and salt products for preparing ORS. There has also been much interest in the use of complex carbohydrates, such as those found in rice gruel, in these solutions. Solutions with electrolyte concentrations that vary greatly from the WHO recommendations should not be used. For example, in the United States serious complications have resulted from use of nonstandard solutions (such as Gatorade) in unsuspected cases of cholera (26).

Antimicrobial therapy. Antimicrobial agents can shorten the duration of cholera diarrhea and the period of excretion of vibrios. Treatment should be started after vomiting subsides (i.e., after initial rehydration and correction of acidosis). Tetracycline is the drug of choice (24, 441); dosages and alternative therapies are summarized in Table 7. Use of tetracycline in children <8 years of age remains somewhat controversial, because of the possibility of staining of permanent teeth. However, the short courses recommended should pose a minimal hazard. While efficacy data are limited, the new quinolones (norfloxacin and ciprofloxacin) have excellent in vitro activity against *V. cholerae* and may prove to be useful therapeutic agents (295). However, in vitro susceptibility of *V. cholerae* to antimicrobial agents does not always correlate with in vivo activity: for example, despite in vitro susceptibility, ampicilin appears to have minimal efficacy in treatment of cholera (317).

Resistance to antimicrobial agents, driven in part by widespread (and often inappropriate) use of these agents, has become an increasing problem throughout the world (3, 106, 137, 443, 450, 462). Antimicrobial resistance is usually encoded by conjugative plasmids of the IncC incompatibility group (137, 160). Levels of tetracycline resistance in East Africa (106), Bangladesh (3), and parts of India (450) have become high enough that *V. cholerae* isolates from these areas should be assumed to be tetracycline resistant until results of susceptibility testing are available. In many instances, isolates are also resistant to other antimicrobial agents, including chloramphenicol, trimethoprim-sulfamethoxazole, and furazolidone. *V. cholerae* O139 strains have not demonstrated tetracycline resistance, although they do have a characteristic pattern of resistance to trimethoprim-sulfamethoxazole (3).

Tetracycline may provide some protection when given prophylactically within a family in which cases of cholera have occurred (273, 374). However, widespread use of tetracycline prophylaxis has been associated with rapid development of antimicrobial resistance (106, 462) and should be strongly discouraged.

EPIDEMIOLOGY

Overview

Perhaps the most distinctive and salient features of cholera as a communicable disease are its epidemiologic behavior by which it tends to occur in explosive outbreaks (often with several simultaneous foci of onset) (133) and its ability to cause true pandemics extensive in place and time. The occurrence of cholera and its epidemiologic behavior prior to the 19th century are subjects of contentious debate among medical historians, as reviewed by Pollitzer (351). In contrast, a new era in the epidemiology of cholera began in 1817 with the onset of the first pandemic (351). It is generally accepted that seven distinct pandemics of cholera have occurred since the first pandemic was recognized in the early 19th century (Table 8). In each instance, cholera spread from Asia (giving rise to the term Asiatic cholera) to reach other continents in pandemics

TABLE 8. Dates of occurrence of the various pandemics of cholera

Pandemic	Published data by:		Recent
	Pollitzer (350)	Wilson (475)	
1	1817–1823	1817–1823	
2	1829–1851	1826–1837	
3	1852–1859	1846–1862	
4	1863–1879	1864–1875	
5	1881–1896	1883–1896	
6	1899–1923/5	1899–1923	
7			1961–present
8			1992–present

that affected many countries and extended over many years. Except for the seventh pandemic, which originated on the island of Sulawesi in Indonesia (204), the other six pandemics arose from the Indian subcontinent, usually from the Ganges delta in Bengal.

Cholera Pandemics

Vignettes from early pandemics. Some fundamental observations on the pathogenesis and treatment of cholera were made during the second pandemic. It was during the second pandemic, when cholera reached the British Isles in the early 1830s, that O'Shaughnessy (327) first demonstrated that the characteristic rice water stools of patients contained salts and alkali, i.e., were high in electrolyte content. Following on this observation, Latta (234) successfully treated some severely dehydrated patients by administering saline infusions intravenously. Regrettably, Latta's mode of therapy and the physiologic concept upon which it was based would be abandoned for the next eight decades.

The second pandemic was the first to reach the New World, arriving in Quebec, Canada, via ships from Ireland carrying immigrants who sustained cases of cholera during the Atlantic crossing (48). Spreading from Montreal, Quebec, in June, cholera moved south unchecked over the next 10 weeks to reach, successively, New York, N.Y.; Philadelphia, Pa.; Baltimore, Md.; and Washington, D.C. (49).

The fundamental epidemiologic observations of John Snow (416) on the waterborne transmission of cholera were made in London in the period 1847 to 1854 during the (late second and) third pandemic. On the basis of his epidemiologic observations, Snow deduced that cholera must represent a contagion.

Cholera was also rampant in the United States during the third pandemic when it was carried west during the 1850s by wagon trains of pioneers. In the early 1870s, towards the end of the fourth panedmic, New Orleans, La., and cities and towns along the Mississippi, Missouri, and Ohio rivers experienced considerable cholera (29). In the course of field investigations in Egypt in 1883 during the fifth pandemic, Koch (226) isolated a bacterium in pure culture from the rice water stools of patients with cholera which he referred to as "comma bacilli" because of their shape. Moving on to Calcutta in 1884, Koch isolated the same bacteria from cholera patients in India (226). The fifth pandemic extensively affected South America, causing large epidemics accompanied by high mortality in Argentina, Chile, and Peru, among other countries (235). Notably, this represents the last time cholera would affect South America for more than a century.

A notable feature of the sixth pandemic of cholera (1899 to 1923) was the extensive involvement of populations in the Near and Middle East and the Balkan peninsula (351). With the exception of a large epidemic in Egypt in 1947 (404), cholera remained virtually confined to South and Southeast Asia from the mid-1920s until the onset of the seventh pandemic in 1961.

Seventh pandemic. The seventh pandemic of cholera, which began in 1961, is notable for several reasons. First, this pandemic, which is still ongoing, is the most extensive in geographic spread and in time. Second, the causative agent is *V. cholerae* O1 of the El Tor biotype. The sixth pandemic, and probably the fifth as well, was caused by *V. cholerae* O1 of the classical biotype. Third, all previous pandemics originated in the Indian subcontinent (204, 351), whereas the seventh pandemic originates from the island of Sulawesi, in the Indonesian archipelago.

Several features have undoubtedly contributed to allow the extensive geographic spread of El Tor cholera observed during the seventh pandemic. One is the enhanced ability of El Tor vibrios to survive in environmental niches compared with classical vibrios. Thus, El Tor vibrios are demonstrably hardier (98). Another is the fact that the clinical case/inapparent infection ratio is skewed for El Tor such that for each case of cholera gravis there exist many more individuals with mild or inapparent infection than is seen with classical biotype cholera (16, 129) (see above). As a consequence, in communities affected by El Tor cholera there are many individuals circulating who are asymptomatic excretors and who, depending on local sanitary and water supply conditions, can serve to spread the infection. Lastly, modern transport, particularly air travel, is increasingly utilized by individuals of all socioeconomic levels in developing countries. Thus, an individual asymptomatically excreting El Tor cholera or incubating the infection prior to onset of clinical illness can be whisked from one continent to another within hours.

The seventh pandemic can be viewed as being composed of four periods. In the first period, extending from 1961 to 1962, El Tor cholera spread from Sulawesi to involve the other islands of Indonesia, including Java, Sarawak, and Borneo (204). El Tor cholera then spread to the Philippines, Sabah, Taiwan, and Irian Barat, thereby affecting virtually the entire Southeast Asian archipelago.

According to Kamal (204), the second period of the seventh pandemic, spanning 1963 to 1969, was characterized by the dissemination of El Tor cholera to the Asian mainland. Beginning with Malaysia, the El Tor pandemic spread to Thailand, Burma, Cambodia, Vietnam, and Bangladesh. India was invaded in 1964 through the port of Madras, and within 1 year El Tor cholera had disseminated throughout the country. From the time that El Tor cholera reached Pakistan in 1965, its spread became even more accelerated. In the space of a few months, Afghanistan, Iran, and nearby republics within the Soviet Union experienced outbreaks. Iraq reported El Tor cholera in 1966.

It is commonly taught that a third period of the seventh pandemic arrived with the large-scale and explosive outbreaks of cholera that began in the Middle East and West Africa in 1970. By 1970, El Tor had reached the Arabian Peninsula, Syria, and Jordan and a limited outbreak was recorded in Israel (62). At this time El Tor Inaba was also resurgent in Iran and the southern Soviet Union. It is of interest that in Lebanon and Syria the epidemic strain was El Tor Ogawa, whereas in nearby Israel and Jordan and in Dubai and Saudi Arabia the epidemic organism was El Tor Inaba.

The invasion of sub-Saharan West Africa by El Tor Ogawa cholera, which occurred during the third period of the pandemic, was a momentous epidemiologic event (145). Following its introduction in Guinea in 1970, probably by means of a returning traveler, cholera subsequently spread along waterways along the coast and into the interior along rivers. Subsequent further dissemination into the interior of the Sahelian states occurred by land travel fostered by the movement of nomadic tribes. It is estimated that the 1970 to 1971 outbreak in West Africa resulted in more than 400,000 cases. Because of a lack of background immunity in the population, insufficient transport to move severe cases to treatment facilities, and inadequacies in the health care infrastructure, case fatality in West Africa was high.

According to WHO records, of the 36 countries that reported cholera in 1970, 28 were newly affected countries and 16 were in Africa. As epidemic cholera raged in West Africa in 1970, epidemiologists and public health officials in South America and elsewhere in Latin America girded themselves

and their communities for what was deemed to be the inevitable passage of cholera westward across the South Atlantic. The scenario of introduction of cholera into the Americas was considered particularly likely to occur once cholera hit Angola, since an estimated 40,000 Cuban troops were in that country. Yet, inexplicably, cholera did not cross the South Atlantic during the next 20 years.

The fourth period of the seventh pandemic can be said to have begun with the return of cholera to South America, in the form of an explosive epidemic that began in Peru in January 1991 (241, 331, 373, 440). This represents the first time that cholera had entered this continent in more than a century, since the fifth pandemic struck in the 1880s.

The epidemic in Peru began in three distinct foci along the Pacific coast, separated from one another by several hundred kilometers (241, 373, 440). Extraordinarily high attack rates were recorded during the first few months, particularly among adults. Within a few weeks epidemic cholera was reported in neighboring Ecuador to the north and then in Colombia. In each of these countries, cholera struck underprivileged low socioeconomic populations lacking bacteriologically monitored piped water and sanitation (331). By mid-April a small outbreak occurred in metropolitan Santiago, the capital of Chile (241). From these countries along the Pacific coast of South America, cholera began to travel north, east, and south to progressively enter more countries in South and Central America. Notifications of cholera markedly diminished during the cool season in South America, but with the return of warm weather in December 1991, the incidence of cholera once again rose. The Pan American Health Organization estimates that in 1991 and 1992 there occurred 750,000 cases of cholera in the Americas accompanied by 6,500 deaths. More than one-half of these cases occurred in Peru.

An eighth pandemic? Beginning in late 1992 and continuing into 1993, epidemic cholera was reported in Madras and other sites in India and in Bangladesh (53, 357, 442). Although the clinical syndrome was typical cholera, manifested by passage of voluminous stools of rice water character leading to dehydration and hypovolemic shock, the causative agent was not *V. cholerae* O1 but rather *V. cholerae* expressing a distinct surface antigen (now referred to as O139). From these initial sites this organism has since spread throughout India, and outbreaks or cases have been reported in Pakistan, Nepal, China, Thailand, Kazakhastan, Afghanistan, and Malaysia (45, 54, 354). Imported cases have been reported from the United States (45) and from the United Kingdom (354). If outbreaks of cholera due to this new serogroup continue to occur in newly affected countries, this may represent an eighth pandemic (442).

Reservoirs

Environment. *V. cholerae* is part of the normal, free-living (autochthonous) bacterial flora in estuarine areas (66, 67, 205, 375). Non-O1 strains are much more commonly isolated from the environment than are O1 strains, even in epidemic settings in which fecal contamination of the environment might be expected. Outside of epidemic areas (and away from areas that may have been contaminated by cholera patients), O1 environmental isolates are almost always CT negative (285, 304, 375, 464). However, it is clear that CT-producing *V. cholerae* O1 can persist in the environment in the absence of known human disease (78, 396, 478). Periodic introduction of such environmental isolates into the human population through ingestion of uncooked or undercooked shellfish appears to be responsible for isolated foci of endemic disease along the U.S. Gulf Coast and in Australia (31, 257, 260, 396, 478). Environmental isolates (introduced again by seafood vehicles) may also have been responsible for the initial case clusters in the South American epidemic.

The persistence of *V. cholerae* within the environment may be facilitated by its ability to assume survival forms, including a viable but nonculturable state and a "rugose" survival form (described below). *V. cholerae* produces a chitinase and is able to bind to chitin, a semitransparent material, predominantly mucopolysaccharide, which is the principal component of crustacean shells (67, 309); the organism can grow in media with chitin as the sole carbon source (309). *V. cholerae* strains colonize the surfaces of copepods (182, 454), with preferential attachment to the oral region and the egg sac (67). Between 10^4 and 10^5 *V. cholerae* organisms may attach to a single copepod (67). Other aquatic biota, such as water hyacinths from Bangladeshi waters, have also been shown to be colonized by *V. cholerae* and to promote its growth (420).

While the estuarine environment represents an ideal setting for survival and persistence of *V. cholerae*, cholera has also become endemic in arid and inland areas of Africa that are distant from coastal waters (134, 279). The environmental reservoirs in these areas are not well defined; if such reservoirs exist, they may differ substantively from those in "traditional" zones of cholera endemicity, such as India and Bangladesh.

Data on *V. cholerae* O139 in the environment are less complete than those available for O1 strains. In an initial study conducted in Bangladesh, 12% of water samples tested contained CT-producing *V. cholerae* O139. In contrast, *V. cholerae* O1 has traditionally been isolated from less than 1% of water samples, even during an epidemic (185). This may reflect the size and force of the O139 epidemic; alternatively, patterns of distribution of *V. cholerae* O139 within the environment may more closely approximate patterns seen with non-O1 compared with O1 strains.

(i) Viable but nonculturable state. *V. cholerae* is capable of entering a dormant state in which it is still viable but not culturable in conventional laboratory media (reviewed in reference 65). Such a dormant state has been described for a number of bacterial species as a survival strategy in the natural environment (378). In this dormant state, the cells are reduced in size and become ovoid (67). At the macromolecule level, an initial rapid decline in total lipids and carbohydrates and a more gradual decline in proteins and DNA are observed (67, 177). The continued viability of the nonculturable *V. cholerae* can be assessed by a direct viable count procedure in which cells are incubated in the presence of yeast extract and nalidixic acid (480). The antibiotic inhibits cell division so that in response to yeast extract the viable cells elongate and are thus identified microscopically.

The viable but nonculturable state can be induced in the laboratory by incubating a culture of *V. cholerae* in phosphate-buffered saline at 4°C for several days. Although these cells are not culturable with nonselective enrichment broth or plates, Colwell and colleagues (63) reported that culturable cells could be recovered when the suspension was injected into ligated rabbit ileal loops (63). To assess the relevance of these observations in humans, a suspension of viable but nonculturable *V. cholerae* O1 was ingested by volunteers. Preliminary results indicated that culturable *V. cholerae* O1 could be recovered from stools of two of nine subjects ingesting nonculturable cells of *V. cholerae* O1 (68).

The direct viable count procedure was combined with fluorescence microscopy, using a monoclonal antibody directed against the O1 antigen. In one study, Brayton et al. (33) examined pond and water samples in Bangladesh and found that the direct viable count-fluorescent antibody procedure

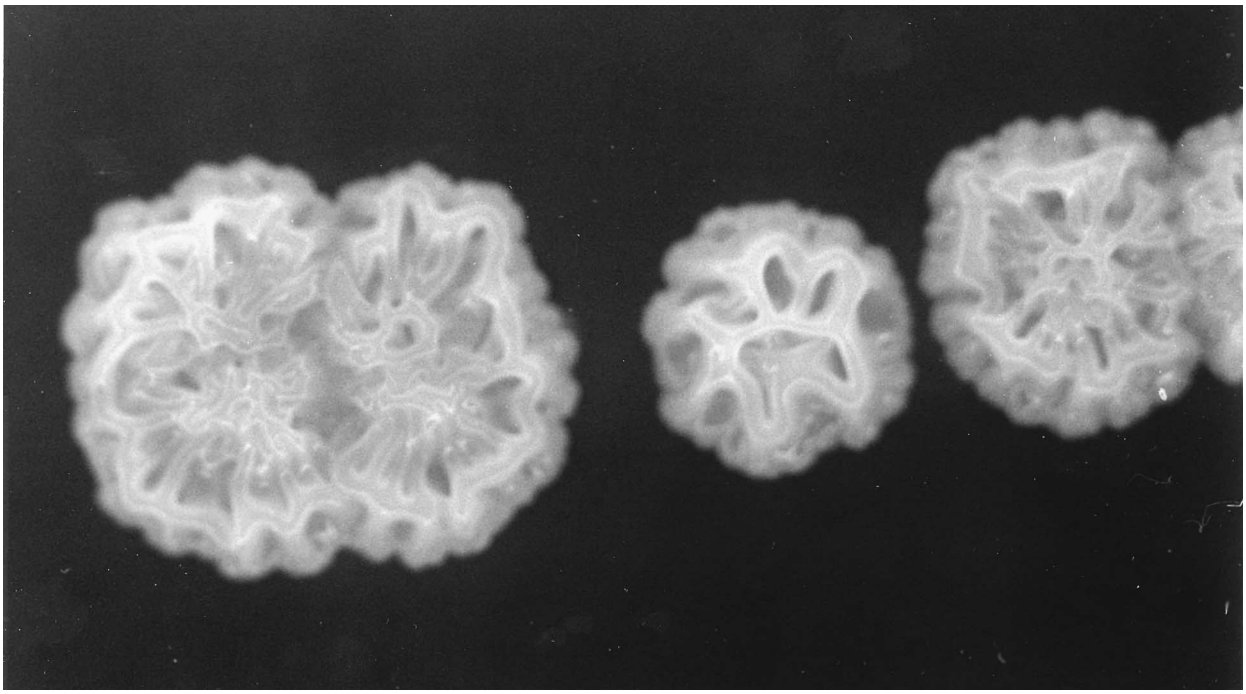

FIG. 4. Appearance of the rugose form of *V. cholerae* on Luria agar.

gave higher counts of viable *V. cholerae* O1 than did conventional culture techniques. Several water samples that were negative for culturable *V. cholerae* O1 were nonetheless positive for *V. cholerae* O1 by the direct viable count-fluorescent antibody technique.

(ii) Rugose survival form. As initially noted by Bruce White in 1938 (473, 474), *V. cholerae* can assume a rugose or "wrinkled" colonial morphology when passaged on a nonselective media such as Luria-agar (Fig. 4) (364, 365). All *V. cholerae* (O1 and non-O1) strains are able to assume this rugose phenotype (291). On Monsur's tellurite-taurocholate gelatin agar, the rugose colony does not appear to be gelatinase positive and so could be discarded as an *Aeromonas* species. On selective agar such as TCBS, the rugose form appears to be smooth. Upon laboratory passage, one in 1,000 smooth strains will shift to a rugose form; when a rugose strain is passaged, approximately 1 in 1,000 of the resultant colonies will shift back to a smooth morphology. The rate of shift to rugose forms can be increased by stress such as passage in alkaline peptone water. In contrast to rough forms, rugose variants are fully virulent in animal models and in volunteers (291).

When examined by light and electron microscopy, cells in a rugose culture are small and spherical and are embedded in an amorphous matrix material (291). The matrix material, or exopolymer, appears to be composed primarily of carbohydrate. Due presumably to the presence of this material, rugose cells tend to aggregate, with 1 to 2% of bacteria within a rugose culture present in clusters of more than two cells (including large aggregates composed of 50 to 100 bacteria). The cell aggregation (and, possibly, the exopolymer itself) provides protection against adverse environmental conditions.

Of particular concern from a public health perspective has been the observation that rugose variants survive in the presence of chlorine and other disinfectants (291, 364). In studies conducted by the U.S. Environmental Protection Agency with clinical isolates from Peru, smooth *V. cholerae* variants were consistently inactivated in less than 20 s when exposed to 0.5 mg of free chlorine per liter. In contrast, disinfection of rugose variants displayed a deviation from first-order kinetics: there was an initial drop in counts (due primarily to killing of single cells), followed by persistence of a resistant subpopulation of cells, presumably within aggregates. In an effort to totally eradicate rugose strains, bacteria were exposed to 2 mg of free chlorine per liter at pH 7.0 and 20°C; starting with an inoculum of ca. 10^6 CFU, *V. cholerae* were still recoverable after 30 min.

Humans and animals. Persons with acute cholera excrete 10^7 to 10^8 *V. cholerae* organisms per g of stool (249, 250); for patients who have 5 to 10 liters of diarrheal stool, total output of *V. cholerae* can be in the range of 10^{11} to 10^{13} CFU. Even after cessation of symptoms, patients who have not been treated with antibiotics may continue to excrete vibrios for 1 to 2 weeks (86). A very small minority of patients ($<<1\%$) may continue to excrete the organism for even longer periods of time (86, 279); for example, there is one often cited report of a patient ("Cholera Dolores") who continued to have positive stool cultures for *V. cholerae* for almost 10 years after an initial case of cholera (12).

As indicated above, a high percentage of persons infected with *V. cholerae* in areas of endemicity have inapparent illness. These persons also excrete the organism, although excretion generally lasts for less than a week (86). Asymptomatic carriers are most commonly identified among household members of persons with acute illness: in various studies, the rate of asymptomatic carriage in this group has ranged from 4% to almost 22% (86). There have also been studies indicating that *V. cholerae* O1 can be carried by household animals, including cows, dogs, and chickens (279).

In the 1970s, it was widely accepted that asymptomatic and convalescent human (and, possibly, animal) carriers were the primary reservoir for cholera (86). With the recognition that *V.*

cholerae can live and multiply in the environment, much greater attention has been given to identification and characterization of environmental reservoirs. Nonetheless, CT-producing *V. cholerae* O1 (i.e., disease-causing strains) continue to be isolated almost exclusively from areas that have been contaminated by human feces or sewage from persons or groups of persons known to have had cholera (285, 375); similarly, in areas of endemicity such as Lima, Peru, rates of isolation from the environment correlate primarily with degree of sewage contamination (117, 452). In Bangladesh, there are also data indicating that severe cases (and inapparent cases) can be present in the community throughout the year, providing a ready source for the organism (133). While arguments can be made that humans do not constitute a true "reservoir" for disease-causing strains, there is clearly a dynamic relationship between human and environmental sources of the organism, with carriage and amplification by human populations playing a critical role in epidemic spread of CT-producing *V. cholerae*.

Seasonality

Once endemicity is established in an area, cholera tends to settle into a clear seasonal pattern. In Bangladesh, for example, the major cholera season (for El Tor cholera) begins after the monsoons, in August or September, with the number of cases reaching a peak in the winter period 1 to 3 months later and then rapidly declining. The beginning of the cholera season coincides with the warmest mean temperature, a fall in the level of river water, and the cessation of rainfall; it ends with the dry, cold weather (129, 133). For reasons that are not well defined, cases caused by classical biotypes tend to occur somewhat later, with onset in November or December (133). Seasonal patterns also differ by geographic area, with peak cholera season in Calcutta, India (less than 200 miles away), occurring in April, May, and June (129). Cholera in South America has developed a similar periodicity, with cases concentrated in the summer months of January and February.

Studies in Bangladesh indicate that when cholera season starts, illnesses occur simultaneously in multiple locations and are attributable to multiple strains (i.e., there is not a discernible pattern of spread of a single strain from one community to another) (133). However, the underlying mechanisms are still not well understood. It is possible that an (as yet undefined) environmental cue triggers a rapid increase in counts of *V. cholerae* in the environment; as human cases start to occur, they, in turn, amplify the number of organisms present, leading to even more cases. Colwell and coworkers have hypothesized that zooplankton blooms play a key role in this process: as noted above, *V. cholerae* colonize copepods and other zooplankton, and zooplankton blooms are known to occur in Bangladesh after the summer monsoon, when cholera season starts (67).

Infectious Dose

As discussed above, doses of 10^{11} CFU of *V. cholerae* were required to consistently cause diarrhea in healthy North American volunteers when the inoculum was given in buffered saline (pH 7.2) (39, 245). When stomach acidity was neutralized with 2 g of sodium bicarbonate immediately prior to administration of the inoculum, attack rates of 90% were seen with an inoculum of 10^6 (245). Food has a buffering capacity comparable to that seen with sodium bicarbonate. Ingestion of 10^6 vibrios with food such as fish and rice resulted in the same high attack rate (100%) as when this inoculum is administered with buffer (245).

Fewer data are available on inoculum size in naturally occurring infections. In one study in Bangladesh, it was almost always necessary to use enrichment techniques to isolate *V. cholerae* from household water samples, suggesting that counts were <500 CFU/ml. At the same time, attack rates in the households surveyed were only 11%, and only half of infected persons had clinical illness, in keeping with the hypothesis that inoculum sizes were relatively low (421). While conditions will obviously vary widely from one community to another, studies such as these have led to the suggestion that the inoculum in nature is in the range of 10^2 to 10^3 (134).

Susceptibility to cholera depends also on largely unknown host factors. Individuals of blood group O are at increased risk of more severe cholera, which has been shown for natural infection (55, 136, 411) as well as with experimental infection (252, 448). Interestingly, the population of South America has one of the highest incidences of blood group O in the world, while the population of Bangladesh has one of the lowest incidences of this blood group.

Age and Sex Distribution of Cases

In areas of endemicity such as Bangladesh, cases of cholera are concentrated in children aged 2 to 9 years, with a secondary peak in women in their childbearing years (15 to 35 years) (133). This appears to be a reflection of underlying immunity in the population, with an initial infection with *V. cholerae* O1 providing protection against subsequent disease. The decreased rates in children under the age of 1 year may relate to decreased exposure, to the protective effect of breast feeding or breast milk (58), or, possibly, to decreased utilization of hospital facilities (and, consequently, to decreased case ascertainment). The increased rates in women of childbearing age may again be a function of case ascertainment or may relate to factors such as decreased immunity or increased risk of exposure to high inoculum levels (which might "override" preexisting immunity) while caring for children (133).

In contrast to the above findings, cholera, when introduced into populations lacking prior exposure to the disease, tends to occur with equal frequency in all age groups (134, 168). This was clearly seen in the South American epidemics: for example, in Trujillo, Peru, attack rates were 0.5% for children less than 1 year of age, 0.5% for children 1 to 4 years of age, and 0.6% for older children and adults (440). A similar pattern was seen in the initial epidemics with O139 strains in Bangladesh. In contrast to findings with endemic O1 strains, the majority of O139 cases occurred in adults (70% or more in persons over the age of 15); rates of severe illness were also higher in men than in women (2). The observation that O139 cases were concentrated in adults suggests that there was not preexisting immunity to the strain in the Bangladeshi population; this, in turn, suggests that immunity to O1 strains is not protective against subsequent infection with O139 strains.

Vehicles of Transmission

The critical role of water in transmission of cholera has been recognized for more than a century. As previously noted, the classic demonstration of this came in 1854, during the second cholera pandemic, when the London physician and epidemiologist John Snow showed that illness was associated with consumption of water from a water system that drew its water from the Thames River at a point below major sewage inflows (416).

This work has been followed by numerous studies highlighting the role of water in spread of the disease (134, 350). In one illustrative study in Bangladesh, 44% of surface water sources

in communities with cholera were culture positive for the organism; not unexpectedly, there was a significantly increased risk of infection associated with use of water from culture-positive sources for cooking, bathing, or washing (but, interestingly, not with drinking) (180). Water has also been implicated in spread of the South American epidemics. In case control studies in Trujillo and Piura, Peru, drinking unboiled water was significantly associated with illness; fecal contamination of municipal water was common, and in Trujillo, the epidemic strain of *V. cholerae* O1 was isolated from the municipal water supply (373, 440).

In these and other recent studies (76, 180, 440, 456), attention has also been given to the potential risks posed by open, wide-mouthed water storage containers, which tend to be ubiquitous in households in cholera-endemic areas. Even if the water received by a household does not initially contain *V. cholerae*, the contents of such containers are easily contaminated, significantly increasing the risk for intrafamilial spread of the disease. Taking this one step further, a study in Calcutta, India, has shown that introduction of narrow-mouthed water storage containers (i.e., containers into which hands, utensils, etc., cannot be placed) results in a significant reduction in disease transmission (76).

Cholera can also be transmitted by food. Seafood may acquire the organism from environmental sources and may serve as a vehicle in both endemic and epidemic disease, particularly if it is uncooked or only partially cooked (14, 31, 78, 107, 223, 257, 260, 464). There are also data suggesting that vegetables irrigated with untreated sewage can harbor and transmit *V. cholerae* O1 (62, 241). Food within households (or institutions [439]) may be contaminated by food handlers (168, 425), or water used in preparation of the food may contain the organism (as has been suggested in an outbreak of cholera that occurred on a U.S. Gulf Coast oil rig [196]). A recent small cluster of cases in the United States was attributed to frozen coconut milk imported from Thailand: among other possible sources of introduction of the organism, canal water was used to wash the floor on which the coconut meat was chopped (457).

As noted above, food buffers *V. cholerae* against killing by gastric acid. It can also provide an ideal culture medium: cooked rice, for example, has been shown to support rapid growth of *V. cholerae* (228), as do neutral sauces such as peanut sauce (more acidic sauces, with a pH of 5.0 and below, appear to provide protection against the organism [425]). While food has been frequently implicated as a vehicle responsible for introduction of cholera into a new area (134, 350), the potential advantages of food-borne transmission (protection against gastric acidity and opportunity for growth of the organism) raise the possibility that food plays a larger role than previously recognized in transmission in areas of endemicity. In keeping with this hypothesis, in studies in Piura, Peru, drinking unboiled water (62% of cases; odds ratio of 6.2), eating food from a street vendor (26% of cases; odds ratio of 17.5), and eating rice >3 h old without reheating (32% of cases, odds ratio of 6.2) were all independently associated with illness (373).

IMMUNE RESPONSE AND VACCINES

Over 100 years of research have been directed towards the development of a successful vaccine against cholera. Development of an effective vaccine is a very realistic goal due to the high degree of long-term protective immunity engendered by an initial clinical infection with *V. cholerae* and the minimal antigenic variation exhibited by this organism. Recent advances in our understanding of the intestinal immune system and in the molecular pathogenesis of *V. cholerae* have brought the goal of an effective vaccine significantly closer.

Infection-Derived Immunity

The strongest evidence for the existence of infection-derived immunity to *V. cholerae* comes from volunteer studies conducted at the University of Maryland. These studies show that volunteers experimentally infected with virulent *V. cholerae* are solidly protected against illness upon subsequent reinfection with *V. cholerae* (39, 251). Levine et al. (240, 245, 251) showed that an initial clinical infection with classical biotype *V. cholerae* O1 provides 100% protection against subsequent disease due to classical biotype vibrios while initial clinical infection with the El Tor biotype provides 90% protection against rechallenge with El Tor organisms. Protection against disease conferred by an initial infection with a classical strain lasts for at least 3 years, the longest interval tested within the same biotype (242). Initial infections with strains of either the Inaba or Ogawa serotype provide protection against reinfection with either serotype; volunteer studies to determine the degree of cross-protection between biotypes have not been performed with virulent strains. The slightly lower protection against disease conferred by El Tor strains correlates with results of bacteriological examination of stool samples. When veterans of a classical strain challenge were rechallenged, it was not possible to culture vibrios directly from the stools of these volunteers (240). However, approximately one-third of volunteers who were challenged and subsequently rechallenged with an El Tor strain yielded positive direct stool cultures for the challenge strain.

Epidemiological studies in Bangladesh support the concept that substantial protective immunity is engendered by infection with *V. cholerae*. Glass et al. (138) concluded that an initial infection severe enough to warrant treatment at a health care facility reduced the risk of subsequent clinical infection by about 90%. These results contrasted with an earlier study by Woodward (477), who reported 14 cases of cholera reinfection, half of which were asymptomatic. Woodward concluded that an initial infection with *V. cholerae* did not confer long-lasting immunity to subsequent infections, but it was not clear in this study whether this rate of reinfection is similar or lower to what was expected in the absence of previous exposure. Recently, Clemens et al. (59) also found evidence of infection-derived immunity. These investigators noted a striking difference between biotypes: an initial infection with a classical strain of *V. cholerae* was associated with complete protection, while initial infections with El Tor strains were associated with insignificant protection.

Components of the Immune Response

Although strong protective immunity is conferred by an infection with *V. cholerae*, the identity of the crucial protective antigens is unclear. The components of the immune response have recently been reviewed (253, 437). Both antibacterial immunity and antitoxic immunity exist, with antibacterial immunity being the most important component. The fact that *V. cholerae* is infrequently recovered from the feces of rechallenged volunteers indicates that strong antibacterial immunity exists, interfering with bacterial survival and/or growth. Recent clinical and field trials have also corroborated the importance of antibacterial immunity. A live recombinant *V. cholerae* vaccine strain, JBK70, which expresses neither the A nor B subunit of CT conferred protective immunity equivalent to that conferred by the toxigenic parent strain (see below) (250). A

recent large-scale field trial in Bangladesh demonstrated that an oral vaccine consisting of killed *V. cholerae* organisms can confer substantial protective immunity (56). In contrast, parenteral vaccines consisting only of toxoids gave little, if any, protection in field trials in Bangladesh (73) and the Philippines (316) or in volunteer studies in North Americans (248).

A synergistic effect has been noted in several animal studies in which the combination of bacterial antigens and toxoids gave higher protection than either component alone (340, 346, 434). The recent field trial in Bangladesh showed that in the initial 6 months of observation following vaccination the combination of whole vibrio and toxoid antigens gave better protection than whole vibrios alone (56). It should be noted, however, that in the last 6 months of follow-up (30 to 36 months after vaccination) the opposite was observed; i.e., the whole vibrio vaccine was significantly more protective than the combination (253).

The best correlation with protection is a serum vibriocidal antibody response. This assay measures the killing of *V. cholerae* cells in the presence of immune sera and complement. Studies in Bangladesh have shown that the prevalence and geometric mean titer of vibriocidal antibody increases with age and that, for every twofold increase in geometric mean vibriocidal titer, the incidence of cholera falls by approximately one-half (298, 299). In contrast, the prevalence and titer of serum antitoxin are not correlated with protection from cholera (22, 139). It is not believed that vibriocidal antibodies in the serum are the actual mediators of protective immunity, but rather the presence of such antibodies serves as a marker for the presence of secretory IgA intestinal antibodies which may be directed against the same antigens. It is believed that the secretory IgA intestinal antibodies mediate the actual protection.

The majority of vibriocidal antibodies can be absorbed with *V. cholerae* LPS (173). The crucial role of LPS in protective immunity is indicated by the significant protection seen in a field trial with a parenteral vaccine consisting of purified LPS (300). In addition, a vaccine consisting of attenuated *Salmonella typhi* expressing genes for *V. cholerae* LPS engendered detectable, albeit low, protection in volunteer studies (445) (see below). Numerous animal studies have also shown an important role for LPS in protective immunity (8, 122, 476). However, seroepidemiological studies in Bangladesh have not shown a relationship between levels of anti-LPS antibodies and protection (138).

In addition to the vibriocidal antibodies directed against the LPS, there is a smaller component of vibriocidal response consisting of antibodies directed against a protein component that has not been definitively identified. Proteins that have been shown to be protective in animal studies include flagellar protein, flagellar sheath protein, cell-associated HAs, a 30-kDa surface protein, OMPs, and TCP (46, 47, 93, 178, 187, 200, 395, 410, 489). A number of lipoproteins regulated by ToxR, including TcpC and AcfD, have been shown to be involved in resistance of *V. cholerae* to the bactericidal effect of immune sera and complement (333). In human convalescent-phase sera, antibody responses against a variety of outer membrane proteins have clearly been demonstrated (393). TCP has clearly for classical *V. cholerae* been proven to be an essential colonization factor in humans (164), yet interestingly, convalescent-phase sera from volunteers did not contain antibodies against TCP (153). Sera from three of six Indonesian cholera patients contained antibodies against TCP, but it appears that long-term protection against cholera can be accomplished in the absence of a detectable anti-TCP immune response. As noted above, there are a number of immunogenic proteins that are expressed only in vivo and not in vitro so that some antibodies in convalescent-phase human sera and jejunal fluids are directed against proteins expressed by *V. cholerae* grown in vivo in rabbit ligated loops but not expressed by *V. cholerae* grown in vitro in broth (201, 366). These results indicate that a complete picture of protective antigens of *V. cholerae* may not be achieved by examination of in vitro-grown bacteria only.

Vaccines

Within a year of the initial isolation of *V. cholerae* by Koch in 1883 (225), a vaccine consisting of broth cultures of *V. cholerae* was administered parenterally (32). A number of parenteral vaccines were tested in the next 90 years; these have been reviewed elsewhere (248, 253). The parenteral vaccine currently licensed in the United States consists of phenol-killed *V. cholerae* organisms and engenders only moderate (ca. 50%) efficacy of short-lived (3 to 6 months) duration. The vaccine is also reactogenic, and a variety of symptoms such as local pain, erythema, induration, fever, malaise, and headache have been noted (444). Because of the modest and short-lived efficacy and the fact that such vaccines do not prevent asymptomatic infection, such vaccines are not recommended by the WHO (135).

In recent years, the importance of the intestinal immune system has been recognized, thus prompting a shift from parenteral to oral vaccines. Since *V. cholerae* colonizes the intestinal mucosal surface without invasion of enterocytes, the protective immune response is believed to reside at the mucosal surface without a major contribution from serum antibody. To maximize the intestinal secretory antibody response, antigens must be delivered directly to the mucosal surface, i.e., by an oral route (253). Parenteral administration of an antigen can result in an increase in intestinal secretory IgA antibodies, but such a response is short-lived and is usually restricted to immunologically primed individuals (253). In contrast, oral immunization stimulates a long-lasting memory so that the primed intestinal immune system can rapidly respond to a subsequent exposure to antigen.

Since 1980, a number of new vaccines have been developed on the basis of recent advances in our knowledge of the intestinal immune system and genetic engineering technology. A more complete review of these vaccines can be found elsewhere (253). The vaccines that are currently being evaluated in clinical trials consist of killed whole-cell vibrio preparations or live attenuated bacterial strains, both of which are delivered orally.

Whole cell-toxoid. Svennerholm and Holmgren developed an oral combination vaccine consisting of 1 mg of purified CT B subunit and killed *V. cholerae* cells (436). The whole-cell component in a dose of vaccine contains three *V. cholerae* strains totalling 10^{11} cells. The strains represent both biotypes and serotypes and are killed by either heat or formalin. This vaccine was evaluated in a randomized, placebo-controlled, double-blinded field trial in Bangladesh (56). In total, 63,000 participants were randomized to receive three doses (6 weeks apart) of the combination of killed whole cells plus B subunit (B-WC), the whole-cell component without B subunit, or a placebo consisting of killed *E. coli* K-12. Both vaccines were safe and protective. For the first 6 months of surveillance, the B-WC vaccine had a significantly higher level of protection (85%) than the whole cell alone (58%). However, after the initial 6 months, the advantage conferred by the B subunit disappeared, so that over the full 36 months of follow-up, B-WC gave 50% and the whole cell gave 52% protection (253). Notably less efficacy was observed for children 2 to 5 years of

age after the first 6 months of surveillance; overall, a protective efficacy of only 23 to 26% was seen, in contrast to the 63 to 68% efficacy noted for those vaccinees greater than 5 years of age. With further analysis of the data, Clemens et al. (55) found that the efficacy of B-WC was substantially lower in persons of blood group O (a recognized host risk factor for cholera gravis) and against El Tor rather than classical biotype.

The B-WC vaccine also provided short-term protection for approximately 4 months against diarrheal disease due to LT-producing ETEC (57). The vaccine not only protected against ETEC expressing only LT (which cross-reacts immunologically with CT), but also against ETEC strains expressing both LT and ST, which shares no immunological similarity with CT. The protection against severe ETEC disease was 86%, while protection against milder disease was 54%. Two spaced doses of B-WC vaccine also conferred ca. 60% protection against LT-producing ETEC among Finnish travelers to Morocco over a period of several weeks of travel (339).

The B-WC vaccine is the best cholera vaccine so far developed for which results are available from a well-designed, large-scale field trial. The oral B-WC vaccine represents a significant advance over the parenteral vaccines. The ultimate usefulness of this vaccine may be limited for a variety of reasons, including (i) the transient protection in young children, (ii) lower protection in persons of blood group O, (iii) the multiple spaced doses required, and (iv) the potential expense of preparing the B subunit and the large number of inactivated bacteria required. Further modifications of the vaccine are in development, including the use of B subunit prepared by recombinant DNA technology (236) and enhanced expression of the TCP and MSHA antigens in the whole-cell component (172).

Live attenuated vaccine strains. (i) Principle. Since oral ingestion of a live, attenuated *V. cholerae* vaccine strain most closely mimics infection-derived immunity, this approach has received great emphasis in recent years. Live strains are more efficiently taken up by M cells, the major antigen sampling cells in the gut, than are killed cells (328). Furthermore, there are a number of antigens recognized during infection that are expressed on cells grown in vivo but not in vitro (201, 366). The feasibility of a live, attenuated vaccine was initially shown with *V. cholerae* Texas Star-SR, which was attenuated by chemical mutagenesis so that it produced the B but not the A subunit of CT (174, 244). Subsequent attenuated strains have been constructed by using recombinant DNA techniques to prepare mutants which are deleted of several hundred nucleotide base pairs, thereby avoiding the potential for reversion of a single-base-pair mutation.

The primary mutation in these attenuated strains involves the in vitro deletion of several hundred base pairs from the cloned *ctxA* gene encoding the A subunit. The *ctx* promoter and the *ctxB* gene encoding the B subunit are retained, and the mutated genes are introduced into a pathogenic *V. cholerae* strain previously shown to confer both disease and protective immunity in volunteers. Homologous DNA sequences flanking the mutated *ctx* genes and the chromosomal wild-type *ctx* genes allow recombination of the mutated genes into the chromosome in place of the wild-type genes. A variety of attenuated vaccine candidates have been developed by investigators at the Center for Vaccine Development of the University of Maryland (209, 210, 249, 250) and at Harvard University (276, 335).

(ii) Attenuated but reactogenic *V. cholerae* O1 strains. The first recombinant attenuated strains to be tested in volunteers were *V. cholerae* O1 El Tor Inaba JBK70, which lacks both the A and B subunits of CT ($A^- B^-$), and *V. cholerae* O1 classical Ogawa CVD101, which lacks only the A subunit ($A^- B^+$) (250). A single dose of either strain elicited strong vibriocidal antibody responses and CVD101 also stimulated antitoxic antibody. When volunteers who were immunized with a single oral dose of 10^6 *V. cholerae* O1 JBK70 were challenged with a virulent El Tor strain, significant protection was observed. Diarrhea occurred in 7 of 8 unimmunized controls but in only 1 of 10 vaccinees, for a vaccine efficacy (89%) equivalent to protection seen following infection with enterotoxinogenic El Tor strains (250). Furthermore, this significant protection occurred in the absence of immunity against cholera enterotoxin, thus demonstrating the importance of antibacterial immunity.

Although these initial attenuated strains were highly immunogenic, they were unexpectedly reactogenic, with more than one-half of the vaccinees developing symptoms such as mild diarrhea, abdominal cramps, anorexia, and low-grade fever (250). Levine et al. (250) reported that ca. 50% of volunteers ingesting doses ranging from 10^4 to 10^{10} Δ*ctx* organisms experienced mild to moderate diarrhea, with total diarrheal stool volumes ranging from 0.3 to 2.1 liters. The severity of diarrhea was not cholera gravis like. To place this reactogenicity in perspective, ingestion of the CT-positive strain Ogawa 395, parent strain of one of the Δ*ctx* strains, has resulted in over 40 liters of diarrhea (240).

While these strains were greatly attenuated compared with the parent strains, the degree of reactogenicity precluded any further clinical studies with these strains. Two hypotheses were advanced to explain this reactogenicity (250). The first hypothesis proposed that the diarrhea and other symptoms were due to additional toxins secreted by *V. cholerae*. The second hypothesis was that mere colonization of the proximal small intestine by adherent vibrios somehow perturbed intestinal function, leading to the diarrhea and other symptoms observed. It is still not clear which of these hypotheses is correct.

Additional potential enterotoxins including the hemolysin/cytolysin, Zot, and Ace have been identified (see above). The role of hemolysin was examined by constructing derivatives of CVD101 and JBK70 specifically mutated in the hemolysin gene. Volunteers who received the hemolysin-negative, CT-negative mutants still experienced diarrhea and other symptoms (250). As noted above, a recent derivative of *V. cholerae* O1 in which genes encoding the hemolysin/cytolysin, Zot, and Ace as well as the A subunit of CT were deleted was constructed. Despite lacking these secretogens, this strain, CVD110, still caused diarrhea and other symptoms in volunteers (446). CVD110 was highly immunogenic. The alternative hypothesis to explain the diarrhea produced by CT-negative strains is that mere colonization of the proximal small bowel by *V. cholerae* may result in diarrhea. The classic experiments of Smith and Linggood (415) demonstrated that in piglets ETEC strains that express both a colonization factor and an enterotoxin caused diarrhea; however, strains that had lost the enterotoxin but still expressed the colonization factor were able to cause mild diarrhea in about one-third of infected animals. Subsequent studies in humans (246) and rabbits (471) showed that a human strain of ETEC that had lost the enterotoxin but still possessed the colonization factor antigen II (CS1 and CS3) could still cause diarrhea. It has been suggested that normal metabolic or enzymatic products of bacteria colonizing the small bowel might act in the gut as secretagogues or as inhibitors of absorption, perhaps via the enteric nervous system (471).

Two volunteer experiments have been conducted to examine the role of colonization in reactogenicity and immunogenicity. In one study, strain CVD102, a thymine-dependent auxotrophic mutant of CVD101, was tested to determine whether

reactogenicity would result with a live but nonproliferating *V. cholerae* O1 strain. The strain was nonreactogenic at a dose of 10^7 but was poorly immunogenic, thus indicating that the auxotrophic mutation was overattenuating (250). In a second study, Herrington et al. (164) infected volunteers with *V. cholerae* O1 strains mutated in either the *tcpA* gene encoding the major subunit of the TCP colonization factor or the *toxR* global regulatory gene (which regulates TCP expression among other virulence factors). Both the *toxR* and *tcpA* mutants strains were nonreactogenic but were also poorly immunogenic. For both studies, the challenge strains were present in stool samples in very low numbers, if at all, suggesting that the strains colonized the intestine poorly. One interpretation of these results is that colonization of the small intestine is the cause of the residual diarrhea and other symptoms seen with the CT-negative strains; it might be difficult to achieve a level of colonization that is nonreactogenic but still sufficiently immunogenic. However, colonization may include two types of interactions of the organism with intestinal mucosal cells. The first type of interaction would be antigenic uptake by M cells overlying the gut-associated lymphoid tissue; such an interaction would lead to an immune response. A second type of interaction would be adherence to enterocytes, an interaction which may lead to reactogenicity. In this perspective, elucidation of the factors responsible for the residual reactogenicity of Δ*ctx V. cholerae* O1 strains could lead to significant insights into the pathogenicity and immunogenicity of *V. cholerae* and can result in highly immunogenic yet well-tolerated live oral vaccines.

(iii) *V. cholerae* CVD103-HgR. Whereas the first few generations of attenuated strains proved to be unacceptably reactogenic or insufficiently immunogenic, there is one recombinant *V. cholerae* O1 vaccine strain that has proven to be well tolerated and immunogenic in extensive clinical trials in multiple countries. *V. cholerae* CVD103-HgR is an attenuated derivative of the classical Inaba strain 569B (220, 249). The strain 569B was first attenuated by deleting 550 bp of the *ctxA* gene, thereby removing 94% of the region encoding the mature A_1 peptide of CT. The resulting strain, CVD103, was further mutated by inserting a gene (*mer*) encoding resistance to mercury into the *hlyA* locus of the chromosome; this marker serves to differentiate the vaccine strain from wild-type *V. cholerae* O1 (220). The vaccine is manufactured in a practical formulation consisting of two aluminum foil sachets, one containing lyophilized vaccine (and aspartame as sweetener) and the other containing buffer (to protect the vaccine from gastric acid).

The vaccine strain *V. cholerae* CVD103-HgR has been tested for safety and immunogenicity in over 4,000 subjects in trials conducted in the United States, Switzerland, Thailand, Indonesia, Peru, Chile, Costa Rica, Italy, Colombia, and Mexico (72, 230, 247, 249, 429, 430). After initial safety and immunogenicity trials in adults, the vaccine has been tested in children, including 1,178 5- to 9-year-old and 610 25- to 59-month-old children. In phase 2 clinical studies of CVD103-HgR using a randomized, placebo-controlled, double-blind trial design, no adverse reactions occurred significantly more in vaccinees than in placebo controls.

With the serum vibriocidal antibody response as the main measure of immunogenicity, CVD103-HgR has proven to be highly immunogenic. A study of North American adult volunteers showed that 92% of individuals who ingested a single dose of 5×10^8 CFU of CVD103-HgR manifested seroconversion (defined as a fourfold or greater rise in vibriocidal antibody titer) (249). In the same study population, a single dose of the live vaccine elicited mean vibriocidal antibodies three- to fivefold higher than those elicited by three doses of the oral inactivated vaccines. Interestingly, the same dose (5×10^8 CFU) of CVD103-HgR administered to low socioeconomic populations in less developed countries where cholera is endemic engenders much lower rates of seroconversion (429, 430). Several factors contribute to the somewhat lower immunogenicity in populations in developing countries. Many individuals in areas of endemicity already have elevated vibriocidal antibody titers prior to immunization and are presumably immune. This preexisting immunity clearly interferes with the "take" of a live oral vaccine, thus preventing significant increases in immune titers. There exists another barrier, however, unrelated to prior immunity. This nonimmune barrier consists of bacterial overgrowth in the proximal small intestine with anaerobic and enteric bacteria. These may inhibit the live vaccine strain. For individuals in less-developed countries, simply increasing the dose to 5×10^9 CFU elicits high rates of vaccine seroconversion. *V. cholerae* CVD103-HgR is recovered from stool samples at much lower levels than earlier, reactogenic *V. cholerae* strains, thus suggesting that CVD103-HgR does not colonize the human intestine as well as the reactogenic strains. However, this strain is highly immunogenic; perhaps the lack of reactogenicity is due to decreased adherence to enterocytes without a noticeable effect on cell uptake.

The ability of CVD103-HgR to confer protective efficacy against disease has been extensively tested in challenge studies in North American volunteers. A total of eight volunteer challenge studies have been conducted with CVD103-HgR or CVD103 (which differs from CVD103-HgR in being sensitive to mercury), and the results of these trials have recently been reviewed (247, 254). Each of these eight trials demonstrated significant protection against disease when volunteers were challenged with fully enterotoxigenic wild-type *V. cholerae* O1. Complete protection against severe diarrhea (defined as ≥5.0-liter total stool volume) and moderate diarrhea (3- to 5-liter stool volume) was observed. Some vaccinees experienced milder diarrhea when challenged with El Tor strains while nevertheless being protected against moderate or severe diarrhea. The overall protection from any diarrhea after challenge with classical biotype was 82 to 100% (irrespective of serotype) and 62 to 67% after challenge with El Tor. These studies demonstrate that *V. cholerae* CVD103-HgR is highly effective against the severe dehydrating diarrhea that makes cholera a significant public health problem.

The duration of protective immunity engendered by CVD103-HgR has not been established, but it is at least 6 months, the longest interval tested (447). A single dose of the vaccine provided 100% protection when volunteers were challenged with classical *V. cholerae* 569B 6 months after ingesting a single dose of the vaccine. Also of importance, the onset of protection is quite rapid; volunteers challenged a mere 8 days after vaccination were completely protected against cholera. The challenge studies in North American volunteers and safety and immunogenicity studies in countries endemic for cholera indicate that CVD103-HgR could be an important public health tool in conjunction with other cholera control measures. A large-scale, randomized, placebo-controlled, double-blind field trial to test the efficacy of *V. cholerae* CVD103-HgR in a country endemic for cholera has recently been initiated in North Jakarta, Indonesia, under the sponsorship of the WHO.

***Salmonella*-based vaccines.** An alternative approach to cholera vaccines using recombinant DNA techniques has been to use attenuated *Salmonella typhi* strains as a carrier of *V. cholerae* antigens. Such an approach could lead to a bivalent cholera-typhoid live oral vaccine. Initial vaccine constructs using this approach have used the attenuated *Salmonella typhi*

vaccine strain Ty21a containing cloned *V. cholerae* genes expressing the O antigen. One such strain was tested in volunteers for immunogenicity and protective efficacy. Three doses elicited modest immune responses in volunteers (9, 115) and conferred 25% protective efficacy against experimental challenge with wild-type *V. cholerae* O1 (445). Further derivatives containing additional *V. cholerae* antigens such as CT B subunit (60) and TCP may prove more successful in conferring protective immunity.

CONCLUDING REMARKS

There is a long history of scientific accomplishments resulting from the study of cholera and *V. cholerae*. This scientific legacy is notable not only for the observations related specifically to cholera but also for the numerous contributions to other fields of medicine. The proposition by John Snow (416), after analyzing cholera attack rates in relation to source of household water supply in London in the mid-19th century, that transmission of this disease was related to water source constituted an early landmark in epidemiology. The therapeutic efficacy of modern intravenous electrolyte solutions to treat severe diarrheal dehydration was presaged by Thomas Latta, who used intravenous injections of saline solution to treat patients with severe cholera in 1832 (234). Clinical trials demonstrating that glucose-coupled transport of sodium and water occurs in cholera patients who drink glucose-electrolyte solution paved the way for the further development and widespread use of oral rehydration in pediatric diarrhea of all etiologies in the developing world. Oral rehydration has been described as one of the most important therapeutic interventions developed during the 20th century. More recently, CT has been a crucial reagent in studying G proteins and mechanisms of eukaryotic cell signalling and regulation. Furthermore, CT, which has been shown to exhibit potent adjuvant activity that can enhance the immune response to concomitantly administered antigens, has become a popular reagent among immunologists who study the fundamental nature of immune responses.

However, despite more than a century of study, cholera still presents surprises and challenges to us. Since 1991 alone, the world has witnessed the extension of the seventh pandemic into South America, the one continent previously untouched by cholera in this century, and the apparent onset of a new (eighth) pandemic caused by a previously unknown serogroup of *V. cholerae* (O139). The O139 epidemic has been occurring in populations assumed to be largely immune to *V. cholerae* O1. These developments show that cholera is not a disease of merely historical interest that has been conquered and vanquished but still remains a fascinating and deadly disease about which microbiologists and physicians should be knowledgeable.

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