



DIARRHOEAL DISEASES CONTROL PROGRAMME

INDEXED



CHOLERA AND OTHER VIBRIO-ASSOCIATED DIARRHOEAS

Report of a Sub-group of the
Scientific Working Group on Epidemiology and Etiology
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17 diarrhoeal diseases

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1. INTRODUCTION

The seventh cholera pandemic, which began nearly 20 years ago, is continuing to spread. Eight new countries were affected in 1978 and several previously affected countries in Asia and Africa experienced severe recrudescences and extensions of the disease, causing major problems for national health authorities. The pandemic has stimulated extensive and intensive research on cholera and related subjects during the last two decades. This research has made many significant contributions to our understanding of the etiology, epidemiology, pathogenesis, clinical management, and immune mechanisms of all acute diarrhoeas, and has made it possible to reduce cholera-related mortality to less than 1% in well-equipped treatment centres. These developments, particularly the simplification of the treatment for cholera, have led to the creation of the WHO Programme for Diarrhoeal Diseases Control (CDD).

The purpose of the meeting of the Sub-group was to review significant recent information on the epidemiology and bacteriology of diarrhoeal diseases caused by *Vibrio* species and related organisms, to identify remaining gaps in knowledge, and to recommend priority areas for further research in these fields. The related subject of vaccine development was also reviewed briefly and constitutes an updating of the report of the Scientific Working Group on Immunity and Vaccine Development.

2. SOME DEFINITIONS

The Group recognized that the nomenclature and taxonomy of the genus *Vibrio* was in an uncertain state and that change was needed. Until recently, the name *Vibrio cholerae*, or the term cholera vibrio, had generally been restricted to the organism that caused epidemic cholera, and terms such as non-agglutinating vibrios (NAGs) and non-cholera vibrios (NCVs) had been used rather ambiguously to describe either all vibrios, including the halophilic vibrios, that did not agglutinate in cholera O-Group 1 antiserum (polyvalent O-1 antiserum), or only the group of vibrios that were biochemically similar to the epidemic cholera strains. Uncertainty about which meaning authors gave these terms made communications between scientists difficult and impeded the development of knowledge about vibrios other than the epidemic strains. When taxonomists recently sought to clarify the nomenclature of the genus *Vibrio*, they unfortunately placed all vibrios that were similar biochemically and by DNA homology to the epidemic strain into one species: *V. cholerae*. The epidemic strains were then classified as *V. cholerae* O-Group 1, and the others as different serotypes of the same species.

The Group expressed its strong disfavour of the inclusion of these other vibrios, many strains of which are non-pathogenic or of doubtful pathogenicity, in the species *V. cholerae* which to the public, microbiologists, physicians, and public health personnel has an implied epidemic potential. Another species name that did not already have a well-established and emotive meaning should have been selected. As reflected in this report, there are important epidemiological and biological distinctions between the epidemic strains and these other vibrios. However, in the absence of a more satisfactory alternative, the Group elected to use the nomenclature recommended by the taxonomists, with some modifications, but emphasized that this should not be considered as constituting WHO approval of the nomenclature.

Thus in this report the following definitions will be used:

(1) The epidemic strain will be referred to as *V. cholerae* O-Group 1 (epidemic strains), or *V. cholerae* O1.

(2) As will be detailed below, a few vibrio strains have been isolated that agglutinate in one or more lots of polyvalent O-1 antisera but are apparently non-pathogenic in that they do not produce enterotoxin in *in vivo* and/or *in vitro* assay systems. Some of these also have a few atypical biochemical properties. These strains will be referred to in the report as atypical *V. cholerae* O-Group 1, or atypical *V. cholerae* O1, in recognition of the concept that they share with the vibrios responsible for epidemic cholerae an antigenic mosaic that should be recognizable by virtually any polyvalent *V. cholerae* antiserum of reasonable quality.

(3) Organisms that are similar biochemically to the epidemic strains but do not agglutinate in polyvalent O-1 antiserum will be referred to as non-O-Group 1 V. cholerae (non-epidemic strains), or non-O1 V. cholerae. These organisms do not appear to be responsible for epidemics of severe diarrhoea, although they have been associated with individual cases and small outbreaks of diarrhoea, and some appear to produce a cholera-like enterotoxin.

(4) Other vibrios, such as V. parahaemolyticus, V. alginolyticus, and "Group F vibrios", are clearly distinct species and will be so regarded in the report.

A summary of some of the characteristics of these groups is given in Table 1.

3. REVIEW OF RECENT KNOWLEDGE ON THE EPIDEMIOLOGY AND BACTERIOLOGY OF VIBRIOS

3.1 Epidemiological significance of vibrios

3.1.1 Vibrio cholerae O-Group 1

3.1.1.1 Global cholera situation

The number of countries affected during the present pandemic has increased in two major phases: a gradual increase from 1961 until 1966, and a major upsurge in 1970, when countries in Africa were infected. Since then, the number of affected countries has levelled off, although the number of new countries infected in 1978 (8 countries) was the highest recorded in any one year since the extension of cholera to the African continent. A source of continuing concern is that if cholera should reach any of the countries of South and Central America, which are considered to be "receptive", there could be another dramatic increase in the number of affected countries.

Since 1961, the El Tor biotype of V. cholerae O1 has rapidly replaced the classical biotype so that, in the 1970s, the global cholera programme has been virtually exclusively caused by the El Tor biotype; however, rare and isolated cases due to the classical biotype do still occur, such as that reported from India in 1978 and 1979 and Bangladesh in 1979 for reasons that are not known. The El Tor biotype appears to have a greater "endemic tendency" than classical V. cholerae O1. Also, in infections with the El Tor biotype, there has been a higher infection-to-case ratio, making surveillance, outbreak investigation, and control potentially more difficult.

The strains causing the present pandemic were initially differentiated as the El Tor biotype because of their ability to haemolyze sheep erythrocytes. It was also shown that, unlike the classical biotype, they agglutinated chicken and sheep erythrocytes and were resistant to classical phage IV and to polymyxin B - 50-unit disc (the Voges-Proskauer reaction was not as reliable). As the pandemic has progressed, the proportion of strains isolated that are haemolytic has gradually decreased, and now for routine practical purposes El Tor strains are indistinguishable in this test from the classical biotype. Generally, however, these isolates still behave as El Tor in the other biotyping tests, although these tests need to be done with great care to give consistent results.

3.1.1.2 The carrier state

It is generally agreed that the true long-term carrier state is extremely rare in cholera. Occasional cholera patients - such as "Cholera Dolores" in the Philippines - become long-term carriers, but secondary outbreaks associated with such individuals have never been documented. It is not known if pre-existing inflammation of the gallbladder can predispose to the carrier state, or if a normal gallbladder can be infected. Either the biotype of V. cholerae O1 or the prior immune status of the host may be important in predisposing to carriage; when Iran was newly infected with El Tor V. cholerae O1 in this pandemic and convalescent cholera patients with culture-negative stools were purged with magnesium sulfate, cultures of the last portion of purged stool were frequently positive. However, when the same procedure was carried out in convalescent cases in an endemic area, Bangladesh, at a time when the classical biotype still prevailed, the cultures were usually negative.

3.1.1.3 The infectious dose in man

It is becoming increasingly evident that the number of vibrios required to cause symptomatic infections is lower than previously believed. When a strain of the classical biotype was administered to adult volunteers in water, the ID₅₀ was 10⁸-10⁹ vibrios. Prior administration of sodium bicarbonate reduced the infectious dose to approximately 10⁴⁻⁶. However, in recent studies using an El Tor strain, administration of 10³ vibrios with bicarbonate resulted in symptomatic infection in 4/6 volunteers challenged. It is clear from these volunteer studies and other epidemiological and clinical studies that gastric acidity is a major factor in host resistance, the disease being more common in persons with hypochlorhydria. Susceptibility may also be increased by more rapid gastric emptying following intake of large amounts of food and water. Volunteer studies with the El Tor biotype have established that the infectious dose is lower when organisms are administered with food than when they are given in a small amount of water. It is not yet known if food itself acts by neutralizing gastric acid or if vibrios are protected from acid by adhering to food particles. One related observation is that vibrio adhesion to chitin (crabshell) particles enhances vibrio survival in an acid environment. It is thus not unreasonable to assume that a dose as low as 10² or 10³ viable vibrios may be able to cause symptomatic infections, and one study of cholera in rural Bangladesh suggests that such doses may lead to symptomatic infection under natural conditions.

3.1.1.4 Mechanisms of transmission

Vibrio cholerae O1 can survive for varying periods and even multiply in various foods and water. Water and a variety of contaminated foods have been epidemiologically incriminated as vehicles of cholera transmission. In general, vigorous efforts have been needed to determine the mode of transmission in cholera outbreaks, especially in newly infected areas (see section 5). In some instances, epidemics have been aborted by rapid rational and instinctive measures to control suspect vehicles such as immediate chlorination of water supplies.

Man is the major source of infection and the disease is usually spread geographically by symptomatic and asymptomatic persons whose evacuations reach and contaminate food and water. The organisms are probably also transported between adjoining areas by moving bodies of water that have been contaminated with infected faeces. The possibility that other reservoirs of infection, such as infected shellfish or coastal waters, can maintain the vibrio for prolonged periods of time cannot, however, be excluded (see section 3.2).

There have been a number of other recent important epidemiological findings. For example, in Bahrain, the incidence of cholera has been found to be higher in bottlefed than in breastfed infants. It has been observed in both Calcutta, India, and in rural Bangladesh that the seasonal incidence of cholera can change dramatically over a short time. The disease used to be most common in the summer in Calcutta and the early winter in Bangladesh; now in both places it is most frequent in the autumn. Recent studies in rural Bangladesh have also shown that access to adequate supplies of pure drinking water may not lessen the incidence of cholera in some areas, especially those where sanitation is poor and impure water is used for other purposes (e.g., cooking).

3.1.1.5 Antibiotic resistance and treatment

The problem of the development of antibiotic resistance among V. cholerae O1 has been well documented in an epidemic of cholera which began in Tanzania in October 1977. All isolates during the first month following recognition of the disease were fully sensitive to tetracycline, but after 5 months of extensive therapeutic and prophylactic use of the drug (1788 kg during the first 6 months) 76% of isolates were found to be resistant. The resistance has been found to be mediated by at least 2 closely related plasmids belonging to the C incompatibility group, one of the few groups of enteric plasmids that can exist stably in V. cholerae O1. Both of these plasmids confer multiple antibiotic resistance.

This appears to be one of the first examples of an independent outbreak of R plasmid-carrying V. cholerae O1 strains. Despite a substantial reduction in the use of tetracycline after the detection of this phenomenon, resistance continues to be common among V. cholerae O1 isolates in Tanzania. The potential for spread of resistant strains to neighbouring countries is a matter of grave concern. The events in Tanzania should lead to a reappraisal of the use of antibiotics, particularly as a mass-prophylactic measure, in the control of cholera epidemics.

3.1.2 Atypical Vibrio cholerae O-Group 1

Before 1961, when the present cholera pandemic began, diarrhoeal illness caused by the El Tor organism was thought to occur only in Indonesia. However, El Tor vibrios had been isolated from surface waters in the Eastern Mediterranean and India long before that date. These El Tor "water vibrios" have subsequently been isolated from surface and well-waters both in cholera-free areas and in endemic areas during seasons when cholera was not occurring. Some of these isolates were studied extensively during the early 1960s as possible vaccine strains and were found to produce little if any cholera enterotoxin.

During the 1970s, such atypical V. cholerae O1 strains have been isolated in at least 8 areas from man and/or other sources. The available information about these strains is summarized below.

3.1.2.1 Recent isolates

- (a) In Guam in 1974, during an investigation of a small outbreak of cholera caused by V. cholerae El Tor, serotype Ogawa, 7 strains of atypical V. cholerae O1 were isolated from sewage (1), storm drains (4), a river (1), and a bay (1). Despite extensive culturing of persons with diarrhoea as part of the cholera investigation, no atypical strains were isolated from humans.
- (b) In April, 1977, a 61-year-old truck driver in Alabama, USA, suspected of having acute cholecystitis of 12 days' duration, underwent a cholecystectomy. V. cholerae El Tor, serotype Inaba, was isolated from a culture of the bile from the gallbladder obtained at surgery. The patient had not had a recent diarrhoeal illness, and except for a brief trip into Mexico 30 years previously, he had not travelled outside the USA. The patient had an Inaba vibriocidal titre of 1/320. His 3 family members were culture-negative and did not have elevated vibriocidal antibody titres. None of the 4 had serum antitoxic antibodies detectable by the rabbit-skin assay.
- (c) In Bangladesh in 1977, an attempt was made to detect atypical V. cholerae O1 strains in man and the environment. One of 82 isolates from the environment was non-toxicogenic, while all of 1275 clinical isolates were toxicogenic.
- (d) In 1977, one of 65 isolates of so-called Vibrio cholerae from Chesapeake Bay, on the eastern coast of the USA, proved to be atypical V. cholerae O1. At least one large hospital near Chesapeake Bay has been using TCBS agar* routinely for all stool cultures for at least 5 years, and has not detected any isolates of V. cholerae O1.
- (e) Since 1974, Brazil has been carrying out routine surveillance of sewerage systems for V. cholerae using Moore swabs. All tests were negative until May 1978, when 2 strains of V. cholerae O1 were isolated from sewage from Santos, a city near São Paulo. Although sewage surveillance was then intensified and TCBS agar introduced for culturing stools from persons with diarrhoea, no more V. cholerae O1 strains were detected. In October 1978, 2 strains of V. cholerae O1 were detected in sewage from the Rio de Janeiro sewerage system. There again, no human infections were identified.

* Thiosulfate Citrate Bile Salts Sucrose agar

(f) At least 11 sporadic cases of cholera caused by V. cholerae O1 El Tor Inaba occurred in Louisiana, USA, in 1978. During extensive culturing of persons with diarrhoea, raw crabs, shrimp, oysters, sewage and surface water, a few strains of V. cholerae El Tor Inaba were found; all were toxigenic and of the same phage type.

During 1979, surveillance of cases of diarrhoea and of sewerage systems throughout the southern half of Louisiana continued, and oysters from commercial distributors were routinely cultured. As of 17 September 1979, 11 apparently atypical V. cholerae O1 strains had been isolated, none of which had the same phage type as the 1978 toxigenic strains. Of these strains, one came from a canal with high faecal coliform counts, and 8 from oysters taken from supposedly clean areas. The remaining 2 isolates were from New Orleans and may represent the same strain, being identical in every laboratory test performed, including phage sensitivities. One of these was isolated from a large, necrotic ulcer on the leg of a vagrant. The man flushed the soiled bandages down his toilet daily and the second isolate was from a sewage line which drained an area that included the patient's toilet. This was the only isolate of V. cholerae O1 from New Orleans sewage during a period of more than 8 months of routine surveillance with Moore swabs.

(g) For 5 weeks during 1977, atypical V. cholerae O1 Ogawa strains were continuously isolated from a brackish agricultural drainage ditch in England where the possibility of sewage contamination was considered to be negligible.

(h) In May 1978, V. cholerae El Tor Inaba was isolated from a river near Yokohama Port in Japan. Environmental studies revealed that the source of this isolate was the septic tank of a hospital used for the disposal of artificial kidney dialysate. No cholera cases or carriers were found. Tests to date of these isolates indicate that they are non-toxigenic.

Finally, workers in the USSR have reported finding that 270 strains of V. cholerae O1 El Tor isolated over 11 years from various sources could be subdivided into 3 groups by using the suckling rabbit assay. One group, which was highly enterotoxigenic (choleraogenic), failed to lyse sheep erythrocytes. Another group was haemolytic and caused death in test animals without choleraogenic effects. The third group was also haemolytic but was avirulent in suckling rabbits, even when high doses were administered.

It is seen from this review that atypical V. cholerae O1 have been isolated primarily from environmental sources, and that, despite extensive searches for these strains in the stools of diarrhoea cases (e.g., in Guam, Brazil, and Louisiana) during and/or shortly after the time when atypical strains were being isolated from environmental sources, to date such isolates have been associated in man only with extraintestinal disease (cholecystitis, wound infection). It is also evident that these atypical V. cholerae O1 strains have a global distribution (Asia, the Pacific Islands, North America, Europe, and the Eastern Mediterranean). There is also some suggestion that these strains may be free-living; the strains in England and those from oysters from Louisiana appear to have come from areas without sewage contamination.

3.1.2.2 Bacteriological characteristics

Difficulties have been encountered in serotyping some of these strains, and particularly in using polyvalent antisera. Variable results were found using four different batches of polyvalent (O-Group 1) antiserum.

The biochemical reactions of some strains were also atypical. The majority of the strains from Guam fermented sucrose slowly. Seven of 8 isolates from oysters in Louisiana did not ferment mannitol; by the criteria of Hugh and Sakazaki, in fact, these strains were not Vibrio species, although in other respects they had typical characteristics.

Although all the recent strains were of the El Tor biotype, many had atypical biotype patterns. Those from Brazil were all sensitive to polymyxin B. The strain from Alabama and 3 strains from Louisiana were sensitive to classical phage IV in one laboratory; however, another laboratory found the Alabama strain to be resistant to this phage. The results of the chicken red blood cell agglutination and Voges-Proskauer tests also varied from strain to strain.

Most of these atypical strains were not markedly sensitive to any of the classical or El Tor phages. They were almost uniformly resistant to the phages 13, 14, 16, and 24 of the Public Health Laboratory, Maidstone, Kent, England. In contrast, all known V. cholerae O1 associated with human infections have, so far, been sensitive to one or more of these phages (see section 3.3).

3.1.2.3 Pathogenicity*

With the exception of the mouse lethal assay (intraperitoneal injection of living cells), which is of doubtful relevance, the results of tests for pathogenicity on these atypical strains have been almost uniformly negative. In the ligated rabbit ileal loop assay, the only suggestion of activity occurred in a few cases when live organisms were used; culture filtrates (unconcentrated) have all been negative. Some of the strains have produced haemorrhagic lesions in the rabbit intracutaneous assay.

In the Y-1 adrenal cell assay, the 2 strains from Santos, Brazil, gave some weakly positive reactions, but they were non-toxigenic in the ELISA and rabbit intracutaneous assays. They have been reported to produce the A but not the B portion of cholera toxin. Both strains have been given orally to volunteers. Strain 1074-78 was given to 7 volunteers in a dose of 10^6 organisms with bicarbonate: none became ill and their stools were culture-negative. Strain 1196-78 was given to 8 volunteers in a dose of 10^6 with bicarbonate: the stools of 6 were culture-positive and none became ill. Sera from these volunteers have not yet been tested to determine whether they developed antitoxic antibodies. Ten isolates from each of the 6 volunteers who excreted strain 1196-78 (a total of 60 isolates) were tested in the adrenal cell assay. Only one isolate was toxigenic at low titres, but a clone of this isolate was found to be non-toxigenic. Four of those who excreted the organism were subsequently challenged with a toxigenic V. cholerae O1 strain from Bahrain, and all became ill. Thus, colonization by the Brazilian strain did not confer protection. This observation and the previous finding that oral administration of living hypotoxigenic or killed cholera vibrios affords protection against subsequent challenge with virulent V. cholerae suggest that there may be significant differences in somatic antigens between the atypical V. cholerae O1 and toxigenic O1 strains. Strain 1196-78 has also been given in a higher dose (10^8 organisms) with bicarbonate to 5 volunteers: the organism was isolated from the stools of 2 of the 5, but none became ill.

3.1.3 Non-O-Group 1 Vibrio cholerae

3.1.3.1 Clinical features

These organisms have been associated with outbreaks and sporadic cases of gastro-intestinal illness, but their clinical features cannot be characterized with any confidence. From the descriptions of sporadic cases one cannot be certain that the organism isolated was always the cause of the illness. In the reported outbreaks, the clinical features have varied, possibly reflecting different characteristics of the responsible strains. In general, the patients have diarrhoea, nausea, and vomiting. Some have fever and abdominal cramps, and a few have blood or mucus in their stools. The illness usually lasts less than 3 days.

* There are no internationally accepted and standardized methods for measuring pathogenicity. Such factors as inoculum size, time of incubation, source of laboratory animals, and definition of a positive response, which can profoundly affect the observed results, may vary from laboratory to laboratory. Thus, the results summarized here should be interpreted cautiously.

In a comprehensive study conducted in Bangladesh, patients whose isolates produced a cholera-toxin-like enterotoxin had more severe illness than those whose isolates were non-toxigenic. Some significant rises in serum agglutinating titres against the homologous strain were demonstrated in both groups, and significant antitoxin titre rises occurred in some patients with toxigenic isolates.

Extraintestinal non-O1 V. cholerae infections have been reported in debilitated or immunosuppressed hosts, and some have been fatal.

3.1.3.2 Epidemiology

Non-O1 V. cholerae has been isolated from the stools of persons with diarrhoeal illnesses in Asia (Bangladesh, Hong Kong, India, Malaysia), Africa (South Africa, Sudan), Europe (Bulgaria, Czechoslovakia, England, Federal Republic of Germany, Hungary, Sweden, USSR), North America (Mexico, USA), South America (Brazil) and on board ships and airplanes. The frequency of infections with these organisms in these areas has not been comprehensively studied. The organisms can undoubtedly be isolated in other countries where investigations have not so far been done. Large epidemics and pandemics like those caused by V. cholerae O1 have not been reported.

Little is known about the seasonality of non-O1 V. cholerae disease. In the USA, most infections occur during the warmer months. In Bangladesh, most of the cases occur during the spring and summer before the annual increase in V. cholerae O1 cases in the autumn.

Transmission is probably exclusively by contaminated food and water. Studies have shown that non-O1 V. cholerae can multiply in a variety of foods. In the USA, patients frequently have a history of consumption of molluscs, especially raw oysters, within 48 hours before onset of diarrhoea. In outbreaks in Czechoslovakia and Australia, the vehicle of transmission was food (potatoes, and an egg and asparagus salad, respectively). The incubation periods in these outbreaks were 20-30 hours, and 5-37 hours. In a large outbreak in the Sudan in 1968, polluted well-water was responsible; cases ceased to occur 4 days after the suspect well had been closed. Despite the large number of exposed persons, no secondary cases were observed and no person-to-person transmission was evident.

Non-O1 V. cholerae strains have been found to be widely distributed in the environment wherever they have been looked for, namely in some countries of Europe and the USA. They have been found in sewage, sewage-contaminated surface water, estuarine waters (both sewage-contaminated and apparently free from faecal contamination), seafoods, and animals. In ecological studies done in the Federal Republic of Germany, the USA, and England, the organisms were generally found in brackish surface waters (rivers, marshes, bays, and coastal areas), were more numerous during the warmer months, and were not associated with sewage contamination. The organisms are thus usually considered aquatic organisms and are widely thought to be free-living in the environment. However, whether free-living strains cause disease in man is not known; pathogenicity may be restricted to strains adapted to the human intestine.

3.1.3.3 Pathogenicity

Using a battery of assays (rabbit ileal loop, infant rabbit, rabbit skin permeability, suckling mouse, Chinese hamster ovary cell) 4 patterns of biological activity of non-O1 V. cholerae have been observed which may be useful in describing, classifying, and comparing these organisms. These patterns are: (1) production of a cholera-toxin-like enterotoxin; (2) production of a heat-stable toxin; (3) "enteritis" (positive infant rabbit and/or positive ileal loop assay with use of whole bacterial culture, without evidence of production of either toxin); and (4) no activity in any of these assays. In the study done in Bangladesh (see section 3.1.3.1), 98% of 43 strains from patients with diarrhoeal disease had some activity in at least one of these assays, while only 33% of 18 strains isolated from surface waters with low coliform counts had such activity. Production of a cholera-toxin-like enterotoxin has also been found in isolates from outbreaks in the Sudan and Czechoslovakia, and studies in the USSR and Japan have strongly suggested that some strains of non-O1 V. cholerae produce toxins other than the cholera-toxin-like enterotoxin. Despite these

results, it must be borne in mind that there is no assay (other than human feeding experiments) or group of assays that can reliably determine if a given strain is a potential pathogen for man.

3.1.3.4 Serotyping

Two serotyping systems for non-O1 V. cholerae are in use: one developed by Dr Sakazaki and the other by Drs Smith and Goodner. Both systems are based on the O antigen (somatic antigens) and in both the classical and El Tor biotypes of V. cholerae are designated O-group 1. By 1979, the system developed by Sakazaki et al. included 60 serotypes, while 72 serotypes had been recognized by Smith and Goodner. In spite of the differences in the methods used to prepare antisera and to perform agglutinations, many serotypes correspond in the two systems, although each system includes some unique serotypes. Neither system has so far determined any marked differences in the serotypes of isolates from human and non-human sources or in their diarrhoeagenic potential. The United States and Japanese Cholera Panels of the United States-Japan Cooperative Medical Science Programme are currently attempting to develop a uniform serotyping system.

In the USSR, serotyping has been attempted with 2008 strains of non-O1 V. cholerae isolated from human and environmental sources between 1968 and 1975 in 18 territories of the country. Only 40.5% of strains from humans and 16% of strains from the environment could be serotyped by the Sakazaki system. The investigators demonstrated 15 serotypes that are not included in the Sakazaki system. Sakazaki serotype 5 appeared to be the predominant type in persons with acute gastrointestinal disease, whereas serotype 8 predominated in the strains from environmental sources.

3.1.4 Vibrio parahaemolyticus

3.1.4.1 Clinical features

V. parahaemolyticus, a halophilic marine vibrio, was first recognized as a cause of food poisoning in Japan in the early 1950s. Two clinical syndromes have been described. In the one more commonly observed, the cardinal manifestation is watery diarrhoea, although abdominal cramps, nausea, vomiting, and fever may occur. The other is a dysenteric syndrome which has been described in several countries; in Calcutta, for example, 60% of the reported cases have had dysentery. In the watery diarrhoea syndrome, the modal incubation period is 15 hours. However, in some cases of the dysenteric syndrome, the incubation period has been reported to be shorter (as short as 2½ hours). The reason for this apparent difference has not been explained. In both syndromes the illness is usually self-limited, with a median duration of about 3 days. Although severe cases of V. parahaemolyticus enteric infection requiring hospitalization and even causing death may occur, the illness is usually of mild or moderate severity.

V. parahaemolyticus wound infections have also been reported from Australia, Canada, and the USA in persons with wounds exposed to sea-water.

3.1.4.2 Epidemiology

During the last decade, V. parahaemolyticus enteric infection has been reported from North America (USA), Central America (Panama), Africa (Togo), Europe (Romania, USSR, and United Kingdom), and Asia (Bangladesh, India, Indonesia, Japan, Korea, Malaysia, Philippines, Singapore, Thailand, Viet Nam). The frequency of isolation of the organism from diarrhoea cases in different countries varies widely: India (Calcutta), 11%; Indonesia, 2.6-3.7%; Thailand, 10.7%; Viet Nam, 8.5-15%; and Korea, 1.5%. In Japan, about 24% of reported cases of food poisoning are attributed to V. parahaemolyticus. In many countries the importance of the disease is unknown because most laboratories do not use culture media, like ICBS, that are appropriate for isolating these vibrios from stools.

Marked seasonality of the disease has been noted in several countries, most of the cases occurring during the warmer months. This may reflect both enhanced opportunity for V. parahaemolyticus to multiply in unrefrigerated foods and increased prevalence of V. parahaemolyticus in the environment during the warmer months. However, in Calcutta, where the ambient temperature varies less, there is little seasonal variation in incidence. Over 65% of the cases in Calcutta occur in females, and most cases are over 15 years of age. Although asymptomatic infections have been found, there have been no reports of long-term carriage of the organism.

V. parahaemolyticus enteritis appears to be transmitted exclusively by food. The vehicle of transmission is usually seafood, although other foods contaminated by raw seafood or surface water are thought also to be vehicles of transmission. In Calcutta, V. parahaemolyticus has been found in stools, food, and water in strictly vegetarian households, and many cases give no history of seafood consumption or other marine exposure. The generation time of V. parahaemolyticus is reported to be as short as 9 minutes under ideal conditions, enabling the organism to multiply very rapidly in mishandled foods and quickly reach the rather large infectious dose; the ID₅₀ has been determined by volunteer studies to be about 10^5 - 10^7 organisms in persons given antacids. Growth of V. parahaemolyticus is inhibited at temperatures below 15°C and the organism tends to die out at lower temperatures. It is quite sensitive to high temperatures as well, succumbing after exposure at 65°C for 10 minutes.

V. parahaemolyticus is part of the normal flora of estuarine and other coastal waters throughout most of the world. It has been isolated from sea-water, sea mud, or seafoods in Asia (Hong Kong, Japan, Korea, Singapore, Sri Lanka), North America (Canada, USA), Oceania (Australia, Hawaii, New Zealand), Africa (Togo), and Europe (Baltic Sea, Black Sea, Mediterranean Sea, North Sea, Denmark, Greece, Italy, Netherlands, Spain, Turkey, United Kingdom, Yugoslavia). It has been isolated from fresh water and fresh fish, crab, and shrimp in India. In estuarine waters in temperate regions, it has been observed that V. parahaemolyticus passes the winter in sediment, is released from the bottom in the spring and becomes attached to zooplankton, and then proliferates as the water temperature rises.

3.1.4.3 Pathogenicity

Almost all V. parahaemolyticus isolates from patients with diarrhoea, but only about 1% of isolates from seafoods and sea-water, are Kanagawa-positive on Wagatsuma agar. However, it is not certain that all Kanagawa-positive strains are pathogenic. The Kanagawa reaction is caused by a heat-stable direct haemolysin with a molecular weight of approximately 42 000 daltons. The haemolysin is cytotoxic to human FL cells in cell culture, and is cardiotoxic for mice. Although minor changes have been described in the electrocardiograms of acutely ill patients, the clinical importance of the heat-stable haemolysin is not known.

Kanagawa-positive and Kanagawa-negative organisms have been studied intensively, and their activity in a number of laboratory assays differs greatly. Kanagawa positivity is associated with penetration of the intestinal epithelium of infant rabbits, rapid cytotoxicity in HeLa cell cultures, rapid adhesion to HeLa and human fetal intestinal cells, and production of a heat-labile factor that produces a cholera-toxin-like reaction in CHO cells.

Despite extensive study of the pathogenic mechanisms of V. parahaemolyticus, it is still not known how the 2 gastrointestinal syndromes described in section 3.1.4.1 are produced.

3.1.4.4 Serotyping

By 1976, 12 O antigens (heat-stable somatic antigens) and 59 K antigens (capsular or envelope antigens) had been identified. Thus far, there has been no strong, worldwide association of any particular serotypes with the Kanagawa phenomenon or with illness in man, although in Calcutta one third of the cases have been associated with isolation of serotype O1 K56. It thus appears that serotyping may be useful for epidemiological investigations but not for diagnostic purposes.

3.1.5 Group F (EF6) vibrios

A group of vibrio-like organisms designated Group F by the Public Health Laboratory, Maidstone, probably constitutes a new species (Table 1). These organisms are identical to organisms designated as Group EF6 by the US Center for Disease Control. They are often mistakenly identified as *Aeromonas*, but can be distinguished from them by their sensitivity to the vibriostatic compound O/129 (150- μ g disc) and their ability to grow in 6% NaCl. Group F vibrios can be divided into 2 biotypes: biotype 1 includes only anaerogenic strains of clinical and environmental origin, while strains of biotype 2 are generally aerogenic and have only been found in the environment. Biotype 1 strains have so far been isolated from patients with diarrhoea in Bahrain, Bangladesh, Egypt, India, Indonesia, Iran, Iraq, Jordan, Kenya, Philippines, Saudi Arabia, Spain, Tanzania and Tunisia.

The clinical features and epidemiology of disease associated with isolation of this group of organisms have not yet been well defined, but some information is available from Bangladesh where, in 1976-1977, there was an increase in isolations of Group F organisms from cases attending a rural treatment centre. Prior to and after this period, there were very few isolations of the organism. About half of the isolates were from children below 5 years of age. In studies of family members of infected persons in Dacca, Group F organisms were found in the stools of less than 1%. The clinical symptoms of the cases were cholera-like, except that some persons had blood and mucus in their stools, abdominal pain, or fever. No agglutinating antibodies against the homologous organisms were detected. Nine isolated strains were tested at the US Center for Disease Control and found negative in the adrenal cell and suckling mouse assays, and in the Serény test. However, another institution has reported that whole cultures and culture filtrates of most strains tested produced fluid accumulation in ligated rabbit ileal loop, regardless of their source of isolation (faeces, sewage, or environment).

While the large increase in the numbers of these organisms in the stools of diarrhoea cases was noteworthy, it must be concluded from the available epidemiological and laboratory data that it is uncertain at present whether this organism is a diarrhoea-producing pathogen.

3.1.6 Other vibrio species and related organisms

Other vibrio species occasionally isolated from man - *V. alginolyticus*, *V. metschnikovii* (enteric Group 16), and *V. vulnificus* (lactose fermenting "vibrio") - are not believed to cause diarrhoeal illness in man. *V. anguillarum* has not been isolated from man, but is an economically important pathogen of salmonids and other marine fish; virulent strains have been shown to harbour plasmids not found in avirulent strains.

Aeromonas hydrophila and *Plesiomonas shigelloides* have been isolated from the stools of children and adults with diarrhoea and a possible causative role has been suggested by a number of workers. Strains of *Aeromonas* have been shown to produce various toxins, and both cultures and filtrates cause accumulation of fluid in rabbit ileal loops.

3.2 Environmental surveillance for *V. cholerae* O-Group 1

In the last decade there has been considerable interest and research in the behaviour of *V. cholerae* O1 in the environment and the use of environmental surveillance as a means for early detection of the organism in a non-infected area. Recent information in this field is summarized below.

3.2.1 Survival of *V. cholerae* O-Group 1 outside the human intestine

Most evidence suggests that *V. cholerae* O1 depend on the human intestinal tract as their primary multiplication site and reservoir, and that they are aliens in aquatic environments and are ultimately eliminated. Several apparent exceptions indicate that this may not always be the case: (a) In the cholera outbreak in Louisiana during 1978 (see section 3.1.2.1(f)),

the incriminated organism was a haemolytic El Tor Inaba strain with the same unusual phage sensitivity pattern as the haemolytic El Tor Inaba strain isolated from a cholera case in Texas in 1973. Although cases could easily have been missed, there is no evidence that other human cholera infections occurred in the intervening years, suggesting that the organisms may have persisted in the environment. (b) In Australia, since 1977, El Tor Inaba vibrios have been isolated repeatedly over a 25-month period from a freely flowing river. Peak incidence coincides with the warmest part of the year. No human source of contamination has been found, and only 2 human infections have been detected, both before discovery of the vibrios in the water. The organism has also been isolated, in 2 out of 60 instances, from the gut contents of common sea mullets taken from the river. Isolates that have been tested appear in general to be toxigenic, though a number fail to elicit a positive ileal loop or are negative in the Y-1 adrenal cell assay. (c) In the USSR, following a cholera outbreak in Astrakhan in 1970, V. cholerae O1 El Tor were isolated from two small water basins near the Volga river for periods of up to 14 months. These waters were not subjected to known human faecal contamination. In 1975, El Tor vibrios were isolated from sulfurous spring waters in the region. The sources of contamination may have been tourists visiting the region from cholera-infected areas, but this was not proved.

In addition, extensive environmental surveillance carried out in Bangladesh to identify an extraintestinal habitat for V. cholerae O1 revealed that these organisms were sometimes associated with the root surface of plants, particularly the water hyacinth (Eichhornia crassipes). Laboratory studies carried out in isolated tanks of fresh pond water indicated that El Tor vibrios and many other heterotrophic bacteria are concentrated on the root surface of these plants within a few hours after immersion. Vibrios adhering to plants remained viable longer than those left free in the water column.

3.2.2 Methods used in environmental surveillance

3.2.2.1 Sampling

Moore swabs have been shown to be an effective way of monitoring flowing water and sewers for V. cholerae and are the most sensitive technique available when counts are <1/litre. In still water, it is usually necessary to collect multiple water samples of 1 litre or more. Such samples should be enriched directly or after filtration through Celite. As surface plants and plankton have sometimes yielded V. cholerae O1 when the surrounding water column has not, sampling of such aquatic organisms may increase the rate of detection of vibrios.

It is important that samples be processed as soon as possible after collection and be maintained at ambient temperature during transport to the laboratory. Enrichment culture can be started in the field. V. cholerae O1 isolates from water sometimes appear to have suffered sub-lethal injury that increases their sensitivity to cold shock. If samples must be cooled for transport, still air cooling (no direct contact with coolant) should be employed.

3.2.2.2 Enrichment

Alkaline peptone water (APW) without NaCl is the best available enrichment, though it permits the growth of too many competitors to be optimal. Cultures can be streaked at 6-8 hours and at 18-20 hours or, alternatively, it may be better to sub-culture after 6-8 hours and then streak the original and sub-culture after overnight incubation. The 6-8 hour incubation can be carried out at 35-37°C or at ambient temperature; the optimum temperature for overnight incubation is 35-37°C. Various other enrichment broths have been suggested, but they have no real advantage.

3.2.2.3 Plating

TCBS agar is recommended for plating enrichment cultures. The efficiency of this medium has varied widely between brands and lots, and the Eiken brand appears to be the most reliable. Environmentally stressed toxigenic V. cholerae O1 grow very poorly on this highly selective medium and direct plating of water samples onto TCBS is not recommended.

3.2.2.4 Identification

An efficient procedure for identifying V. cholerae O1 is to pick up smooth yellow colonies (atypical O1 and non-O1 strains occasionally form green colonies) from TCBS onto gelatin neopeptone agar containing no NaCl. Isolates that grow and produce gelatinase zones should be tested for oxidase reaction and slide agglutination in polyvalent V. cholerae O1 antiserum and, if positive, in type-specific Ogawa and Inaba antisera. Non-agglutinating isolates can be picked up on to Kligler's iron agar, and also tested for lysine and ornithine decarboxylase and arginine dihydrolase production in order to screen for non-O1 V. cholerae (see Table 1). Some difficulties in obtaining typical agglutination reactions with colonies selected off TCBS agar have been reported.

3.2.2.5 Quantification

Quantification of V. cholerae O1 in water samples is best done by a 3-tube MPN* procedure using APW and follow-up streaking on TCBS agar. Membrane filtration and incubation of the membrane on TCBS is not satisfactory. Pre-incubation of membranes on starch agar for 2-3 hours before transfer to TCBS has been found to be as efficient as the MPN procedure only when assaying water with high ($\geq 10^2/100$ ml) concentrations of V. cholerae O1, or water with low turbidity and few competitors.

The extent to which counts are affected by the organism's adherence to particulate matter has not been studied sufficiently. Blending has been shown in some cases to increase the apparent concentration of V. cholerae O1 in water samples.

3.2.2.6 The problem of overgrowth of V. cholerae O1 by non-O1 V. cholerae

There is no selective procedure available that permits the outgrowth of V. cholerae O1 from a population of non-O1 strains. If low numbers of O1s are present (as is frequently the case) in a sample with a large number of non-O1s, the O1 strains can be unrecoverable in practice and thus exist for long periods of time as a "silent" population.

3.2.3 The role of environmental surveillance in cholera control

Sewer surveillance using only Moore swabs (in South Africa and Louisiana, USA) and sampling of pooled night soil and of latrines (in Hong Kong) have proved to be sensitive indicators of the presence of V. cholerae O1 in a community. In cholera-affected areas served by sewer systems, sampling with Moore swabs can be a cost-efficient adjunct to diarrhoeal disease surveillance for the monitoring of V. cholerae O1. In cholera-free areas, routine surveillance should be limited to common sewer lines. If V. cholerae O1 are detected, additional Moore swabs can then be placed in branch lines to assist in locating the source. In areas where cholera is sporadically present, surveillance at sewage disposal sites may be the least expensive and least difficult way to detect the occurrence of cholera in the community.

* Most Probable Number

3.3 Phage and vibriocin typing of *V. cholerae*

This has also been an area of expanding interest, though the information available, as summarized below, is still limited.

3.3.1 *V. cholerae* O-Group 1

It is agreed that a phage-typing scheme would be very helpful for epidemiological studies of toxigenic and atypical human and environmental *V. cholerae* O1 isolates. The practical value of the original scheme of Basu and Mukerjee¹ is limited because recent isolates have fallen into only 3 types. In fact, the only published work demonstrating the epidemiological use of this scheme has been that describing an outbreak of cholera in Togo, in which it was used along with the scheme of Gallut and Nicolle².

Recently, however, an expanded phage-typing scheme has been developed at the Public Health Laboratory, Maidstone, where a collection of freeze-dried control cultures has been started. A number of phages have been screened and 14 have been shown to be useful for typing purposes: Mukerjee's classical phages I to IV; Basu and Mukerjee's phages 4 and 5; Nicolle's β ; 4996, 13, 14, 16 and 24 isolated in Bangladesh; and 32 and 57 which are derived from Basu and Mukerjee's 3 and 5, respectively. Testing of 1135 strains revealed 25 patterns of sensitivity to these 14 phages (Table 2). The types are numbered 1-25 for the purposes of this discussion. They appear to be reasonably stable, although occasionally strains sensitive to only one or two phages may give variants of wider sensitivity on repeated sub-culturing. This does not appear to be a problem with freshly isolated strains and is not significant enough to interfere with the use of the scheme for routine typing.

Almost all (99%) of the strains were typable, but 64% of strains again fell into 3 types (11, 6 and 7). The selection of strains was biased: some countries, notably Bangladesh, India, and Indonesia, were much more heavily represented than others. Included in the 1135 strains were 114 which had previously been shown to be of Basu and Mukerjee's type 4 and sensitive to β . They were selected because it was known from earlier studies that this was the most common type and it was hoped that they could be subdivided with the new phages. In fact, all except one of them fell into type 11. The predominance of a few types, in particular type 11, may be explained by the fact that the majority of the typing phages were isolated in one endemic area (the Ganges delta). Phages isolated from the environment have so far proved to be of greater value than those from lysogenic strains. These factors suggest that new typing phages should be sought in the environment in other cholera-endemic areas.

The Public Health Laboratory (PHL), Maidstone, phages show considerable promise as an epidemiological tool, as is illustrated by the following 3 examples:

- (a) During the Portuguese outbreak in 1974, type 16 predominated in Portugal and southern African states, but has not yet been detected in other Mediterranean countries. This correlates with the belief that cholera was introduced into Portugal from southern Africa.
- (b) The *V. cholerae* O1 strains isolated from humans and the environment in Louisiana, USA, during 1978 were all of type 17, as was the strain isolated in Texas in 1974 (see section 3.2.1); this type has not been detected in any other country.
- (c) Environmental *V. cholerae* O1 isolates known to be atypical (non-toxigenic) and/or not associated with clinical cases have so far all been resistant to phages 13, 14, 16 and 24.

¹ Basu, S. and Mukerjee, S. (1968) Experientia, 24, 299-300

² Gallut, J. and Nicolle, P. (1963) Bull. Wld Hlth Org., 28, 389-393

Sensitivity to one or more of these phages may thus be a marker of potential pathogenicity.

In addition to this work, two groups of Russian workers have also isolated new phages active against V. cholerae O1 strains which may be useful for typing. One of these groups, working in conjunction with the National Institute of Cholera and Enteric Diseases, Calcutta, divided O1 strains into about 20 types using Mukerjee's classical phages, Basu and Mukerjee's E1 Tor phages, Nicolle's β phage, and 3 new phages.

3.3.2 Non-O-Group 1 V. cholerae

A scheme developed in Calcutta for phage typing non-O1 V. cholerae has not been widely applied and appears to have fallen into disuse. Fifteen percent of 433 non-O1 strains recently typed with the PHL, Maidstone, phages were sensitive to at least one of the phages. The sensitivity patterns of non-O1 strains and some atypical (non-toxigenic) V. cholerae O1 strains were the same. Sixteen of 54 non-O1 strains examined were lysogenic. Since some of these phages are also active on V. cholerae O1 strains, non-O1 strains may be a source of new typing phages for O1 strains.

3.3.3 Vibriocin typing of V. cholerae O-Group 1

A scheme was developed in the early 1970s and limited attempts for using it for routine typing have given variable results. The scheme has not gained acceptance for epidemiological studies.

4. CHOLERA ENTEROTOXIN AND ITS RELEVANCE TO PATHOGENESIS, IMMUNITY, AND VACCINE DEVELOPMENT*

It is now 20 years since experimental evidence first suggested that cholera could be a toxin-mediated disease. Today, cholera is recognized as the prototype of a number of previously unrecognized diarrhoeal diseases that are also mediated by enterotoxins, some of which are immunologically related to the cholera enterotoxin. Perhaps the most important of these is the Escherichia coli heat-labile toxin (LT). Collectively, the other enterotoxic enteropathies by far exceed cholera as causes of morbidity and mortality in the world. Thus, if appropriate means of inducing antitoxic immunity against cholera can be developed, they might be applicable to prevention of these diseases as well. Similarly, an understanding of the mechanism of action of the cholera enterotoxin at the molecular level may lead to rational methods of pharmacological intervention, and these too may be applicable to the other, similar diarrhoeal diseases.

The cholera enterotoxin (called cholera toxin in earlier works) has been isolated to homogeneity, its structure has been elucidated, and its mode of action has been defined at the molecular level. It is a protein of 84 000 MW consisting of 2 immunologically distinct regions designated A (active) and B (binding). The B region (previously called cholera toxinogenin), of approximately 56 000 MW, is composed of non-covalently associated B sub-units of about 11 500 MW. This region of the toxin is responsible for binding the holotoxin to host-cell membrane receptors that contain a particular glycolipid, the GM1 ganglioside. This binding enables the 28 000-MW A region to penetrate the host cell where it acts, enzymatically, to cleave NAD and to transfer ADP-ribose to the GTP-binding protein associated with the host-cell enzyme, adenylate cyclase. This ADP-ribosylation of GTP-binding protein prevents the breakdown of GTP to GDP, and effectively locks adenylate cyclase in its active state. The net result is the continuous formation of excessive amounts of cyclic-AMP (cAMP). This leads

* This section brings up to date the information provided in the Report of the Scientific Working Group on Immunity and Vaccine Development, published under the title Intestinal Immunity and Vaccine Development: a WHO Memorandum in Bull. Wld Hlth Org., 1979, 57(5), 719-734 (previously issued as unpublished document WHO/DDC/78.2). For further information, especially on the development of killed bacterial cholera vaccine, the reader is referred to that report.

to a cascade of events, as yet uncertain, that cause intestine epithelial cells to over-secrete electrolytes followed by water - the cholera stool.

Because of the ubiquity of G_{M1} ganglioside in eukaryotic cell membranes, cholera toxin can activate adenylate cyclase in a variety of cells and tissues with which normally the toxin never comes into contact. The cholera enterotoxin has thus become widely used as an adenylate cyclase/cAMP probe by researchers interested in a variety of cAMP-mediated effects unrelated to cholera. A number of sensitive assays for cholera enterotoxin based on this property have been developed as well. Cultured Chinese hamster ovary (CHO) or Y-1 mouse adrenal tumour cells respond to picogram quantities of enterotoxin with characteristic morphological changes. Cholera toxin also causes a characteristic skin reaction in rabbits and guinea pigs which serves as a basis for assays. Related enterotoxins are also active in these assays, though exceptions may occur. It is important to recognize that apparently similar activity in organisms of other species or genera may be due to very dissimilar factors, and to exercise caution in the interpretation of these assays.

The cholera enterotoxin is a very effective antigen. In addition to their serological response to the somatic antigens of the cholera vibrios, cholera patients also usually exhibit antitoxic antibody responses. In many experimental animal studies, antitoxic immunity has been shown to be protective. Antibodies stimulated by the cholera enterotoxin are primarily directed against the B portion of the molecule. The immunologically distinct A region is a relatively poor antigen as compared to B. It might be predicted that a V. cholerae O1 strain that produces only the B region of the toxin (an $A^- B^+$ mutant), or produces B with an inactive A region (an $A^X B^+$ mutant), could, without causing diarrhoea, effectively deceive the host into responding with an immune response similar to that induced by the disease itself, which, as proved in volunteer studies, is highly protective.

A hypotoxigenic mutant developed several years ago was found to be avirulent in human volunteers fed doses of 10^{10} live vibrios (after sodium bicarbonate). These volunteers were found to be resistant to subsequent challenge with the virulent wild parent V. cholerae O1 strain. However, because the resistance was not as solid as that induced by convalescence, and because the mutant (a) was apparently unstable in that, from one of the volunteers, a colony was isolated that produced larger amounts of cholera toxin; (b) did not colonize extremely effectively; and (c) could not be expected to induce substantial antitoxic immunity that might extend to the cholera-related enterotoxic enteropathies, further evaluation of this strain as a vaccine was not undertaken.

Recently an $A^- B^+$ mutant has been isolated from a strain of V. cholerae O1, El Tor Ogawa. Tests in several laboratories indicate that this mutant colonizes but is avirulent in experimental animal models following extensive serial passages of large inocula. The mutant has induced immunity against challenge with virulent cholera vibrios at 3 weeks in the chinchilla model, although it is not yet clear whether an anti-B antitoxic antibody response is induced. Evaluation of this strain for safety and efficacy as a live vaccine in volunteers is imminent. Another recent mutant ($A^+ B^-$) is also ready to be tested in volunteers. If these mutant strains are found to induce effective immunity against cholera, subsequent tests in volunteers will determine whether significant immunity is also produced against related enterotoxic enteropathies, especially LT-producing E. coli.

Other approaches to effective immunization against cholera could involve the use of non-viable antigens, alone or in combination, administered perorally or parenterally. Volunteer studies suggest that strictly peroral immunization could be effective. A natural toxoid vaccine of only the B region has been developed and is currently being evaluated.

Little is known of other virulence factors that might play a role in immunity. The factor or factors responsible for adherence have not been well characterized. Motility of cholera vibrios appears to be an attribute of virulence in some experimental animal models,

and it has been postulated that it helps the vibrios to penetrate the mucus layer of the small bowel. The role, if any, of other factors such as mucinase, neuraminidase, protease, etc. is not understood. A haemolysin has recently been isolated from a strain of *V. cholerae* O1, El Tor, which is cytotoxic and lethal in mice. As cholera caused by haemolytic El Tor vibrios is clinically identical to that caused by non-haemolytic El Tor and classical biotypes, this haemolysin probably does not play a role in pathogenesis.

5. CHOLERA CONTROL AS AN INTEGRAL PART OF A DIARRHOEAL DISEASES CONTROL PROGRAMME

Cholera control activities were undertaken in the past by many countries as an ad hoc special set of actions, initiated only when cholera threatened or struck. A number of countries that have experienced cholera in recent years and are aware of the potential of the recent scientific advances are developing programmes for the control of all diarrhoeal diseases. The essential strategies for such programmes have been outlined elsewhere.* The Group recommends the adoption of such a programme in all cholera-infected or cholera-threatened areas, as it offers the best chance of controlling cholera. If such a programme is operative, patients with cholera are unlikely to die and panic, which often occurs in newly infected areas following the first deaths, can be prevented.

One of the essential strategies is epidemiological surveillance; if a surveillance programme is functioning properly, outbreaks of cholera and other diarrhoeal diseases can be detected early and investigations and control measures can be initiated before extensive spread occurs. Effective surveillance requires not only the systematic collection of information on the occurrence of cases, but also the analysis and interpretation of data to provide a rational basis for public health interventions and to enable health officials to determine priorities in allocating limited resources.

The data providers (those who report cases) may be anyone delivering primary health care, such as village health workers, traditional healers, pharmacists, village and religious leaders, teachers, and physicians in secondary and tertiary care facilities. Simple reporting forms and a simple case definition should be used. For example, for cholera surveillance in non-endemic areas, the occurrence of an unusually high number of dehydrating diarrhoea cases in persons over 10 years of age is probably enough to suggest that cholera may be present. A report of such cases by primary data providers should trigger immediate action to strengthen treatment facilities, to confirm the presence of cholera, to determine the vehicle(s) of transmission, and to implement control measures to prevent further spread of the disease.

Diagnostic laboratories are important but not absolutely essential. Available laboratory facilities should not be overburdened with a large number of specimens; instead, selectivity in the collection of epidemiologically relevant specimens should be emphasized. In a given point-source epidemic it should not be necessary to collect more than a few specimens for laboratory analysis from cases and items of food and water suspected of being involved in transmission.

A well thought-out plan of action for epidemiological surveillance and epidemic control is essential, as are the personnel and facilities to carry out the plan. Diarrhoeal diseases surveillance should, wherever possible, be integrated into other national communicable diseases surveillance programme.

* For more details the reader is referred to Resolution WHA31.44 adopted by the World Health Assembly in May 1978, and to WHO unpublished document "Development of a Programme for Diarrhoeal Diseases Control", WHO/DDC/78.1.

6. RECOMMENDATIONS FOR RESEARCH

Taking into account current knowledge and laboratory methodology, and keeping in mind the overall Programme objective of the control of cholera and diarrhoea caused by related vibrios, the Group made recommendations for further research. In view of the widely different aspects considered during the meeting, no attempt was made to establish an overall list of priorities for research. Instead, the recommendations are listed separately for each of the main topics discussed.

6.1 Epidemiology and bacteriology6.1.1 V. cholerae O-Group 1

- The modes of transmission of cholera at the community level require more precise studies, coupling the most effective techniques of epidemiology and environmental microbiology. Such studies should identify methods for specific interventions that are most likely to be effective in controlling cholera.

- Coordinated epidemiological, microbiological and sociological studies need to be carried out to identify the determinants of cholera endemicity, persistence in the environment, and seasonality.

- Programmes for continuous surveillance of antibiotic resistance of strains of V. cholerae O1 and research into the nature of the R factors involved need to be accelerated.

- Volunteer studies are needed to further define the infectious dose of the biotypes of V. cholerae O1 and to determine the influence of food and water on the infectious dose.

- The reasons for the low incidence of cholera among infants need to be explained by careful studies in different endemic areas, including several areas where breastfeeding is universal.

- Research into the genetic basis for differences between the classical and El Tor biotypes needs to be encouraged so that more stable markers than those used currently to differentiate these biotypes can be established.

6.1.2 Atypical V. cholerae O-Group 1

- Studies are needed to determine whether there is any association between the lack of toxin production in vitro of the atypical V. cholerae O1 and other biochemical reactions.

- More volunteer studies should be undertaken to find out whether atypical V. cholerae O1 strains that do not produce toxin under laboratory conditions - as measured in a variety of assays - can cause disease in man.

- Efforts should be made to test for toxin production in V. cholerae O1 isolated from cases and from the environment in the absence of cases, so as to determine the frequency of atypical V. cholerae O1 strains.

- The antigenic structure of V. cholerae O1 and atypical V. cholerae O1 should be examined with the aim of preparing a better antiserum for V. cholerae O1 for use as an international standard.

6.1.3 Non-O-Group 1 V. cholerae, Group F vibrios, and related species

- It is important to identify the pathogenic members of these groups and the factors related to pathogenicity. The results of studies in animal and other laboratory models to distinguish variations in virulence potential should ultimately be confirmed in volunteers. Characteristics of these isolates that can be used for their laboratory identification should then be determined.
- The pathogenic members of these groups should be looked for in outbreaks and in prospective studies on diarrhoea to gather information on their incidence, means of transmission, and clinical features.
- A single, internationally accepted serotyping system for non-O1 V. cholerae should be developed to facilitate international communication and the quest for better understanding of the ecology, epidemiology, pathogenicity, and clinical features of this group.

6.1.4 V. parahaemolyticus

- Studies are needed to elucidate the determinants of virulence in V. parahaemolyticus and the relationship of the Kanagawa phenomenon to human enteropathogenicity.
- Studies should be done to determine the pathogenesis of V. parahaemolyticus gastroenteritis, particularly of the syndrome with the short incubation period.
- The mode of transmission in sporadic cases and in outbreaks in which seafood is not involved needs to be clarified, as does the ecology of V. parahaemolyticus strains reported to be indigenous to fresh-water areas. Further research is also needed to define conditions or means of processing seafood to prevent the multiplication of V. parahaemolyticus.

6.2 Environmental surveillance

- The ecology of V. cholerae O1 in surface waters should be better characterized. If the organisms are truly indigenous to certain habitats, this should be demonstrated. If they are aliens in aquatic environments, much more work is needed to identify factors that influence their ability to survive in these environments.
- Improvements are needed in the methodology for isolating V. cholerae from water and sewage, particularly in enrichment techniques, which currently fail to suppress many common aquatic competitors of V. cholerae. An even greater need is for a technique that facilitates the isolation of V. cholerae O1 from non-O1 V. cholerae. An immunological procedure may be a feasible approach, but efforts to identify exploitable physiological differences should also be encouraged.
- Basic studies are needed on the physiological differences between the different types of V. cholerae (classical, El Tor, atypical, non-O1) that influence their ability to survive in aquatic environments. These should include survival studies carried out in situ.

6.3 Laboratory procedures and phage typing

- There is a need for simple, carefully defined methods for determining the pathogenic potential of vibrio species isolated from various sources. International agreement on the test(s) that will be accepted as the minimum criteria for establishing pathogenicity should be sought.
- The value of the Maidstone phage-typing system as an epidemiological tool should be assessed in field studies. The isolation of additional phages from various geographical areas may improve the sensitivity of this system and should be encouraged. The feasibility of expanding this system as a basis for developing an international standard system should also be examined.

- Studies to determine whether phage typing can distinguish atypical V. cholerae O1 from V. cholerae O1 strains should be expanded and encouraged.

- Simpler and more rapid methods for the laboratory diagnosis of cholera and related infections are still needed. The establishment of a minimum standard of quality for TCBS agar should be encouraged.

6.4 Pathogenesis, immunity, and vaccine development*

- Further studies are needed to define the factors essential to virulence of V. cholerae with the hope that they may lead to additional means of pharmacological intervention. In this regard, research on factors and mechanisms involved in colonization, and in toxin synthesis and transport, could be very productive. The genetic basis of virulence needs to be elucidated, particularly the role of extra-chromosomal elements, plasmids, and lysogenic phage in mediating specific virulence factors.

- Immunizing agents (e.g., B-subunit toxoid, an A⁻ B⁺ mutant, and an A⁺ B⁻ mutant) that have recently been developed need to be tested for safety, stability, and ability to elicit substantial protection against both serotypes and biotypes of V. cholerae O1 and, if there is protection, against cholera-related enterotoxic enteropathies.

- Further basic research is needed to provide a more complete understanding of the mechanism of action of cholera enterotoxin, particularly of the cascade of events after activation of adenylate cyclase that results in induction of diarrhoea.

- The range of antigenic relatedness between enterotoxins from various species of enteropathogens needs to be defined. Additional studies should be directed toward the isolation and characterization of factors related to enterotoxigenicity in organisms other than V. cholerae O1.

* These recommendations are supplementary to those given in the Report of the Scientific Working Group on Immunity and Vaccine Development, Bull. Wld Hlth Org., 1977, 57(5), 719-734 (previously unpublished document WHO/DDC/78.2).

TABLE 1. CHARACTERS OF VIBRIO SPECIES AND RELATED ORGANISMS
LIKELY TO BE ENCOUNTERED IN THE CLINICAL LABORATORY

	<u>V. cholerae O1</u>	<u>Non-O1 V. cholerae</u>	<u>V. parahaemolyticus</u>	<u>V. alginolyticus</u>	<u>Group F biotype 1</u>	<u>Group F biotype 2</u>	<u>Aeromonas</u>	<u>Plesiomonas shigelloides</u>	<u>V. anguillarum</u>	<u>V. metschnikovii</u>
Oxidase (Kovac)	+	+	+	+	+	+	+	+	+	-
Arginine dihydrolase	-	-	-	-	+	+	+	+	+	+
Lysine decarboxylase	+	+	+	+	-	-	d	+	-	d
Ornithine decarboxylase	+	+	+	+	-	-	-	+	-	-
O/129 sensitivity 10 µg	S	S	R	R	R	R	R	S	S	S
150 µg	S	S	S	S	S	S	R	S	S	S
Gas from glucose	-	-	-	-	-	+	d	-	-	-
VP	d	d	-	+	-	-	d	-	+	+
Indole	+	+	+	+	-/+	-/+	+	+	+	d
Acid from arabinose	-	-	d	-	+	+	d	-	d	-
inositol	-	-	-	-	-	-	-	+	d	d
mannose	+	d	+	+	+	+	+	-	+	+
sucrose	+	d	-	+	+	+	+	-	+	+
Hydrolysis of aesculin	-	-	-	-	d	-	d	-	-	-
Gelatinase	+	+	+	+	+	+	+	-	+	+
Growth at 43°C	+	+	+	+	-	-	d	+	-	d
Growth in % NaCl										
0	+	+	-	-	d	d	+	+	-	-
3	+	+	+	+	+	+	+	+	+	+
6	d	d	+	+	+	+	-	-	d	+
8	-	-	+	+	d	d	-	-	-	d
10	-	-	-	+	-	-	-	-	-	-
Growth in TCBS	+	+	+	+	+	+	d	-	+	+

+ or - = reaction present in \geq 80% of strains.
d = reaction differs from strain to strain.
All strains are Gram-, motile and fermentative.

TABLE 2. PHAGE TYPING OF V. CHOLERAE 01

Basu and Mukerjee type	Type number	Sensitivity at RTD ^φ to bacteriophage														Number in type	% in type	
		I	II	III [‡]	IV	e4	e5	B	32	57	4996	13	14	16	24			
3	(1	V	V	V	V	+	+	+	-	+	+	+	+	+	+	20	2	
	(2	V	V	V	V	+	+	+	-	+	+	+	+	+	-	5		
	(3	-	-	-	-	+	+	+	+	-	+	+	+	+	+	4		
	(4	-	-	-	-	+	+	+	-	+	+	+	-	+	-	14		1
	(5	+	-	-	+	+	+	-	-	+	-	-	-	-	-	4		
2	(6	-	-	-	-	-	+	+	+	-	+	+	+	+	+	137	12	
	(7	-	-	-	-	-	+	+	+	-	-	+	+	+	+	122	11	
	(8	-	-	-	-	-	+	+	+	-	-	-	+	+	+	38	3	
	(9	-	-	-	-	-	+	+	+	-	-	+	-	+	+	9		
	(10	-	-	-	-	-	+	+	+	-	+	-	+	-*	-	5		
4	(11	-	-	-	-	-	+	+	-	-	+	+	+	+	+	465	41	
	(12	-	-	-	-	-	+	+	-	-	+	-	+	-*	-*	49	4	
	(13	-	-	-	-	-	+	+	-	-	-	+	+	+	+	13	1	
	(14	-	-	-	-	-	+	+	-	-	-	-	+	-	-	6		
	(15	-	-	-	-	-	+	+	-	-	-	-	-	-	-	6		
	(16	-	-	-	-	-	+	-	-	-	+	+	+	+	+	16	1	
	(17	-	-	-	-	-	+	-	-	-	+	+	-	+	+	50	4	
	(18	-	-	-	-	-	+	-	-	-	+	-	-	-	-	7		
	(19	-	-	-	-	-	+	-	-	-	-	-	+	-	-	14	1	
	(20	-	-	-	-	-	-	-	-	-	-	-	+	-	-	15	1	
-	(21	-	-	-	-	-	-*	-*	+	-	-	-	+	-*	-	33	3	
	(22	-	-	-	-	-	-*	-*	+	-	+	-	+	-*	-	10		
	(23	-	-	-	-	-	-*	-	-	-	+	-	-	-*	-	23	2	
	(24	-	-	+	+	-	-	-	-	-	-	-	-	-	-	4		
	(25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8		
															Others	58	5	
															Total	1135		

φ = Routine Test Dilution

+ = sensitive

‡ III gives variable weak reactions with many El Tor strains in the common types. These have not been shown.

* occasionally may give weak reaction of just a few plaques.