МИНИСТЕРСТВО ЗДРАВООХРАНЕНИЯ УКРАИНЫ
Харьковский национальный медицинский университет

CHOLERA

Methodical instructions on the subject «Microbiology, virology and immunology» for the II and III year English media students of medical and dentistry faculties

ХОЛЕРА

Методические указания по дисциплине «Микробиология, вирусология и иммунология» для студентов II и III курсов медицинского и стоматологического факультетов с английским языком преподавания

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Theme: Microbiological diagnosis of cholera

Actuality of the theme.

Cholera has smoldered in an endemic fashion on the Indian subcontinent for centuries. There are references to deaths due to dehydrating diarrhea dating back to Hippocrates and Sanskrit writings. Epidemic cholera was described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India. The mode of transmission of cholera by water was proven in 1849 by John Snow, a London physician. Vibrio cholerae was first isolated in pure culture by Robert Koch in 1883, although it had been seen by other investigators, including Pacini, who is credited with describing it first in Florence, Italy, in 1854.

Cholera is a life-threatening secretory diarrhea induced by an enterotoxin secreted by V.cholerae. Cholera and the cholera enterotoxin are increasingly recognized as the prototypes for a wide variety of non-invasive diarrheal diseases, collectively known as the enterotoxic enteropathies; of these, diarrhea due to enterotoxigenic strains of Escherichia coli is the most important.

Cholera remains a major epidemic disease. There have been seven great pandemics. The first long-distance spread of cholera to Europe and the Americas began in 1817, such that by the early 20th century, six waves of cholera had spread across the world in devastating epidemic fashion. Since then, until the 1960s, the disease contracted, remaining present only in southern Asia. In 1961, the "El Tor" biotype reemerged and produced a major epidemic in the Philippines to initiate a seventh global pandemic. Since then, this biotype has spread across Asia, the Middle East, Africa, and parts of Europe.

El Tor broke out explosively in Peru in 1991 (after an absence of cholera there for 100 years), and spread rapidly in Central and South America, with recurrent epidemics in 1992 and 1993. From the onset of the epidemic in January 1991 through September 1, 1994, a total of 1,041,422 cases and 9,642 deaths (overall case-fatality rate: 0.9%) were reported from countries in the Western Hemisphere to the Pan American Health Organization.

V. cholerae serogroup O139"Bengal", which arose in October of 1992 in India and Bangladesh, may become the cause of the 8th great pandemic of cholera. It is derived genetically from the El Tor pandemic strain but it has changed its antigenic structure such that there is no existing immunity and all ages, even in endemic areas, are susceptible. The epidemic has continued to spread. And V. cholerae O139 has affected at least 11 countries in southern Asia. Specific totals for numbers of V. cholerae O139 cases are unknown because affected countries do not report infections caused by O1 and O139 separately.

Other vibrios may also be clinically significant in humans, and some are known to cause diseases in domestic animals. Nonpathogenic vibrios are widely distributed in the environment, particularly in estuarine waters and

seafoods. For this reason, isolation of a vibrio from a patient with diarrheal disease does not necessarily indicate an etiologic relationship.

Goal: Studying of laboratory diagnosis of cholera.

Concrete goals:

1. Study of biological properties and classification of Vibrio.
2. Study pathogenesis and clinical manifestations of cholera.
3. Study of the methods of laboratory diagnosis of cholera.
4. Interpret the results of the bacteriological examination of microorganisms.

Students should be able to:

1. Differentiate of pathogenic vibrios on biochemical and antigenic properties.
2. Isolate pure cultures of V.cholera and examine colonies of on alkaline agar.
3. Identify of isolated pure culture of V.cholera for morphology, culture and biochemical properties, antigenic structure.

Equipment: slides, immersion microscope, brouth culture of Vibrio, plate culture on MPA. basic dyes, inoculating loops, tables, atlas.

CHOLERA

Vibrios are among the most common bacteria in surface waters worldwide. They are curved aerobic rods and are motile, possessing a polar flagellum. V.cholerae serogroups O1 and O139 cause cholera in humans, while other vibrios may cause sepsis or enteritis (Table 1).

Table 1 – The Medically Important Vibrios

Organism	Human Disease
V.cholerae serogroups O1 and O139	Epidemic and pandemic cholera
V.cholerae serogroups non-O1/non-O139	Cholera-like diarrhea; mild diarrhea; rarely, extraintestinal infection
V.parahaemolyticus	Gastroenteritis, perhaps extraintestinal infection
V.mimicus, V.vulnificus, V.hollisae, V.damsela, V.anginolyticus, V.metschnikovii, V.fluvialis	Ear, wound, soft tissue, and other extraintestinal infections, all uncommon

Structure, Classification, and Antigenic Types

The cholera vibrios are Gram-negative, slightly curved rods whose motility depends on a single polar flagellum. On prolonged cultivation, vibrios may become straight rods that resemble the gram-negative enteric bacteria.

Their nutritional requirements are simple. Fresh isolates are prototrophic (i.e., they grow in media containing an inorganic nitrogen source, a utilizable carbohydrate, and appropriate minerals). In adequate media, they grow rapidly with a generation time of less than 30 minutes. Although they reach higher

population densities when grown with vigorous aeration, they can also grow anaerobically. Vibrios are sensitive to low pH and die rapidly in solutions below pH 6; however, they are quite tolerant of alkaline conditions. This tolerance has been exploited in the choice of media used for their isolation.

There are 155 known serogroups, differentiated by their O antigen. Only two serogroups, O1 and O139, are responsible for all epidemic and endemic cholera. The O1 serogroup can be further differentiated into three serotypes, Ogawa, Inaba, and (rarely) Hikojima, that can themselves be divided into two biotypes (genotypes), classical and El Tor. These serotypes are differentiated in agglutination and vibriocidal antibody tests on the basis of their dominant heat-stable lipopolysaccharide somatic antigens. The cholera group has a common antigen, A, and the serotypes are differentiated by the type-specific antigens, B (Ogawa) and C (Inaba) (see Table 2). An additional serotype, Hikojima, which has both specific antigens, is rare. V.cholerae O139 appears to have been derived from the pandemic El Tor biotype but has lost the characteristic O1 somatic antigen; it has gained the ability to produce a polysaccharide capsule; it produces the same cholera enterotoxin; and it seems to have retained the epidemic potential of O1 strains.

Table 2 – Antigenic Determinants of Vibrio cholerae

Serotype	O Antigens
Ogawa	A, B
Inaba	A, C
Hikojima	A, B, C

Because of DNA relatedness and other similarities, other vibrios formerly called "nonagglutinable". The term nonagglutinable is a misnomer because it implies that these vibrios are not agglutinable; in fact, they are not agglutinable in antisera against the O antigen group 1 cholera vibrios, but they are agglutinable in their own specific antisera (Fig. 1).

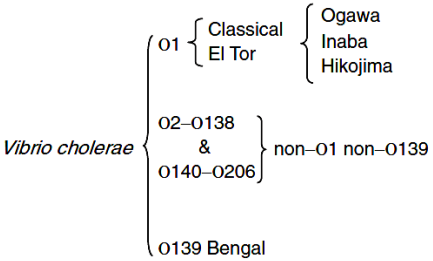

Fig. 1. Classification of V. cholerae with special reference to O serogroup

More than 200 serogroups are so far idenitified. A summary of the classification is as shown in Figure 1. Some strains of non-O group 1 vibrios cause diarrheal disease by means of an enterotoxin related to the cholera enterotoxin and, perhaps, by other mechanisms, but these strains have not been associated with devastating outbreaks like those caused by the true cholera vibrios.

Culture. V.cholerae produces convex, smooth, round colonies that are opaque and granular in transmitted light. V.cholerae and most other vibrios grow well at 37°C on many kinds of media, including defined media containing mineral salts and asparagine as sources of carbon and nitrogen. V.cholerae grows well on thiosulfate-citrate-bile-sucrose (TCBS) agar, on which it produces yellow colonies that are readily visible against the dark-green background of the agar. Characteristically, vibrios grow at a very high pH (8.5–9.5) and are rapidly killed by acid. Cultures containing fermentable carbohydrates therefore quickly become sterile.

In areas where cholera is endemic, direct cultures of stool on selective media such as TCBS, and enrichment cultures in alkaline peptone water are appropriate. However, routine stool cultures on special media such as TCBS generally are not necessary or cost-effective in areas where cholera is rare.

Growth Characteristics. The cholera vibrios cause many distinctive reactions. Vibrios are oxidase-positive, which differentiates them from enteric gram-negative bacteria. The O group 1 cholera vibrios almost always fall into the ***Heiberg I fermentation pattern***; that is, ***they ferment sucrose and mannose but not arabinose, and they produce acid but not gas***. Freshly isolated agar-grown vibrios of the El Tor biotype, in contrast to classical V.cholerae, produce a cell-associated mannose-sensitive hemagglutinin active on chicken erythrocytes (table 3). This activity is readily detected in a rapid slide test. In addition to hemagglutination, numerous tests have been proposed to differentiate the classical and El Tor biotypes, including production of a hemolysin, sensitivity to selected bacteriophages, sensitivity to polymyxin, and the Voges-Proskauer test for acetoin. El Tor vibrios originally were defined as hemolytic. They differed in this characteristic from classical cholera vibrios; however, during the most recent pandemic, most El Tor vibrios had lost the capacity to express the hemolysin. Most El Tor vibrios are Voges-Proskauer positive and resistant to polymyxin and to bacteriophage IV, whereas classical vibrios are sensitive to them. As both biotypes cause the same disease, these characteristics have only epidemiologic significance. Strains of the El Tor biotype, however, produce less cholera enterotoxin, but appear to colonize intestinal epithelium better than vibrios of the classical variety. Also, they seem some what more resistant to environmental factors. Thus, El Tor strains have a higher tendency to become endemic and exhibit a higher infection-to-case ratio than the classical biotype.

Table 3 – Different features of classical and El-Tor vibrios

Feature	Classical	El-Tor
Hemolysis of sheep RBC	-	+
Hwmagglutination of chick RBC	-	+
VP reaction	Weak or -	Strong +
Susceptibility to polymyxin	+	-
Sensitivity to phage IV	+	-
Sensitivity to phage V (Mukherjee and Basu)	-	+

Virulence factors. The vibrios never invade the epithelium but instead remain within the lumen and secrete an enterotoxin.

Bacterial Colonization: Fllagelar proteins mediate chemotaxis based motility which helps virulent V.cholerae to reach the luminal epithelium in small intestine. Proteolytic enzymes help to dissolve glycoprotein coating of the intestinal cells. Coregulated pili along with accesory colonization factor "adhesin" mediate adherence of the organism to the epithelium of microvilli at the brush border where they multiply.

Hemagglutination-protease (mucinase) is important for detachment of Vibrio from epithelial cells.

Vibrio cholerae Enterotoxin is the primary virulence factor responsible for the rice water diarrhea. The toxin gene is carried by a lysogenic bacteriophage ensuring that this mobile toxin gene will continue to create new pathogenic strains.

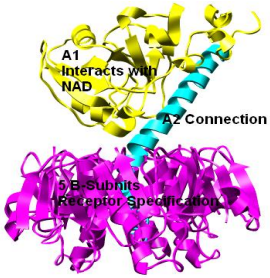

Fig. 2. Cholera toxin structure

V.cholerae produce a heat-labile enterotoxin with a molecular weight of about 84,000, consisting of subunits A (MW 28,000) and B (Fig. 2). Ganglioside GM1 serves as the mucosal receptor for subunit B, which promotes entry of subunit A into the cell. Activation of subunit A1 yields increased levels of intracellular cAMP and results in prolonged hypersecretion of water and electrolytes. There is increased sodium-dependent chloride secretion, and absorption of sodium and chloride is inhibited.

Diarrhea occurs—as much as 20–30 L/d—with resulting dehydration, shock, acidosis, and death.

Epidemiology. Cholera appears to exhibit three major epidemiologic patterns: heavily endemic, neoepidemic (newly invaded, cholera-receptive areas), and, in developed countries with good sanitation, occasional limited outbreaks. These patterns probably depend largely on environmental factors (including sanitary and cultural aspects), the prior immune status or antigenic

experience of the population at risk, and the inherent properties of the vibrios themselves, such as their resistance to gastric acidity, ability to colonize, and toxigenicity. In the heavily endemic region of the Indian subcontinent, cholera exhibits some periodicity; this may vary from year to year and seasonally, depending partly on the amount of rain and degree of flooding. Because humans are the only reservoirs, survival of the cholera vibrios during interepidemic periods probably depends on a relatively constant availability of low-level undiagnosed cases and transiently infected, asymptomatic individuals. Long-term carriers have been reported but are extremely rare. The classic case occurred in the Philippines, where "cholera Dolores" harbored cholera vibrios in her gallbladder for 12 years after her initial attack in 1962. Her carrier state resolved spontaneously in 1973; no secondary cases had been associated with her well-marked strain. Recent studies, however, have suggested that cholera vibrios can persist for some time in shellfish, algae or plankton in coastal regions of infected areas and it has been claimed that they can exist in "a viable but nonculturable state."

In neoepidemic cholera-receptive areas, vigorous epidemiologic measures, including rapid identification and treatment of symptomatic cases and asymptomatically infected individuals, education in sanitary practices, and interruption of vehicles of transmission (e.g., by water chlorination), may be most effective in containing the disease. In such situations, spread of cholera usually depends on traffic of infected human beings, although spread between adjacent communities can occur through bodies of water contaminated by human feces.

Reservoir. Humans apparently are the only natural host for the cholera vibrios. V.cholerae lives in aquatic environments. V.cholerae lives attached to algae, copepods, and crustacean shells. It can survive for years and grow, but when conditions are not suitable for growth it can become dormant.

There are two reservoirs for V.cholerae O1 and O139: humans and the aquatic environment. Humans are considered the primary reservoir and can be asymptomic carriers. These bacteria are considered hyperinfective immediately upon release from the human body as defined by a significantly lower infectious dose required to cause an infection.

Portal of Exit. The portal of exit from the human reservoir is from the anus in fecal waste. Humans shed fragments of V.cholerae biofilm into the environment. Symptomatic patients begin shedding hyperinfectious bacteria from before diarrhea begins and continue to shed for one to two. Asymptomatic cases are believed to shed bacteria for only a day. The portal of exit from the aquatic reservoir is in water used for drinking or food preparation, or in contaminated shellfish.

Mode of Transmission. Whether the reservoir is human or aquatic, the primary mode of transmission is ingestion of water or food prepared with water

containing V.cholerae. During an outbreak, there can be hand-to-mouth communication of V.cholerae.

In many instances, only 1–5% of exposed susceptible persons develop disease. The carrier state seldom exceeds 3–4 weeks, and the importance of carriers in transmission is unclear. Vibrios survive in water for up to 3 weeks.

Portal of Entry is through the mouth in contaminated water or food.

Susceptible hosts. Vulnerable populations include anyone put at risk by dehydration. This would include all infants, young children, pregnant women, the elderly, and people with other health conditions where hydration levels must be closely monitored. Fluid losses can be has high as 1 liter per hour causing rapid, severe dehydration and metabolic acidosis.

A person with normal gastric acidity may have to ingest as many as 10^{10} or more V.cholerae to become infected when the vehicle is water, because the organisms are susceptible to acid. When the vehicle is food, as few as 10^{2}–10^{4} organisms are necessary because of the buffering capacity of food. Any medication or condition that decreases stomach acidity makes a person more susceptible to infection with V.cholerae.

Pathogenesis & Pathology. Cholera is not an invasive infection. The organisms do not reach the bloodstream but remain within the intestinal tract. Virulent V.cholerae organisms attach to the microvilli of the brush border of epithelial cells. There they multiply and liberate cholera toxin and perhaps mucinases and endotoxin.

Colonization of the Small Intestine. There are several characteristics of pathogenic V.cholerae that are important determinants of the colonization process. These include adhesins, neuraminidase, motility, chemotaxis and toxin production. If the bacteria are able to survive the gastric secretions and low pH of the stomach, they are well adapted to survival in the small intestine. V.cholerae is resistant to bile salts and can penetrate the mucus layer of the small intestine, possibly aided by secretion of neuraminidase and proteases (mucinases). They withstand propulsive gut motility by their own swimming ability and chemotaxis directed against the gut mucosa.

Specific adherence of V.cholerae to the intestinal mucosa is probably mediated by long ***filamentous fimbriae*** that form bundles at the poles of the cells. These fimbriae have been termed Tcp pili (for toxin coregulated pili), because expression of these pili genes is coregulated with expression of the cholera toxin genes.

Two other possible adhesins in V.cholerae are a surface protein that agglutinates red blood cells (hemagglutinin) and a group of outer membrane proteins which are products of the acf (accessory colonization factor) genes. acf mutants have been shown to have reduced ability to colonize the intestinal tract. It has been suggested that V.cholerae might use these nonfimbrial adhesins to mediate a tighter binding to host cells than is attainable with fimbriae alone.

V.cholerae produces a protease originally called ***mucinase*** that degrades different types of protein including fibronectin, lactoferrin and cholera toxin itself. It has been suggested that the mucinase might contribute to detachment rather than attachment. Possibly the vibrios would need to detach from cells that are being sloughed off of the mucosa in order to reattach to newly formed mucosal cells.

Cholera Toxin. The toxin has been characterized and contains 5 binding (B) subunits, an active (A1) subunit, and a bridging piece (A2) that links A1 to the 5B subunits. They complement each other to create a potent enterotoxin.

Drifting in the intercellular space, cholera toxin's B subunits eventually binds to the cell surface of a target cell due to the target cell's expression of certain GM1 gangliosides. The binding causes a conformational alteration and causes presentation of the A subunit to the cell's surface. Intracellular glutathione reduces the disulfide bond of the A subunit separating A1 from A2. A1 is then able to hydrolyze NAD, forming ADP-ribose and nicotinamide. Once it has entered the cell, the A1 subunit enzymatically transfers ADP ribose from NAD to a protein (called Gs), that regulates the adenylate cyclase (AC) system which is located on the inside of the plasma membrane of mammalian cells (Fig. 3).

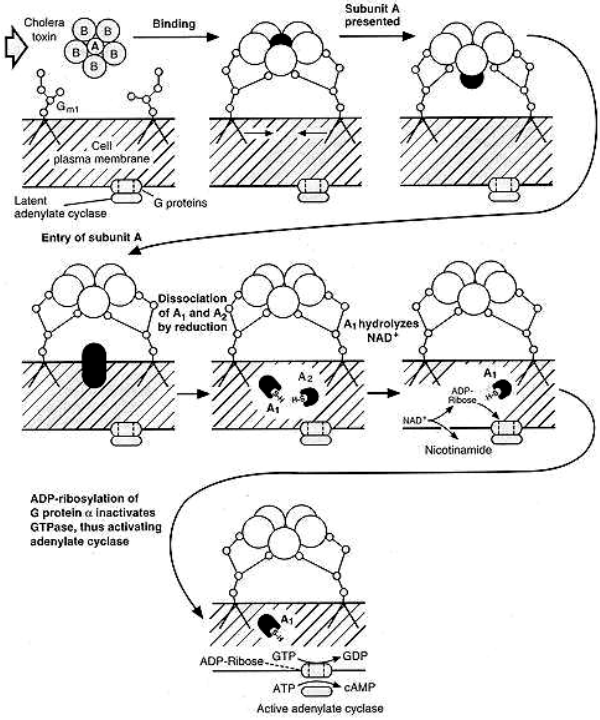

Fig. 3. Mechanism of action of cholera toxin

Activation of the AC system leads to increase levels of intracellular cAMP, and eventually leading to the secretion of H_2O, Na+, K+, Cl-, and HCO_3- into the lumen of the small intestines.

AC is normally activated by a regulatory protein (Gs) and GTP. Activation is normally brief because another regulatory protein (Gi) hydrolyzes the GTP. (Fig. 4).

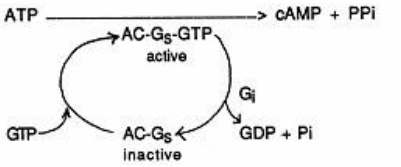

Fig. 4. Regulation of adenylate cyclase

Cholera toxin causes a change from normal actions. The A1 fragment of cholera toxin catalyzes the attachment of ADP-Ribose (ADPR) to the Gs forming the Gs-ADPR form which GTP cannot be hydrolyzed. The enzyme therefore becomes continually activated because GTP hydrolysis is the event that inactivates the adenylate cyclase (Fig. 5).

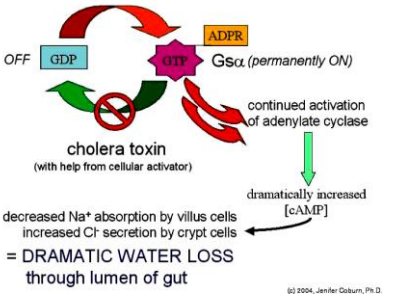

Fig. 5. Mechanism of action of cholera toxin

The effect of ending inactivation is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of Cl- into the intestinal lumen. H_20, Na+ and other electrolytes follow due to the osmotic and electrical gradient caused by the loss of Cl-. The lost H_20 and electrolytes in the mucosal cells are replaced from the blood. Essentially the affected cells become pumps for water and electrolytes out of the body, causing the dehydration characteristic to cholera.

If dehydration is not remedied, the patient will eventually experience liquid stool. From the first liquid stool, it takes 4–12 hours to experience shock. From shock death will follow within 18 hours to several days.

Because the stool can contain 10^8 viable vibrios per ml, such a patient could shed 2×10^{12} cholera vibrios per day into the environment. Perhaps by production of CT, the cholera vibrios thus ensure their survival by increasing the likelihood of finding another human host.

Signs and symptoms. About 75% of people infected with cholera do not develop symptoms. Of those who do develop symptoms, 80% are considered mild to moderate cases and 10 to 20% are severely affected. Children and the elderly are most susceptible. In its extreme manifestation, cholera is one of the most rapidly fatal illnesses known. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2–3 hours if no treatment is provided. More commonly, the disease progresses from the first liquid stool to shock in 4–12 hours, with death following in 18 hours to several days.

Incubation ranges from 12–72 hours depending on the dose of ingested organisms. Symptoms include acute, profuse watery diarrhea and often vomiting. Tachycardia, loss of skin turgor (Fig. 6), dry mucous membranes, hypotension and thirst are often signs of dehydration.

Fig. 6. Loss of skin turgor

The clinical description of cholera begins with sudden onset of massive diarrhea. The patient may lose gallons (10–20 liters/day) of protein-free fluid and associated electrolytes, bicarbonates and ions within a day or two. This results from the activity of the cholera enterotoxin which activates the adenylate cyclase enzyme in the intestinal cells, converting them into pumps which extract water and electrolytes from blood and tissues and pump it into the lumen of the intestine. This loss of fluid leads to dehydration, anuria, acidosis and shock. The watery diarrhea is speckled with flakes of mucus and epithelial cells ("rice-water stool") and contains enormous numbers of vibrios. The loss of potassium ions may result in cardiac complications and circulatory failure. In extreme cases and if left untreated, severe dehydration can lead to kidney failure and death. Untreated cholera frequently results in high (50–60%) mortality rates.

Consequences of severe dehydration: intravascular volume depletion, severe metabolic acidosis, hypokalemia, cardiac and renal failure.

Immunity. Infection with V. cholerae results in a spectrum of responses ranging from life-threatening secretory diarrhea to mild or unapparent infections of no manifestation except a serologic response. The reasons for these differences are not known. One idea is that individuals differ in the availability of intestinal receptors for cholera vibrios or for their toxin, but this has not been proven. Prior immunologic experience is certainly a major factor.

Resistance is related to the presence of circulating antibody and, perhaps more importantly, local immunoglobulin A (IgA) antibody against the cholera bacteria or the cholera enterotoxin or both. Intestinal IgA antibody can prevent attachment of the vibrios to the mucosal surface and neutralize or prevent binding of the cholera enterotoxin. For reasons that are not clear, individuals of blood group O are slightly more susceptible to cholera. Breastfeeding is highly recommended as a means of increasing immunity of infants to this and other diarrheal disease agents.

After natural infection by V. cholerae, circulating antibodies can be detected against several cholera antigens including the toxin, somatic (O) antigens, and flagellar (H) antigens. Antibodies directed against Vibrio O antigens are considered "vibriocidal" antibodies because they will lyse V.cholerae cells in the presence of complement and serum components. Vibriocidal antibodies reach a peak 8–10 days after the onset of clinical illness, and then decrease, returning to the baseline 2–7 months later.

After natural infection, people also develop toxin-neutralizing antibodies but there is no correlation between antitoxic antibody levels and the incidence of disease in cholera zones.

Since cholera is essentially a topical disease of the small intestine, it would seem that topical defense might be a main determinant of protection against infection by V.cholerae. Recurrent infections of cholera are in fact, rare, and this is probably due to local immune defense mediated by antibodies secreted onto the surfaces of the intestinal mucosa. Moreover, in children who are nursing cholera is less likely to occur, presumably due to protection afforded by secretory antibody in mother's milk.

Secretory IgA, as well as IgG and IgM in serum, can be detected in the intestinal mucosa of immune individuals. Although these antibodies presumably have to function in the absence of complement they still bring about protective immunity. Motility is important in pathogenesis, and antibodies against flagella could immobilize the vibrios. Antibodies against flagella or somatic O antigens could cause clumping and arrested motion of cells. Antitoxic antibodies could react with toxin at the epithelial cell surface and block binding or activity of the toxin. Since the process by which the vibrios attach to the intestinal epithelium is highly specific, antibodies against Vibrio fimbriae or other surface components (LPS?) could block attachment.

Diagnosis. Rapid bacteriologic diagnosis offers relatively little clinical advantage to the patient with secretory diarrhea, because essentially the same treatment (fluid and electrolyte replacement) is employed regardless of etiology. Nevertheless, rapid identification of the agent can profoundly affect the subsequent course of a potential epidemic outbreak. Because of their rapid growth and characteristic colonial morphology, V.cholerae can be easily isolated and identified in the bacteriology laboratory, provided, first, that the presence of cholera is suspected and, second, that suitable specific diagnostic antisera are available.

The classic case of cholera, which includes profound secretory diarrhea and should evoke clinical suspicion, can be diagnosed within a few minutes in the prepared laboratory by finding rapidly motile bacteria on direct, bright-field, or dark-field microscopic examination of the liquid stool. The technician can then make a second preparation to which a droplet of specific anti-V.cholerae O group 1 antiserum is added. This quickly stops vibrio motility. Another rapid technique is the use of fluorescein isothiocyanate-labeled specific antiserum (fluorescent antibody technique) directly on the stool or rectal swab smear or on the culture after enrichment in alkaline peptone broth.

Microscopy:

Direct microscopy: Hanging drop preparation to see the motility of the bacteria.

Gram staining: Gram-negative, short-curved (comma-shaped) rods.

Fluorescent antibody staining: for identifying V.cholerae O1.

Culture:

Specimen: watery stool and mucus flakes from stool; rectal swab; vomitus.

Preservation and transport media: When there may be delay in transmission of specimen to laboratory, it must be refrigerated at 8° to 10° for 24 hours, else following transport media may be used: Vekataraman-Ramakrishnan (V.R) medium and Cary-Blair medium.

Subcultures are made from transport media to TCBS or BSA within 12–18 hours since other organisms may begin to overgrow the media after prolonged incubation.

Enrichment media: alkaline peptone water (pH 8.6); Monsur's taurocholate tellurite peptone water (pH 9.2).

- *Thiosulphate citrate bile sucrose agar (TCBS) aka Indicator-bromothymol blue medium:* After overnight incubation at 37 °C, the colonies are large, moist, translucent, round and yellow due to sucrose fermentation and turns green on continuous incubation.
- *Alkaline Bile salt agar (BSA):* colonies resembling that in the nutrient agar.
- *Monsur's Gelatin Taurocholate Trypticate tellurite Agar (GTTA):* small translucent colonies with a greyish black center.

Biochemical tests:

- Ferments sucrose, glucose and mannose with production of acid and gas but fails to ferment arabinose.
- Catalase and oxidase positive.
- Cholera red reaction: When V.cholerae are grown for 24 hours in peptone water medium containing adequate amount of tryptophan and nitrate, they produce indole and reduce nitrate to nitrite. On adding a few drops of sulphuric acid, nitroso-indole is formed, which is red in color.
- Hemolysis reaction: the classical biotype do not hemolyse sheep or goat RBCs but El Tor biotype can hemolyse it.
- Agglutination test: slide or tube agglutination test with V.cholerae O1 antiserum. Agglutination is rapid (<2 min.), fine and granular.
    - Positive test result: Test is repeated using monspecific Ogawa and Inaba sera. Hikojima strain react equally well with both Ogawa and Inaba sera.
    - Negative test result: Tested with other O antiserum to establish their identity (non O1 strains).

Although demonstration of typical agglutination essentially confirms the diagnosis, additional conventional tests such gelatinase, lysine, arginine, and ornithine decarboxylase reactions may be helpful. Tests for chicken cell hemagglutination, hemolysis, polymyxin sensitivity, and susceptibility to phage IV are useful in differentiating the El Tor biotype from classic V.cholerae. Tests for toxigenesis may be indicated.

Diagnosis can be made retrospectively by confirming significant rises in specific serum antibody titers in convalescents. For this purpose, conventional agglutination tests, tests for rises in complement-dependent vibriocidal antibody, or tests for rises in antitoxic antibody can be employed. Convenient microversions of these tests have been developed. Passive hemagglutination tests and enzyme-linked immunosorption assays (ELISAs) have also been proposed.

Cultures that resemble V. cholerae but fail to agglutinate in diagnostic antisera (nonagglutinable or non-O group 1 vibrios) present more of a problem and require additional tests such as oxidase, decarboxylases, inhibition by the vibriostatic pteridine compound 0/129, and the "string test." The string test demonstrates the property, shared by most vibrios and relatively few other genera, of forming a mucus-like string when colony material is emulsified in 0.5 percent aqueous sodium deoxycholate solution. Additional tests for enteropathogenicity and toxigenesis may be useful. Genetically based tests such as PCR are increasingly being used in specialized laboratories.

Treatment of cholera involves the rapid replacement of the lost fluid and ions. Rehydration is the key form of treatment. Mild or moderate dehydration can be treated with simple oral rehydration solutions containing salts and

glucose. Severe cases of dehydration require treatment with intravenous fluids and antibiotics. Following this replacement, administration of isotonic maintenance solution should continue until the diarrhea ceases. By this simple treatment regimen, patients on the brink of death seem to be miraculously cured and the mortality rate of cholera can be reduced more than ten-fold.

WHO recommends that antibiotics are only used in severe cases as overuse of antibiotics has led to the emergence of multiresistant strains, some of which where found to be highly virulent. Doxycycline is the antibiotic of choice for adults (except pregnant women) because only one dose is required. TMP-SMX is the antibiotic of choice for children. Tetracycline is equally effective; however, in some countries it is not available for paediatric use. Furazolidone is the antibiotic of choice for pregnant women. Erythromycin or chloramphenicol may be used when the antibiotics recommended are not available, or where V.cholerae O1 is resistant to them.

Vaccine. The observation that natural infection confers effective and long-lasting immunity against cholera has led to efforts to develop a vaccine which will elicit protective immunity. The first attempts at a vaccine in 1960s were directed at whole cell preparations injected parenterally. At best, 90% protection was achieved and this immunity waned rapidly to the baseline within one year. Purified LPS fractions from different biotypes have also been given as vaccines with variable success. The cholera toxin can be converted to toxoid in the presence of formalin and glutaraldehyde. The toxoid is a poor antigen, however, and it elicits a very low level of protection.

The oral vaccines are made from a live attenuated strains of V. cholerae. The ideal properties of such a "vaccine strain" of the bacterium would be to possess all the pathogenicity factors required for colonization of the small intestine (e.g. motility, fimbriae, neuraminidase, etc.) but not to produce a complete toxin molecule. Ideally it should produce only the B subunit of the toxin which would stimulate formation of antibodies that could neutralize the binding of the native toxin molecule to epithelial cells.

A new vaccine has been developed to combat the V.cholerae Bengal strain that has started spreading in epidemic fashion in the Indian subcontinent and Southeast Asia. The Bengal strain differs from previously isolated epidemic strains in that it is serogroup O139 rather than 01, and it expresses a distinct polysaccharide capsule. Since previous exposure to O1 V.cholerae does not provide protective immunity against O139, there is no residual immunity in the indigenous population to the Bengal form of cholera. The noncellular vaccine is relatively nontoxic and contains little or no LPS and other impurities.

Probably the natural disease should be simulated to induce truly effective immunity although a parenterally administered conjugate vaccine consisting of the polysaccharide of the vibrio LPS covalently linked to cholera toxin has

given promising results in preliminary studies. Combined preparations of bacterial somatic antigen and toxin antigen have been reported to act synergistically in stimulating immunity in laboratory animals; that is, the combined protective effect is closer to the product than to the sum of the individual protective effects.

Cholera is essentially a disease associated with poor sanitation. The simple application of sanitary principles protecting drinking water and food from contamination with human feces, control rests on education and on improvement of sanitation would go a long way toward controlling the disease. Patients should be isolated, their excreta disinfected, and contacts followed up. However, at present, this is not feasible in the underdeveloped areas that are afflicted with epidemic cholera or are considered to be cholera receptive. Antibiotic or chemotherapeutic prophylaxis is feasible and may be indicated under certain circumstances. Repeated injection of a vaccine can confer limited protection to heavily exposed persons (eg, family contacts) but is not effective as an epidemic control measure.

Practical tasks, being carried out during practical classes:

1. Study microslides: V.cholera (stained after Gram) and motility (in phase contrast microscope).
2. Study "stool culture of a patient with cholera" on alkaline brouth and agar.
3. Prepare a microslide from the isolated colorless colony. Stain the smear after Gram. Examination of the smear with immersion microscope.
4. Performing of slide agglutination test with O-1 cholera serum.
5. Studying of the scheme of laboratory diagnosis of cholera.

Therminology: Vibrio cholera, El Tor biotype, Ogawa, Inaba, and Hikojima types, thiosulfate-citrate-bile-sucrose (TCBS) agar, Alkaline Bile salt agar (BSA), Monsur's Gelatin Taurocholate Trypticate tellurite Agar (GTTA), Heiberg I fermentation pattern, Mukherjee and Basu cljassification, adenylate cyclase, "rice-water stool".

Theoretical questions for control:

1. Genus Vibrio, major characteristics, antigenic structure.
2. Culture properties of Vibrio.
3. Laboratory diagnosis of cholera.
4. Routes of transmission and pathogenesis of cholera.
5. Mechanism of action of choleratoxin.
6. Control of cholera.

Test tasks for control:

1. A patient with complaints to diarrhea, vomiting, muscles pain, weakness was brought to a department of infectious disease. A doctor diagnosed cholera presumptively. Which of the following methods should be used for express-diagnosis?

A. RIF. *C. Biological.* *E. Allergic.*
B. AT. *D. Bacteriological.*

2. Mechanism of action of V.cholera:

A. Activation of adenylcyclase. *D. All of the above.*
B. Invasion of intestine. *E. None of the above.*
C. Endotoxin release.

3. Faeces of patient with suspicion on cholera were delivered to bacteriological laboratory. Which of the following methods should be used to confirm the diagnosis?

A. Allergic. *C. Biological.* *E. Virological.*
B. Bacterioscopical. *D. Serological.*

4. Characteristic properties of causative agent of cholera (serotype O139) are:

A. Resistance to polymixin. *D. Agglutination with O1 serum.*
B. Haemolysis absence. *E. All of the above.*
C. Sensitivity to phages of El Tor.

5. Determine the characteristic property of the cause of classical cholera, distinguished from the one of cholera El-tor:

A. Susceptibility to polymixin. *D. Exotoxin production.*
B. Agglutination with O1-serum. *E. Endotoxin production.*
C. Hemolytic activity.

6. Cholera is a toxicogenic dysenteric disease common in many parts of the world. In the treatment of patients who have cholera, the use of a drug that inhibits adenyl cyclase would be expected to:

A. Kill the patient immediately. *D. Block the action of toxin.*
B. Eradicate the organism. *E. All of the above.*
C. Increase intestinal motility.

7. Which of the following media on which the colonies are colourless, transparent and flat is useful for cultivation of V.cholera:

A. Blood agar *D. Egg yolk agar.*
B. MacConkey agar. *E. Alkaline bile salt agar.*
C. Meat pepton agar.

8. The vibrio is classified based on antigenic characters into six groups of which the Vibrio cholera belongs to:

A. O-1 subgroup. *C. O-3 subgroup.* *E. O-5 subgroup.*
B. B. O-2 subgroup. *D. O-4 subgroup.*

9. Vibrio cholera was discovered by:

A. Mechnikiff. *C. Ivanivsky.* *E. Koch.*
B. Erlich. *D. Paster.*

10. The first seeding of water into the 1% pepton base water the thin film appeared on the surface of the medium in 5 hours. Which of the following of the causative agent of the infectious diseases has such cultural properties?

A. Plaque. *C. Cholera.* *E. Salmonelosis.*
B. Tuberculosis. *D. Dysentery.*

Answers to test tasks: **1** – A; **2** – A; **3** – B; **4** – A; **5** – A; **6** – D; **7** – E; **8** – A; **9** – E; **10** – C.

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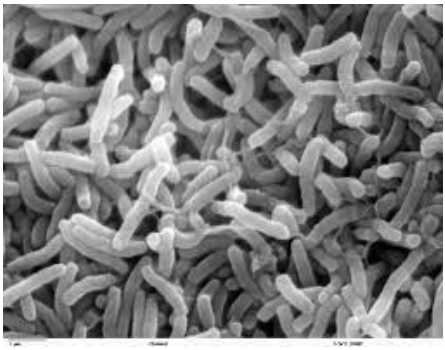

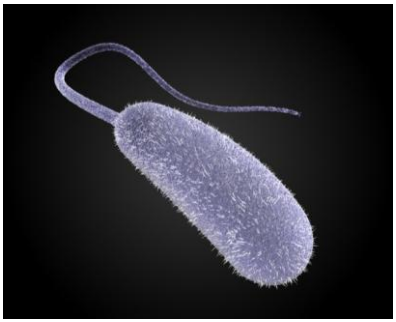

CHOLERA

Methodical instructions on subject "Microbiology, virology and immunology" for the II and III year English media students of medical and dentistry faculties

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