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LABORATORY DIAGNOSIS OF CHOLERA *

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Cholera may be, and usually is, difficult to distinguish on clinical grounds alone from other acute diseases characterized by a purging diarrhoea, vomiting and associated symptoms resulting from extreme dehydration and concomitant haemoconcentration. Precise diagnosis of the individual case is probably not of great significance during the course of an epidemic already established as cholera, but assumes very considerable importance at the endemic level and at the initiation of epidemic prevalence. As in many other infectious diseases, the diagnosis of cholera may be established unequivocally only through isolation of the causative micro-organism, its differentiation from related but non-pathogenic forms, and its identification as *Vibrio cholerae*.

The diagnosis of cholera has been discussed in detail elsewhere (Pollitzer, 1956); the present communication is concerned with the minimal procedures required to isolate and establish the identity of *V. cholerae*, and hence the laboratory diagnosis of the human disease.

Collection and Preservation of Specimens

In the naturally occurring human disease the cholera vibrio proliferates in, and is almost always confined to, the lumen of the bowel, invasion of the deeper tissues being a rare occurrence. While the micro-organism may occasionally be found in the vomitus, it is constantly present in the dejecta, and for diagnostic purposes is isolated from a stool specimen.

Collection

The collection of stool specimens is to be considered under two headings—namely, collection from patients subject to examination by trained

* This is one of a series of studies on the laboratory diagnosis of various diseases which, it is hoped, will eventually be revised and published in monograph form. An effort is made to ensure that the diagnostic methods recommended in these studies are as internationally representative and acceptable as possible by securing the co-operation of a number of experts from different countries. A list of the reviewers of the study presented here is given in the Annex on page 288. To all of these, and to the two authors, the World Health Organization is greatly indebted. — ED.

personnel and collection from infected persons by the individual himself or by some untrained person.

In the first instance rice-water or liquid stool specimens may be taken by insertion of a sterile soft rubber catheter (such as French No. 12), lubricated with sterile liquid petrolatum, 4-5 cm into the rectum, and collection of the specimen in a sterile tube. When the stool is sufficiently formed for it not to flow readily through a catheter (and a freshly voided stool specimen is not available), the specimen may be taken by the usual rectal-swab method. The sterile swab of absorbent cotton is enclosed in a short length of gum-rubber tubing, cut at an angle at the end to be inserted. The rubber tube is lubricated with sterile liquid petrolatum and inserted 3-5 cm into the rectum, and the swab is pushed through the open end of the tube, moved about to collect material from the bowel wall, and withdrawn into the rubber tube before the assembly is removed from the anus. The swab is then taken from the rubber tube and placed in a sterile test-tube. The test-tube may be dry when the specimen is to be cultured within an hour or so, or may contain a small amount of sterile physiological salt solution or preserving fluid (see below). It should not contain a nutrient solution such as broth. When a stool specimen cannot be obtained directly, a piece of clothing soiled with faeces may be used; such a specimen is not as satisfactory as a faecal specimen, but may be of value in establishing a retrospective diagnosis.

When the specimen is taken by the infected individual or by untrained personnel, or when a large inoculum is desired because the occurrence of vibrios in only small numbers is expected, a portion of freshly voided faeces is put into a leak-proof container such as a wide-mouthed bottle. Pasteboard or wooden spoons (such as tongue depressors) should be provided to facilitate transfer of the faecal material to the container. The containers should have tightly fitting tops or screw caps to avoid spilling, access of flies, and drying of the specimen. The containers should be previously boiled or sterilized by autoclaving. It is essential to prevent the specimen and/or its container from coming in contact with disinfectants or ordinary water; in addition to not being sterile, the latter may contain cholera-like vibrios.

Preservation

While it is preferable that specimens be cultured within one to three hours after collection, some form of preservation may be required for transmission to distant laboratories. If refrigeration is available, specimens stored at 8°C to 10°C for 24 hours show no apparent diminution in the number or proportion of cultivable vibrios over those obtained on immediate culture. In the absence of refrigeration facilities the preserving fluid of Venkatraman & Ramakrishnan (1941) may be used. It is prepared as follows:

In 800 ml of hot distilled water, dissolve 12.4 g of boric acid and 14.9 g of potassium chloride, cool the solution and make up to 1 litre. To 250 ml of this stock solution,

add 133.5 ml of M/5 sodium hydroxide and 20 g of dried sea-salt (or other unrefined table salt) and filter through paper. This borate-buffered saline is dispensed appropriately, for instance in 10-ml quantities in 1-oz screw-capped bottles, and sterilized in the autoclave.

If time or facilities for preparing this preserving fluid are not available, a 2% solution of sodium chloride may be substituted and a few drops of N sodium hydroxide solution added immediately before inoculation with stool specimen to neutralize the usually acid reaction of the stool.

When specimens are forwarded to the laboratory by mail it is essential that the container be surrounded by sufficient absorbent material, such as sawdust or cotton wool, to take up the contents in the event of breakage, and be enclosed in another durable leak-proof container, and conform in all respects to the postal regulations of the country.

Culture Media and Reagents

The successful isolation of *V. cholerae* from infected stool specimens is dependent upon exploitation of the appropriate physiological properties and activities of the micro-organism. These include the ability to grow very rapidly in simple culture media such as peptone water, a preference for aerobic conditions, similarity to other enteric pathogens in failure to ferment lactose promptly and in resistance to bile salts, and the ability to grow at relatively high alkalinities. Within the large group of pathogenic enteric bacilli, none of these properties is unique to *V. cholerae* except growth in highly alkaline media.

While it is commonly assumed that *V. cholerae* requires an alkaline reaction for optimal growth, this micro-organism in fact grows quite as rapidly at neutrality as at an alkaline reaction, though it is relatively sensitive to acidity and a pH of 6 is definitely harmful. The use of alkaline media for the purposes of isolation, however, is a consequence, on the one hand, of the necessity for neutralization of the normally acid faecal material used as inoculum and, on the other, of an at least partial inhibition of the growth of contaminants by alkalinities which are not inhibitory to the cholera vibrio. *V. cholerae* multiplies at alkalinities as high as pH 9.5, though pH 9.6 is appreciably inhibitory, while most coliform bacilli are inhibited at pH 9.5 but not at pH 9.4. Thus, if the extreme alkaline range is to be used to inhibit the growth of contaminants, the reaction must be adjusted with some precision.

Fluid media

Fluid media are used in the isolation of *V. cholerae* largely for enrichment purposes, and may be required when the specimen is taken a week or more after onset of symptoms or in attempts to isolate the micro-organisms from stool specimens of persons showing no symptoms of the disease.

Peptone water. A medium containing 1% peptone and 0.5-3.0% sodium chloride and adjusted to pH 9.2 has been quite generally used since its introduction by Dunham (1887) and its recommendation by Koch and his co-workers (1902). The brand of peptone to be chosen for this and other peptone-containing media used for the isolation of *V. cholerae* is of some importance, for all peptones are not equally effective in supporting growth. The peptones described in the older literature as " peptonum siccum ", etc. are no longer available and, like all such preparations, are not subject to precise chemical characterization. It is highly desirable, then, that the peptone used be known to be suitable for this purpose.[a] Alkaline peptone-water medium is selective in the sense that *V. cholerae* grows more rapidly in it than most other micro-organisms, and characteristically as a surface film where there is maximal access to free oxygen. After growth for a few hours in this medium, *V. cholerae* is present in the surface growth in proportionately greater numbers than are the other micro-organisms in the inoculum.

Bismuth-sulfite medium. This medium was originally recommended for isolation of the cholera vibrio by Read (1939), and in the opinion of a number of workers is preferable to peptone water as an enrichment medium. As modified by Wilson & Reilly (1940), the medium is prepared in the following way:

Bismuth ammoniocitrate, if not available in dry form (scales), may be prepared as a stock solution as follows: put 60 g of bismuth citrate in a 500-ml glass-stoppered bottle, add 50 ml of distilled water and stir the mixture into a smooth paste with a glass rod. Add 20 ml of a 12% solution of ammonium hydroxide (liquor ammonii, specific gravity, 0.880), stir to mix, and replace the glass stopper and shake until the bismuth citrate has almost entirely dissolved. Then add distilled water to a final volume of 500 ml.

Bismuth-sulfite stock solution is prepared by dissolving 20 g of anhydrous sodium sulfite in 100 ml of boiling water, and adding to it *either* 0.1 g of bismuth ammoniocitrate dissolved in 10 ml of water *or* 0.16 ml of bismuth ammoniocitrate solution prepared as described above. Prepare a solution of 20 g of glucose in 100 ml of boiling water, and when this is cool mix the two solutions.

Bismuth-sulfite medium is prepared for use by adding 10 ml of bismuth-sulfite stock solution to 100 ml of saline peptone water at pH 9.1, and then 1 ml of absolute ethanol. The medium is used in the same way as peptone water, but when handling stool specimens from convalescents or asymptomatic persons suspected of harbouring the vibrios, it may be used at double strength and inoculated with an equal volume of faeces suspended in saline or in Venkatraman & Ramakrishnan's preserving fluid.

Potassium-tellurite medium. This medium as described by Gohar & Makkawi (1948) and by Gohar (1951) was used successfully in the Egyptian epidemic of 1947. It is prepared as follows:

Peptone water containing 1% peptone and 0.5% sodium chloride is treated with sufficient sodium carbonate (about 0.2%) to give a reaction of pH 9.0. To this 0.5%

[a] Of the peptones currently available, Bacto peptone (Digestive Ferments Company, Detroit, Mich., USA) is one of the most satisfactory.

sodium taurocholate is added, and the medium is distributed in appropriate containers. The authors recommend 25-ml Erlenmeyer flasks filled almost to the neck to give a small surface with concentration of growing vibrios, but culture tubes containing 10-ml quantities of medium may be used.

It is recommended that three series of cultures be made in this medium to which potassium tellurite has been added to give concentrations of 1: 100 000, 1: 200 000, and 1: 400 000, respectively. Alternatively, a single concentration of 1: 200 000 of tellurite may be used.

Solid media

A variety of agar media have been used for the isolation of *V. cholerae* in pure culture; many of them are to some degree selective and/or differential for this micro-organism. As indicated above, the nutritive requirements of the cholera vibrio are relatively simple and it will grow rapidly on peptone or on meat-extract/peptone agar, though the cell crop is much smaller than that obtained with richer media. The vibrio may be isolated by culture on meat-extract/peptone agar containing 0.5% sodium chloride and adjusted to pH 8 when the specimen contains very large numbers of micro-organisms. For diagnostic purposes, however, it is preferable to use a medium more specifically favouring the growth of *V. cholerae*.

Bile-salt agar. This is a widely used, though unpublicized, medium for the isolation of the cholera vibrio. It has the great advantage of simplicity, for it is the standard meat-extract/peptone agar containing 0.5% sodium chloride, but adjusted to pH 8.0 and containing, in addition, 0.5% sodium taurocholate. If *Proteus* is troublesome, the concentration of taurocholate may be increased to 1%.

Aronson's medium. This is a medium of the Endo type containing fermentable carbohydrate and decolorized fuchsin (Schiff's reagent); the colour is restored to the fuchsin in the presence of the aldehyde intermediates formed in the fermentation process. It was developed by Aronson (1915) and is still used. It is prepared as follows:

An agar medium containing 3.5% agar, 1% peptone, 1% meat extract, and 0.5% sodium chloride is prepared and steamed for 4-5 hours. To each 100 ml of the hot medium, add 5-6 ml of a 10% solution of anhydrous sodium carbonate and steam for 10-15 minutes. Then to each 100 ml of the hot medium add 5 ml each of a 20% solution of sucrose and a 20% solution of dextrin previously sterilized by steaming or filtration, 0.4 ml of a saturated alcoholic solution of basic fuchsin, and 2 ml of a freshly prepared 10% solution of sodium sulfite previously sterilized by boiling. After cooling to 40-45°C, plates are poured. If stored in the dark the medium may be used for several days.

Some batches of the medium are unsatisfactory, or even inhibitory, and each batch should be tested before being used for diagnostic purposes.

Modified Wilson-Reilly medium. This is the bismuth-sulfite medium devised by Wilson & Reilly (1940) as modified by Pandit (1941) and

Ahuja et al. (1951). The basic medium is tryptic-digest broth adjusted to pH 8.8 (Douglas, 1914) solidified with 2.5% agar. To each 100 ml of the basic medium are added 4.8 ml of a 20% solution of sodium sulfite, *either* 0.16 ml of the bismuth ammoniocitrate solution prepared as described above *or* 0.1 g of bismuth ammoniocitrate dissolved in 10 ml of water, 0.2 ml of absolute ethanol, and 1 ml of 10% mannose solution. These ingredients are added to the hot liquid basic medium, and plates are poured after it has cooled to 40-45°C.

Media for biochemical tests

Only three cultural characteristics of *V. cholerae* are of proven utility in the identification of this micro-organism. These are: the fermentation of sucrose and mannose and failure to ferment arabinose (*l*(+)-arabinose), giving Heiberg's (1934) fermentation type 1; the positive cholera-red reaction indicating the simultaneous formation of nitrite and indole; and the failure to form acetylmethylcarbinol during the fermentation of glucose, as indicated by a negative Voges-Proskauer reaction.

The fermentation tests are carried out in 1% peptone water containing 0.5% of the carbohydrate and 0.02% alcoholic solution of bromothymol blue as the acid-base indicator. Since only acid, but no gas is formed by *V. cholerae*, the fermentation tubes need not contain inverted vials.

For the cholera-red test it is essential that the peptone used in the peptone-water medium contain sufficient tryptophane to allow the formation of indole in detectable amounts in the nitroso-indole reaction. Batches of peptone may be previously tested with known cholera-red positive vibrios or with indole-producing coliforms, or special peptones devised for the purpose, such as Bacto tryptone, may be used.

Acetylmethylcarbinol is formed by Voges-Proskauer positive bacteria during growth in a liquid medium containing 0.5% glucose, 0.5% dibasic potassium phosphate, and 0.5% peptone; the kind of peptone used is not critical.

Agglutinating antisera

The serological specificity of *V. cholerae* is a function of the heat-stable O-antigenic complex, and the cholera vibrios are members of the serological O group I of Gardner & Venkatraman (1935). The heat-labile antigenic complex of *V. cholerae* is related to the antigenic complexes occurring in a variety of non-cholera vibrios, and antisera containing antibody to this antigen are not specific for O group I vibrios.

Preparation of immunizing antigens. Only first-day isolates of *V. cholerae* should be used as immunizing antigens to avoid R strains, which usually do not appear before the third day of the disease. Established Inaba and

Ogawa serotypes may be cultivated in broth or on agar media and harvested after 18 hours' incubation. In the former instance the organisms may be spun out in the centrifuge and resuspended in physiological salt solution; they need not be washed if the culture medium does not contain antigenic substances. The growth from agar cultures is washed off in sterile physiological salt solution and filtered through cotton to remove any particles of agar that may be present. The suspensions so prepared are then heated to destroy H antigen; heating should be begun as soon as the suspensions are prepared, to minimize autolytic changes in the vibrios.

To destroy the H-antigenic complex completely, so that traces of H antigen do not remain to stimulate antibody formation, requires more than simple immersion in boiling water for two hours. Burrows et al. (1946) showed that boiling under a reflux condenser for two to three hours gave a serologically pure O antigen, Gallut (1949) reported similar results, and Singh & Ahuja (1950) recommend heating the antigen in sealed ampoules in boiling water containing sufficient sodium chloride to raise the boiling-point to 101°C. Autoclaving at a steam pressure of 1-2 pounds per square inch (p.s.i.) (0.07-0.14 kg per cm^2) for two hours also completely destroys the H antigen and is probably the simplest method.

The heated antigen is diluted in physiological saline and standardized, photometrically or gravimetrically, to contain 1 mg (dry weight) of vibrios per ml (2000 million per ml).

Rabbit immunization. Young rabbits weighing 2-3 kg should be used; such animals are not yet full-grown and continue to gain weight. The animals are inoculated at four-day intervals, or twice a week, by the intraperitoneal (IP) route for the first two or three inoculations, and by the intravenous (IV) route for the remainder. The intraperitoneal inoculations stimulate primarily the non-splenic, and slower reacting, antibody-forming mechanisms, while the intravenous inoculations stimulate the splenic mechanisms (Taliaferro, 1956; Draper & Süssdorf, 1957), so that peak response of the splenic and non-splenic sources of antibody approximately coincide to give a maximal serum antibody titre.

An approximate immunization schedule would be 0.5 ml IP, 1.0 ml IP, 2.0 ml IP, 1.0 ml IV, 2.0 ml IV, and 4.0 ml IV. Such a schedule can be only approximate, for the animal should be weighed before each inoculation; if it has gained weight the previous dose should be doubled, but if it has failed to gain or has lost weight, the last dose should be repeated. When the course of immunization is sufficiently rigorous for the animals not to gain more than 100 g per week, the maximal immune response is obtained.

Four days after the last intravenous inoculation the animal may be test-bled. If the agglutinin titre is 1 : 10 000 or more it may be bled out, but if the titre is less than this two more intravenous inoculations should

be given, and the animal bled out four days after the last inoculation. Burrows et al. (1946) reported that of 76 rabbits so immunized, 3 had a titre of 1 : 2 000, 9 a titre of 1 : 5 000, 23 a titre of 1 : 10 000, 28 a titre of 1 : 20 000 and 13 a titre of 1 : 50 000, and Gallut (1949) obtained similar results using the same method of hyperimmunization.

O group I antiserum for the identification of *V. cholerae* regardless of serotype may be prepared by immunizing with bivalent antigen containing equal amounts of the Inaba and Ogawa serotypes (Ahuja et al., 1951). Inaba-specific and Ogawa-specific antisera may be prepared by absorption of the bivalent antiserum with the heterologous type; if a monovalent immunizing antigen is used, the antiserum will still have to be absorbed with the heterologous serotype to remove cross-reacting antibody.

Type-specific antisera. In the preparation of type-specific antiserum by absorption of bivalent or monovalent antiserum with the heterologous serotype, H-O antigen, e.g., living or formolized vibrios, may be used. Antiserum may be absorbed undiluted or diluted 1 : 5, using approximately 1 Roux bottle agar culture of vibrios per ml of undiluted serum or per 5 ml of 1 : 5 diluted serum. The growth is suspended in the serum by washing it directly off the slant, and is incubated at 37°C for two to three hours, either with constant gentle agitation or with shaking every 15 minutes. The bacteria are removed by centrifugation, the supernatant serum is decanted on to the next agar culture, etc. Usually three such absorptions suffice to exhaust the antiserum of antibody homologous to the absorbing antigen, and only rarely are as many as five such absorptions required.

Antisera may be preserved with 1: 10 000 sodium ethylmercurithiosalicylate added as 0.1 ml of a 1 % solution in 1.4 % sodium borate (the stock solution does not keep for longer than three weeks) per 10 ml of serum. All antisera must be standardized for slide agglutination by testing serial dilutions against homologous and heterologous antigens to determine the dilution to be used for diagnostic purposes.

Examination of Specimens

The cholera vibrio is excreted in large numbers, often in practically pure culture, in the rice-water stools characterizing the early untreated stage of the disease, and its isolation is relatively simple. The proportion of vibrios found in stool specimens taken on the second day of the disease, i.e., after the onset of general symptoms, may be greatly reduced and make up as few as one colony in 100 growing up on directly inoculated solid media. Thereafter, the vibrios may continue to be difficult to find on direct culture, or may apparently increase in abundance; in general, they are

disappearing rapidly by the sixth to seventh day. Gilmour (1952), in a study of 113 continuously observed cases, found that 71.6% were negative on culture by the end of the first week, 89.3% at the end of two weeks, and 98.1% after three weeks, with a few showing intermittent excretion for as long as 25 days. Similar results were observed in the Egyptian epidemic of 1947, in which 96% of patients were negative after three weeks.

The administration of antibacterial drugs such as sulfonamides, tetracyclines and chloramphenicol appreciably reduces the period over which positive cultures may be obtained, though the disease process is apparently unaffected (Seal, Ghosal & Ghosh, 1951; Das et al., 1951; Konar & Sengupta, 1951).

Thus two factors affect the successful isolation of *V. cholerae* for diagnostic purposes—namely, the stage in the disease during which the specimen is taken, and whether or not antibacterial substances have been administered for therapeutic purposes before the specimen is taken. In general, the cholera vibrio is isolated in about 50% of cases when the specimen is taken during the acute stage of the disease and inoculated directly on to solid media, and isolation is successful in 90-95% of cases when more than one specimen is taken and/or more than one culture medium is inoculated.

Microscopic examination

Earlier workers attached considerable significance to the demonstration of morphologically characteristic *V. cholerae* in Gram-stained, heat-fixed smears of rice-water stool or a flake of mucus. Such direct microscopic examination is now regarded as of little or no value, in part because *V. cholerae* cannot be differentiated with confidence from cholera-like vibrios or from coliform and related enteric bacilli on morphological grounds alone, and in part because *V. cholerae* in such preparations frequently shows a large proportion of atypical cells.

Enrichment culture

Preliminary culture in fluid media to give a relative increase in the proportion of *V. cholerae* over that of extraneous micro-organisms present in the specimen is often desirable, especially when the specimen is taken later than 48 hours after the onset of the disease, and is essential to successful isolation of the micro-organism from specimens taken after four to six days. The fluid media described above—namely, peptone water, bismuth-sulfite fluid medium, and potassium-tellurite medium—may be used for this purpose.

The amount of inoculum is inversely related to the numbers of *V. cholerae* suspected to be present. For example, a flake of mucus from a rice-water stool may suffice, or the medium may be prepared at double strength and

inoculated with an equal volume of faecal suspension. The enrichment culture may be incubated for a few hours only (as little as two hours in the case of specimens taken during the acute stage of the disease) or for as long as 6 or even 12 hours (in the case of specimens from convalescents), and when the incubation time is extended it is desirable to subculture at more than one time-interval. The vibrios grow rapidly in the form of a thin film on the surface of the medium, and a loopful of this material is used as inoculum for culture on solid media.

Isolation in pure culture

Agar media should be inoculated directly with specimens taken during the acute stage of the disease, as well as with an inoculum from an appropriately incubated enrichment culture, if the latter has been made. One or more of the solid media described above—namely, bile-salt agar, Aronson's medium, and modified Wilson-Reilly bismuth-sulfite agar—are used for this purpose.

After 18 hours' incubation *V. cholerae* appears on bile-salt agar as small colonies, 1 mm or less in diameter, that are raised, smooth and completely translucent, and literally dewdrop-like in appearance, thus being readily distinguishable from coliform and similar bacilli. Colonies of cholera-like vibrios and *Alcaligenes faecalis* are closely similar, but may show a very slight opalescence.

On Aronson's medium minute colonies of *V. cholerae* appear as early as after 10 hours' incubation, and after 15-20 hours not only increase in size but also take on a bright-red colour. This coloration is not specific for *V. cholerae*, but is only indicative of fermentation of the sugars contained in the medium.

On bismuth-sulfite medium *V. cholerae* appears after 12 to 18 hours as yellowish-brown colonies that, in the case of some strains, may acquire a dark metallic lustre on continued incubation. This appearance, while characteristic, is not specific for *V. cholerae* in that *Proteus* species give closely similar colonies.

Heat-fixed smears should be prepared from the characteristic growth on one or another of these media and stained by Gram's method. The colonial growth appearance, coupled with the demonstration of the Gram-negative curved rods characteristic of *Vibrio* morphology, provides evidence consistent with the assumption that the micro-organisms are *V. cholerae*, but identification of the latter can be regarded as no more than presumptive at this point.

Identification

The identification of *V. cholerae* is based upon: (*a*) its biochemical characteristics, i.e., the fermentation of sucrose and mannose but not

arabinose, the reduction of nitrate to nitrite and the formation of indole from tryptophane to give the cholera-red reaction, and a negative Voges-Proskauer reaction; (*b*) its failure to haemolyse goat or sheep erythrocytes under appropriate conditions; and (*c*) its agglutination in O group I antiserum.

A subculture of the colonially and microscopically typical *V. cholerae* is prepared from the isolation plate culture and used to inoculate media for biochemical tests. These include one tube each of the sugar broths, sucrose, mannose and arabinose, a tube of peptone water for the cholera-red test, and a tube of glucose/phosphate/peptone-water for the Voges-Proskauer test. In addition, a tube of isotonic Douglas broth is inoculated for the haemolysis test (see below).

Biochemical reactions. The fermentations should be read after 18-24 hours' incubation to avoid the late fermentation of arabinose that occurs with some strains. The nitroso-indole reaction, or cholera-red test, is carried out by adding concentrated sulfuric acid, about 1 drop per ml of culture, to the peptone-water culture after 24 hours' incubation; a positive reaction is indicated by the development of a crimson to ruby colour, appearing more or less rapidly on the surface and then spreading to the whole of the mixture two to three hours after the addition of the reagents.

The glucose/phosphate/peptone-water culture is incubated for two to four days, although according to Taylor et al. (1937) positive reactions may be obtained with cultures incubated for 24 hours. To 1 ml of the culture are added 0.6 ml of a 5% solution of α-naphthol in absolute ethanol and 0.2 ml of a 40% solution of potassium hydroxide. The reagents are added in the order indicated, and it is important to shake for about five seconds after the addition of each reagent. A positive reaction is indicated by the development of a crimson to ruby colour in the mixture two to four hours after addition of the reagents. Standard procedures commonly specify that the test should be read not later than four hours, but some workers with *V. cholerae* read the test as late as 24 hours afterwards.

Haemolytic activity. In assaying the haemolytic activity of *V. cholerae* and related vibrios it is of primary importance to distinguish between haemolysis as observed on blood-agar culture, and the lysis of suspended erythrocytes in admixture with a suspension or culture of the micro-organisms. Thus many strains of true *V. cholerae* show the zones of complete clearing of β-haemolysis around colonies on a blood-agar plate while others do not, though neither lyse red blood cells in suspension under the conditions indicated below. The apparent contradiction was resolved by van Loghem (1913), who established that the haemodigestion observed on

blood-agar cultures and the haemolysis of suspended erythrocytes were basically different processes.

The test for haemolytic activity of vibrios, or Greig test, has been studied exhaustively by Krishnan & Gupta (1949) and by Ahuja et al. (1951). It is carried out by adding 1 ml of a 3% suspension of erythrocytes to 1 ml of either a 24-hour culture of the micro-organisms in isotonic Douglas broth or a suspension of the micro-organisms harvested from an agar culture in isotonic saline and containing about 2000 million vibrios per ml. Of the two, the broth culture is regarded as preferable. Goat erythrocytes were used in the test as originally devised, but sheep erythrocytes are at least equally satisfactory, if not preferable (Pollitzer, 1955). It has been reported by De, Bhattacharya & Roychaudhury (1954) that typical *V. cholerae*, negative to the haemolysis test using sheep cells, are nevertheless haemolytic when human erythrocytes are used; human red blood cells are not suitable for the haemolysin test for differentiating *V. cholerae* from the haemolytic (to goat and sheep cells) El Tor vibrios. The mixture is incubated at 37°C for two hours, read, stored overnight in the refrigerator, and read again. A positive reaction is indicated by clearing of the red-cell suspension and liberation of free haemoglobin. The haemoglobin is frequently reduced and the haemolysis is usually not a complete sparkling haemolysis, but the test can be read without difficulty.

Agglutination. Since *V. cholerae* is agglutinated by O group I antiserum it is said to be " agglutinable " while vibrios of other O-antigenic specificity are " inagglutinable ". This terminology is not to be taken to imply that serologically unrelated vibrios are not agglutinated in their homologous antisera.

The agglutination test may be carried out as a rapid slide-agglutination, either using morphologically typical colonies taken from the isolation plate or growth from an agar-slant subculture. Alternatively, the agglutination may be the usual tube titration, using a suspension of the micro-organisms in isotonic saline and serial dilutions of 2^n of antiserum.

In the rapid slide-test a loopful of isotonic saline and a loopful of antiserum, appropriately diluted as determined by prior test against known strains of *V. cholerae*, are placed side by side on a clean glass slide. Bacterial growth from an agar culture is then suspended in the saline to give a heavy milky suspension, and this drop of suspension is then stirred thoroughly into the drop of antiserum. A positive reaction is indicated by the development of a curdled appearance, usually within one to two minutes, which is apparent to the naked eye and, if desired, may be examined under a hand lens or dissecting microscope. It is essential that the test be controlled with a suspension of the bacteria in saline without antiserum. By the third day of the disease, R forms of the vibrio may be encountered that are spontaneously agglutinable in salt solution; obviously this control

should be negative. In the event of saline-agglutinable forms being found, it is often possible to obtain a stable suspension by reducing the salt concentration to 0.5%.

Bacterial agglutination in serial dilutions of antiserum in the tube titration to titres within a dilution or two of that obtained with homologous antigen are more dependable evidence of the serological identity of the organisms tested. Normal rabbit serum frequently agglutinates *V. cholerae* in dilutions as high as 1 : 50, and it is preferable that the serum dilution series begin at a 1 : 100 dilution. High-titred sera prepared by hyperimmunization as described above are more satisfactory than sera with agglutinin titres of 1 : 2000 or less. It is essential here also that saline control tubes be included in the titration. A suspension containing 2000 million vibrios per ml (1 mg (dry weight) per ml), corresponding approximately to 5 units of the International Reference Preparation for Opacity (Maaløe, 1955), is a satisfactory agglutinating antigen.

Either of the above methods may be used to type *V. cholerae* as the Inaba or Ogawa serotype. Such typing is not essential to the laboratory diagnosis of the disease, but if desired is readily carried out by substituting absorbed antisera for the bivalent diagnostic serum.

Summary and evaluation

The characterization of *V. cholerae* in terms of the foregoing tests may be summarized as follows:

sucrose	mannose	arabinose	cholera-red	Voges-Proskauer	haemolysis
+	+	—	+	—	—

Vibrios conforming to this pattern are usually found to be of serological O group I. Such agglutinable vibrios conform to the definition of *V. cholerae* and the micro-organism may be regarded as identified.

Two deviations from this pattern are encountered with some frequency. First, in an appreciable proportion of cases, perhaps as much as 1%, vibrios are isolated from clinically typical cases of cholera in practically pure culture which may or may not differ culturally from *V. cholerae*, but which do not agglutinate in O group I antiserum. Whether such organisms are etiologically related to the acute diarrhoeal disease or represent contamination in the cholera stool specimen has not been satisfactorily determined.

Secondly, the vibrios isolated may conform in all respects to the characterization of *V. cholerae* with the exception that they are haemolytic. These are the so-called El Tor vibrios of O group I, and the test for haemolytic activity thus assumes primary significance in their differentiation from *V. cholerae*. Such haemolytic forms, because they are frequently present

in surface waters, may be met with as contaminants in human stools, most often those of healthy individuals. But they have been found to be the specific etiological agent in acute epidemic diarrhoeal disease, clinically indistinguishable from cholera in Celebes. Haemolytic vibrios have also been found in India in connexion with acute diarrhoeal disease (Mukherji, 1955). Whether these El Tor vibrios are distinct pathogens differentiable from *V. cholerae*, as maintained by de Moor (1948, 1949), or are atypical variants of the cholera vibrio, as suggested by Doorenbos (1936, 1937), has not yet been determined.

However this question may eventually be resolved, only those vibrios conforming to the pattern described above are generally regarded as *V. cholerae* and reported as such.

Annex

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