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Laboratory Protocols for Isolation and Identification of Toxigenic Strains of *Vibrio cholerae*: A Review

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ABSTRACT

***Vibrio cholerae* is a pathogenic bacteria that accounts for diarrheal illness which can be life-threatening in lack of treatment and accounts for great morbidity and mortality. The common mode of transmission includes fecal-oral route transmission mediated through contaminated food and water. The strains 01 and 0139 have been associated with epidemic outbreak of cholera with severe clinical manifestation whereas the infection caused by non O1 and non O139 strains are recognized as non-epidemic cholera characterized by mild-severe diarrheal syndrome without epidemic potential. Laboratory diagnosis through Culture and determining Antimicrobial susceptibility tests are recommended for diagnosis of cholera in all clinical laboratory settings. The simple conventional laboratory diagnosis through culture should be effective for diagnosis of cholera in the resource (PCR) limited laboratories. However, *Vibrio cholerae* in VBNC (Viable but non-culturable) state can only be detected through molecular methods. Therefore, molecular methods need to be extended in all epidemiological laboratories for preventing misidentification of *Vibrio cholerae* especially from environmental samples. Epidemic outbreak of cholera has explained the need of immediate preparedness with preventive, diagnosis and curative improvement in all health sectors. Therefore, immediate health and sanitation related awareness are necessary for prompt control of the disease before it leads community to global burden.**

Keywords- *Vibrio cholerae,* Culture, Pathogenesis, Epidemiology, Faecal sample, food, sewage, water, PCR.

I. INTRODUCTION

Vibrio is short, curved, Gram-negative rods measuring 2×0.5µm and that is motile by its single polar flagellum. It is a facultative anaerobe that produces acid from carbohydrate fermentation[1]. Around 12 species of Vibrio are identified to cause gastrointestinal and extra gastrointestinal infections that include *V. cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus, and V. alginolyticus. Vibrio cholerae* (strain O1 and O139) is reported in the number of epidemic cholera outbreak whereas other serogroups known as non 01 and non 0139 strains that cause diarrheal illness are less severe than epidemic cholera[2]. *V. cholerae* O1 serogroup is divided into two biotypes, classical and El Tor. Among them, classical biotype includes serotype Inaba, Ogawa or Hikojima and El Tor biotype includes serotype Inaba, Ogawa or Hikojima[3]. The O139 serogroup emerged due to multi-gene substitution in the O antigen-coding region of a progenitor O1 El Tor strain[4].

The growth of most species of Vibrio is facilitated by Na^+ ion but not for *Vibrio cholerae* and *Vibrio mimicus* as both of these species can grow well in the absence of NaCl[5]. *Vibrio cholerae* is non-halophilic that can grow in media without NaCl and fails to grow in media containing >6% NaCl.The optimum temperature for its growth is 37°C and pH is 8.2[6]. This selectivity enhances rapid isolation and identification of *V. cholerae* from other interfering vibrio species. *Vibrio capable* of growing on nutrient agar without added NaCl with a positive oxidase reaction helps in presumptive identification of *V. cholerae*[7]. In addition, a conventional biochemical test that includes oxidase test, arginine dihydrolase activity and esculin hydrolysis offer rapid biochemical technique to identify *Vibrio cholerae* with highest sensitivity and specificity[8]. Thus, the main aim of this review is to study the physiological characteristics, epidemiological profile and pathogenesis of *Vibrio cholerae*. The review is aimed to combine the different laboratory findings, protocols to prepare review for isolation and identification of *Vibrio cholerae* from fecal and environmental samples through conventional and molecular methods.

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II. EPIDEMIOLOGY

It is estimated that each year there are 1.3 million to 4.0 million cases of cholera, causing 21000 to 143000 deaths worldwide[9]. Epidemic and endemic cholera outbreaks have been reported time and again from different parts of Nepal. In the year 2011, cholera outbreak was reported from Tilathi VDC of Saptari, Nepal with 2 deaths where *V. cholerae* 01 E1 Tor and Ogawa serotype was isolated from suspected stool samples and pond water[10]. Most of the studies suggest that cholera is water borne[11]. However, many studies have revealed that contaminated foods such as meat, fish, sweet, dairy products and vegetables act as a source of the *Vibrio* species through improper handling, under cooking, washing with unhygienic water etc.[12,13,14]. Cholera is endemic in Nepal and causes massive morbidity and mortality in every monsoon in both urban and rural areas[15]. In Nepal every year 30,000 to 40,000 people die due to diarrheal diseases, majority of deaths occurring due to cholera[16]. Recently, emergence of multi drug resistance conferred by *ctx* gene in *V. cholerae* has become a serious problem which can only be addressed by prescribing appropriate antibiotics after antibiotic susceptibility test[17]. Studies have reported that *V. cholerae* bear ability of forming biofilm like structure that play crucial role in pathogenesis and pose a higher virulence during fecal-oral route transmission[18].

Cholera outbreak occurred in India/Bangladesh in 1993, which was associated with serogroup O139 was unknown before[10]. Except for O antigen and presence of polysaccharide capsule, this strain is similar to seventh pandemic strength of *V. cholera* O1. This 0139 strain has become endemic in Bengal region and is associated with Eighth Cholera pandemic[19].

III. PATHOGENESIS

Vibrio cholera infiltrates itself to gastro intestinal tract. Mucinase production allows penetration of mucous layer. Toxin co-regulated pilli (TCP) helps in attachment to mucosal cells. Cholera toxin released from bacteria disrupt junction of intestinal cells that leads to diarrhea. Cholera toxin (CT) is responsible for disease cholera. CT causes mucosal cells to hyper secrete water and electrolytes in lumen of gastrointestinal tract resulting watery diarrhea[1]. Fluid loss and dehydration accounts for hypotension and death. Since, cholera is toxin mediated disease, no blood cells are seen in stool rather there is outlet of fluids and mucous flicks. Symptoms of cholera include abrupt onset of watery diarrhea (rice watery diarrhea), vomiting, and abdominal cramps[20]. Dehydration presents clinical manifestation of thirst, dry mucous membranes, decreased skin turgor, sunken eyes, hypotension, weak or absent radial pulse, renal failure, seizures, coma, and death[21,20].

IV. LABORATORY DIAGNOSIS OF *Vibrio cholerae* FROM STOOL SAMPLE

Laboratory identification of *Vibrio cholerae* from fecal sample should be performed as explained by CDC[22,23,24].

- Thiosulfate citrate bile salts sucrose (TCBS) agar should be prepared according to manufactures instruction. Suspend the thiosulfate citrate bile salts sucrose (TCBS) agar in distilled water and heat to dissolve the media completely. Do not autoclave. Pour the media in sterile plates.
- Alkaline Peptone Water (APW): Measure 5gm of peptone and 5gm of NaCl and dissolve it in 500ml distilled water. Adjust the pH 8.6 to 9.0 using 1mol/L sodium hydroxide. Dispense the medium in a 10ml screw-cap tube and sterilize by autoclaving at 121°C for 15 minutes.

Specimen: The most required specimen for *Vibrio spp.* is a fecal matter. Rectal swabs are most accepted during the initial phase of the disease. The specimen should be collected in a sterile container and delivered as soon as possible to the microbiology lab. In the case of delay, 5ml feaces can be added in 20ml Transport medium like alkaline transport medium with boric acid or Cary-Blair medium.

Microscopy: Direct microscopy of fecal sample is not preferred.

Culture of Vibrio cholerae from fecal sample

Suspend 2ml feaces in 20ml Alkaline Peptone Water (APW) with pH-8.6 and then incubate APW at 37°C exactly for 5 hrs.

- Take loopful of the surface aliquot from APW and inoculate into thiosulfate citrate bile salts sucrose (TCBS) agar and perform streaking on the agar surface. Incubate the plate at 37°C for overnight.
- The next day, examines the TCBS plate for Vibrio like colonies (Table 1). Pick up the yellow colony of Vibrio from TCBS and inoculate into nutrient agar (NA) without NaCl. Incubate the media at 37°C for overnight.
- After incubation, observe the nutrient agar (NA) plate for the growth of the culture. *Vibrio cholerae* can grow with colorless, glistening, translucent colonies in nutrient agar without NaCl. *Vibrio cholerae* should be confirmed by its sufficient growth on nutrient agar without salt, either on 1% tryptone water without NaCl or in a CLED plate. This conventional test is reliable enough for the presumptive isolation and identification of *Vibrio cholerae* from other *Vibrio sp.*

- For further confirmation, perform Gram staining, oxidase test, and slide agglutination test. For these test, culture should always be taken from non-selective media like nutrient agar.

V. LABORATORY IDENTIFICATION OF *Vibrio cholerae* FROM WATER SAMPLE

Isolation of *V. cholerae* from water sample is performed according to CDC guideline[25]. Membrane filters technique is most appropriate for clear sample that do not contained debris, mud or silt. Clean, non-cloudy and sediment free water from tap and tube well should be filtered through membrane filtration technique. In this method, 100ml of water should be filtered through 0.1-0.22µm membrane filter. Membrane filter should be placed in 10ml alkaline peptone water (APW) (pH-8.6) tube and vortexed for 30 seconds. The enrichment of bacteria from pond and sewage should be performed by inoculating 1 ml of sample water in 9ml alkaline peptone water. The enriched culture tubes should be incubated at 37°C for 6 hours and then the topmost layer of enriched aliquot should be streaked on thiosulfate citrate bile salts sucrose (TCBS) agar media. The plates should be incubated at 37°C for overnight[11]. After overnight incubation, the Vibrio like yellow colony, 2-4mm in diameter, slightly flattened with opaque centers should be picked and streaked on nutrient agar without NaCl and incubate at 37°C for 16-24 hours.

VI. LABORATORY IDENTIFICATION OF *Vibrio cholerae* FROM FOOD SAMPLE

Transportation of food sample

After the collection of food samples, processing should be done as soon as possible within 24 hours[26]. For transportation specimens should be refrigerated at 4°C in an insulated box with frozen refrigerant packs. If wet ice cubes are only available for refrigeration of specimens then water from the melted ice should not enter into the specimens[25]. The samples should be stored in a cool box at about 10 to 15°C temperature for enumeration of *Vibrio cholerae* prior to processing (not to exceed 8 hours)[27]. In fact, according to a recent study conducted, if specimens are transported at ambient air temperature before processing then the growth of *Vibrio cholerae* in the sample can be increased[28].

Isolation of Vibrio cholerae

About 25gm of food sample should be weighed aseptically and added to a sterile blender jar. Larger food samples should be cut into smaller pieces and blending should be done adding little APW (alkaline peptone water) in the jar. After blending is completed, APW should be added till the total volume reaches 225ml (10^{-1} dilution). Two tenfold dilution (10^{-2} and 10^{-3}) of blended food samples should be prepared in APW. If enumeration of *Vibrio cholerae* is required then dilutions should be made up to 10^{-6}. However, if the food sample is oyster then quick dilution of samples is most as it will decrease the number of competent microorganisms and reduce the toxic effect of oyster meat on *Vibrio cholerae*. Also, if possible duplicate samples should be prepared, diluted and incubated at both 37°C and 42°C. Incubation should be usually done for 6 to 8 hours for enrichment. After incubation, a loopful of sample should be spread on TCBS from the surface from topmost portion of the APW broth, as Vibrio's specially migrate to this part. If subculture is not done within 8 hours then after 18 hours maintain sub cultured to another fresh APW. The second APW is then inoculated into TCBS after 6 to 8 hours of incubation. Finally, TCBS plates should be incubated at 37°C for 24 hours [25].

VII. LABORATORY DIAGNOSIS OF *Vibrio cholerae* FROM SEWAGE SAMPLE

Moore swab method is considered to be best technique for isolating *V. cholerae* from sewage. Swab can be easily prepared by using cotton gauze (120cm long and 15cm wide). The piece of gauze is then folded for several times and finally a fishing line is tied at the center. The swab is sterilized by autoclaving prior to its use. Moore swabs should be placed carefully at the main lines and central locations of sewage system by analyzing the factors that can minimize the recovery *V. cholerae.* The swab should be kept at the place for 24 to 48 hours. After collecting swab from the site, it should be placed in sterile plastic container and should be transported to the laboratory as soon as possible in an ice frozen refrigerant packs. If there will be delay of more than 3 hours for the processing of sample then APW (300 to 500ml) should be added to the container containing swab at the collection site to maintain the load of *V. cholerae.* For enrichment, the sewage sample and Mooreswabs should make up nearly 10 to 20% of the total volume of the sample and APW. The broth is then incubated for 6 to 8 hours at 37°C before plating on TCBS[25].

VIII. BIOCHEMICAL TESTS, SEROGROUPING, SEROTYPING, BIOTYPING FOR IDENTIFICATION

Grams Staining: Grams staining should be performed according to standard microbiological procedure[29]. *Vibrio cholerae* is gram negative rod. Gram staining and biochemical tests like MR-VP, Indole, citrate, oxidase, string test, arginine dihydrolase acitivity, esculine hydrolysis etc. should be performed according to CDC guidelines[24]:

String test

This test is very useful presumptive test for suspected *V. cholerae* as these strains are string test positive. A large colony from agar culture should be emulsified in a small drop of 0.5% sodium deoxycholate in sterile water. Within 60 seconds if the cells lyse (loss of turbidity) and forms DNA strings when a loopful is lifted (up to 2 to 3cm) from the slide then report positive string test for *Vibrio cholerae.*

Oxidase reaction

The overnight growth from non-selective media should be transferred using a wood applicator stick to a filter paper saturated with oxidase reagent. A dark purple color developing within 10 seconds indicate a positive test for *Vibrio cholerae.*

Arginine dihydrolase activity

For this test, the media used is Luria-Bertani broth containing 1% (wt/vol) L-arginine (pH 6.8). Phenol red powder (Difco) should be added as an indicator. After inoculation, the medium need to be covered with sterile mineral oil and should be incubated at 37°C for 24 hours. Appearance of a red color should be considered a positive reaction for *Vibrio cholerae*[30].

Esculine hydrolysis

Heart infusion agar containing 0.1% esculin and 0.05% ferric chloride is used for testing esculin hydrolysis. Blackening of the medium after incubation at 37°C for up to 3 days is a positive reaction.

Serogrouping

1. Serogrouping using somatic or O antigens gives important epidemiological evidence.
2. Three sections should be marked on a glass slide. One drop of 0.85% saline solution should be added on the slide. With a sterile loop, overnight incubated culture should be emulsified in saline solution for all three sections on the slide.
3. A drop of polyvalent *V. cholerae* O1 antiserum should be added to one section of emulsified culture and mixed with sterile loop. A drop of anti-O139 should be emulsified into a separate section.
4. The mixtures should be tilted back and forth for one min and observed against a dark background. A positive reaction is indicated by a rapid, strong agglutination in a clear background.
5. Non- agglutinated cultures should be reported as non O1/O139 *V. cholerae.*
6. Agglutinated cultures should be reported as *Vibrio cholerae* strain O1 or O139.

Serotyping

Serotyping of *Vibrio cholerae* provides important epidemiological information. This test is directed for identifying Serotypes viz. Ogawa, Inaba or Hikojima (rare) of Serogroup O1. These serotypes are found in both Classical and E1 Tor biotypes.

1. Slide agglutination test for serotyping is most common. With the help of sterile loop inoculate culture from non-selective medium on two section of glass slide and emulsify it by adding 0.85% saline. Upon examination, if the mix suspension agglutinates, then discard the mix.
2. Add a drop of Ogawa antisera added on one side and Inaba antisera on another and mix by help of sterile needle.
3. The slides need to be tilt forth and back for a minute and check for agglutination. The positive test is indicated by a strong agglutination against clear background.
4. Agglutination on both sections of cultures must be reported as Hikojima strain.

Biotyping

Although, *Vibrio cholerae* O1 Biotyping is not of much importance in clinical settings but it provides evidence for epidemiological and public health studies that should rule the source of infection. *Vibrio cholera* biotype includes classical biotype and El Tor biotype:

1. Voges-Proskauer test (modified with 1% Nacl): The Voges-Proskauer test is used for differentiating El Tor and classical biotype of *V. cholerae* O1. Classical biotype provides negative results whereas El Tor isolates are positive.

2. Beta-hemolysis: In this test the test culture of *Vibrio cholera* should be inoculated on a blood agar plate with incubation at 37°C for 18-34 hours. The clear zone around culture report positive beta-hemolysis. El Tor is β-hemolytic, while classical strains are non-hemolytic. However, Biotype El Tor from Latin America, Asia, Africa, Europe, and Pacific have been identified with non- hemolytic activity.

3. Polymyxin-B sensitivity: Mueller-Hinton agar is used for antibiotic susceptibility test with 50 unit disc polymyxin-B. The agar plate seeded by *Vibrio cholera* should be inoculated by polymyxin-B disc and plates should be incubated overnight at 37°C. The Classical strains are sensitive (>12mm zone); whereas El Tor strains are resistant.

4. Hemagglutination test: In this test the Fresh chicken or sheep red blood cells are used. A 2.5% (vol/vol) suspension of 3 times washed and packed cells should be prepared in normal saline. Heavy loopful of the red cell suspension should be placed on a glass slide and small portion of the culture from a nonselective agar is inoculated to the red cells and mix well with needle. Observe agglutination of the red cells that occurs in a minute. Biotype El Tor shows hemagglutination whereas classical biotype doesn't show hemagglutination.

IX. ANTIMICROBIAL SUSCEPTIBILITY TEST (AGAR DISC DIFFUSION METHOD)

Antimicrobial Susceptibility Test of identified *Vibrio cholerae* should be evaluated against different available antibiotics such as ampicillin, chloramphenicol, sulfonamides, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, cefotaxime, nalidixic acid, imipenem and neomycin by disc diffusion method following CLSI guidelines, (2013)[31]. Colonies should be taken from nutrient agar plates and turbid suspension should be made by emulsifying colonial growth in nutrient broth (NB). The turbidity of the inoculums should be adjusted to the equivalent turbidity of 0.5 McFarland standards. After incubation, a sterile cotton swab should be dipped into NB broth and swabbed entirely on surface of Muller Hinton agar (MHA) three times, rotating the plate. Using sterile tweezers, antibiotic discs should be placed aseptically on the surface of MHA plates. The plates should be incubated at 37°C for 24 hours. After 24 hours of incubations the zone of inhibition should be measured and interpretation should be made as sensitive, intermediate and resistance.

Interpretation:

- If Gram's staining report is a Gram negative rod, the oxidase test is positive then report: cholera.
- If Gram's staining report is a Gram negative rod, the oxidase test is positive and Serogroup slide agglutination test is also positive then present the report as epidemic cholera (either 01 or 0139 strain cholera).
- If Gram's staining report is a Gram negative rod, oxidase test is positive but the slide agglutination test is negative then present report as non-epidemic cholera.
- Provide the antibiotic susceptibility report to physician.

Table 1: Key biochemical and physiological properties of *Vibrio spp.*

Species	Colonies in TCBS	Growth in 0.0% NaCl	Gram's test	Oxidase	Indole
V. cholerae	Yellow	+	–	+	+
V. mimicus	Green	+	–	+	+
V. parahaemolyticus	Green	–	–	+	+
V. alginolyticus	Yellow	–	–	+	variable
V. vulnificus	Green	–	–	+	+
V. fluvialis	Yellow	–	–	+	variable

Serodiagnosis

Agglutination, vibriocidal, antitoxin test and ELISA can be used. However, these tests are not suitable for the current diagnosis of infections in hospital settings.

X. MOLECULAR METHODS FOR IDENTIFICATION OF *Vibrio cholerae*

Preparation of crude DNA template by boiling

An overnight loopful of culture from agar plate or broth culture can be used for extraction of DNA by boiling method. For this, 1ml overnight grown culture bacterial cells grown overnight at 37°C on *Luria-Bertani* (LB) broth supplemented with 3% NaCl should be suspended in 1ml sterile water. In case of culture from agar plate, loopful isolated pure cultures (50-100) colonies must be suspended in 1ml sterile water and vortex vigorously. The suspension should be diluted 1:1000 in sterile water within a sterile micro-centrifuge tube. The tubes should be suspended in a boiling water bath for 10 minutes. The tubes should be slowed to cool to room temperature and should be treated with 10mg/mL bovine serum albumin (BSA) (4µl per 100µl supernatant). A dilution of 1:10 (supernatant: sterile distilled water) can be prepared[26].

DNA extraction using phenol, chloroform, and isoamyl alcohol

DNA is extracted by phenol, chloroform, isoamy alcohol explained by Sambrook, (1989)[32].

1. Centrifuge 1ml aliquot from the upper surface (top 1–2mm) of the APW enrichment or overnight incubated LB culture broth for 5 minutes at 12,000×g, room temperature.
2. Suspend the cell pellet in 567µl TE buffer.
3. Add 30µl of 10% SDS, followed by 3µl Proteinase K solution.
4. Incubate the suspension for 1 hour at 35°C.
5. Add 100µl of 5M NaCl, followed by 80µl of CTAB/NaCl solution.
6. Incubate mixture for 10 minutes at 65°C.
7. Add 800µl phenol/chloroform/isoamyl alcohol (25:24:1), vortex and centrifuge for 5 minutes at 12,000×g, room temperature.

8. Transfer aqueous phase (supernatant) to a new micro centrifuge tube. Add 800µl of chloroform/isoamyl alcohol (24:1). Vortex, and centrifuge for 5 minutes at 12,000×g, room temperature.
9. Transfer aqueous phase (supernatant) to a new micro centrifuge tube and precipitate DNA with an equal volume of isopropanol.
10. Pellet DNA by centrifuging for 5 minutes at 12,000×g, 25°C. Wash DNA with 1ml of 70% ethanol and centrifuge for 5 minutes at 12,000×g, 25°C.
11. Dry pellet and suspend in 100µl TE buffer and store at -20°C until use.

Quick DNA extraction from APW suspension

1. From the upper surface (top 1–2mm) of the APW enrichment media, remove a 1ml aliquot and boil for 10 minutes.
2. Put on ice, and then centrifuge to obtain pellet. Add 700µl supernatant to a sterile 1.5ml micro centrifuge tube.
3. Add 28µl of 10mg/mL bovine serum albumin (BSA) at concentration of 4µl/100-µl supernatant. Make a 1:10 dilution (supernatant: sterile distilled ionized water) [26].

Multiplex PCR (Polymerase Chain Reaction)

The multiplex PCR assay should utilize five primer pairs and should detect the presence of *wbe* (O1) and/or *wbf* (O139), *ctxA*, *tcpA* El Tor or *tcpA* Classical. As described previously, DNA should be extracted by boiling the sample or by phenol chloroform ethyl acetate method followed by storage at -20°C until use. A bacterial cell lysate should be used as the source of DNA. To ensure that each individual primer pair should be adequate for amplification, a single-target PCR assay should be conducted prior to multiplex PCR optimization with the control strains. After confirmation of the specificity of each primer by monoplex PCR, combine primer sets in different ratios and test the control strains in several PCR cycling protocols. The optimized protocol should be carried out with a 35µl reaction mixture that should contain 10X PCR amplification buffer (100mM Tris [pH 9.0], 500mM KCl, 0.1% gelatin), 2.5µl of magnesium chloride (25mM); 2.5µl each of 2.5mM dATP, dCTP, dGTP, and dTTP; 90 pmol each primer for *tcpA* (El Tor) and *tcpA* (Classical); 68 pmol each primer for *ctxA*, *wbe* O1, and *wbf* O139; 1.2 U of Taq DNA polymerase; Milli-Q water to a final volume of 29.5µl; and 5.5µl of cell lysate (template DNA). Finally, the reaction mixture should be overlaid with a drop of sterile mineral oil. Amplification should be carried out for 4 minutes at 94°C for the initial denaturation, followed by 30 cycles of 1.5 minutes at 94°C, 1.5 minutes at 55°C, and 1.5 minutes at 72°C, with a final round of 7 minutes at 72°C in a thermal cycler. Run PCR product out on a 1.5% agarose gel in 1X TAE buffer for 1–2 hrs at 5-V/cm. Stain the gel 15 minutes in 1µg/ml ethidium bromide solution and destain the gel 15 min in distilled water. Visualize the gel products under UV light. The ctxA amplicons is 302-bp in length [33].

Real-time PCR for detection of V. cholerae

Taq Man PCR method is a quantitative, sensitive, and rapid detection protocol for *V. cholerae*[34,26].

Procedure:

1. Set up Real Time PCR with a total amplification reaction mixture of 50µl per sample containing the following: 2.5µl DNA sample (100 ng/µl), 1X Taq Man buffer A, 5mM $MgCl_2$, 200µM (each) dATP, dCTP, and Dgtp; 400µM dUTP, 0.02µM hylA – probe, 0.3µM hylA -F primer, 0.3µM hylA -R primer, 1 U of Amp Erase uracil N-glycosylase, 2.5 U of Ampli Taq Gold DNA polymerase.
2. Amplify the targets with the following cycle conditions:
   Initial hold: 5 min at 50°C
   Initial denaturation: 5 min at 94°C
   40 cycles: 20 sec at 95°C
   1 min at 60°C.
3. Results are interpreted using Real-Time PCR machines software program that report the result in Ct value. The Ct value is the number of amplification cycles. Ct values <29 are considered strong positive reactions and are indicative of abundant target nucleic acid in the sample, while Ct values of 30 to 35 are positive reactions indicative of moderate amounts of the target, and Ct values of 38 to 40 are considered weak reactions with little or no target nucleic acid in the sample.

Immunological Methods for Direct Detection of V. cholera from Environmental Samples

Conventional culture methods are ineffective when bacterial cells have entered into the viable but non-culturable (VBNC) state[26].

Fluorescent In-Situ Hybridization (FISH)

Fluorescence In-Situ Hybridization (FISH) uses a fluorescently-labeled oligonucleotide probe that is visualized under epifluorescence microscopy.

Sample preparation:

1. Sample water (500ml-1000ml) should be filtered through a 0.2µm polycarbonate membrane filter, and suspend cells attached to membrane filter in 5ml 1X PBS.
2. 1X PBS should be centrifuged at 13,000g for 10 minutes in microcentrifuge tube. Supernatant should be discarded and suspend in 250µl of 1X PBS.
3. Add 750µl of fresh 4% paraformaldehyde solution and incubate at room temperature for 1hr.
4. Pellet the cell solution by centrifugation at 13,000g for 5 minutes. Discard the supernatant and then wash the cell twice with 1X PBS. Cells should be

centrifugation and re-suspend in 100µl PBS-ethanol solution. Store cells at −20°C.

Procedure:

1. Multi-well slides should be immersed with 0.01% PLL solution for 10 minutes and air-dry.
2. About 5µl aliquots cells should be released onto slides and air-dry.
3. Slides should be washed for 3 minutes each in successive ethanol solutions (50, 80 and 96%) and then air-dry. Pre-warm humid chamber, slides and filter paper soaked with hybridization solution at 45°C for 10 minutes.
4. Add 5µl aliquot of Hybridization solution containing 5ng/µl of labeled probe to each well.
5. Hybridization is achieved by incubation at 45°C for 24 hours in the humid chamber using hybridization soaked paper.
6. Pre-warm the washing buffer solution at 45°C for 10 minutes before washing the slides and incubate at 45°C for 10 minutes.
7. Sterile deionized water should be used to wash the slides and air-dry at room temperature.
8. Release anti-fading agent to each well and a coverslip is applied.
9. Visualizefluorescence under epifluorescence microscopy.

Direct Fluorescent Antibody–Direct Viable Count (DFA-DVC) Method.

The direct fluorescent antibody staining method is a very useful, direct, and culture-independent method[26,35,36,37].

Procedure:

1. To 1ml concentrated water or homogenized plankton sample, add 10µl yeast extract solution and 10µl nalidixic acid solution.
2. Incubate the mixture at 25°C for 6 hours to overnight.
3. Fix the sample by adding formaldehyde to a final concentration of 3% (v/v) and incubate 30 min at room temperature in the dark.
4. DFA is performed by placing 5-10µl of the fixed sample onto a glass slide and air dry for 15–20 minutes.
5. Fixation is achieved by adding 5µl methanol and air dry for 1–5 minutes.
6. Add 10µl of reconstituted FITC-conjugated specific DFA reagent. Incubate for 30 min at 37°C in humid chamber. Protect slide from light.
7. Slide should be rinsed with 50ml PBS and air dry slides in the dark for 15–20 minutes.
8. Mount slide with one drop of kit-provided Fluorescent Mounting Medium and add cover slip. Observe under an epifluorescent microscope.

Indirect Fluorescent Antibody (IFA) Method

The IFA protocol remains useful for laboratories where commercial DFA kits for *V. cholerae* are not readily available [38,26].

Samples fixation: Add required amount of each sample to a Teflon-coated multi-well slide and air dry (15– 20 minutes) at room temperature. Sample should be fixed by adding 95% ethanol to each well containing sample with air dry for 5–10 minutes at room temperature. Heat slide for 10 min in a 55°C incubator.

Staining procedure:

1. Rinse the slide with 50ml PBS and air dry for 15–20 minutes.
2. To each dry sample well, add 1 to 2 drops of a 1:20 dilution of FA Rhodamine Counterstainand incubate in humid chamber for 30 min at 35°C. Exposure to light from this step should be minimized.
3. Slide in PBS should be gently flooded (50-ml), and then soaked in the same solution for 10 min at room temperature. Remove and rinse again briefly in PBS.
4. Allow slide to air dry for 15–20 minutes.
5. Add 5–10µl of *V. cholerae* O1-specific antiserum. Incubate in humid chamber 30 min at 35°C.
6. Repeat steps 3 and 4. Use fresh PBS for washing.
7. Add 1–2 drops undiluted FITC-conjugated anti-rabbit globulin goat serum and incubate in humid chamber for 30 min at 35°C.
8. Repeat washing steps 3 and 4 using fresh PBS.
9. Mount each slide with a glass coverslip and a low fluorescence, anti-quenching mounting medium, such as Citifluor AF1.
10. Examine samples by epifluorescent microscope with a FITC band-pass filter.

XI. CONCLUSION

In laboratories, other Vibrio species are often confused with *Vibrio cholerae* on the basis of yellow pigmentation of culture in TCBS medium. However, all the sucrose fermenting Vibrio in TCBS are not *Vibrio cholerae*. The growth physiology of *Vibrio cholerae* to produce yellow pigmentation, positive oxidase test and most importantly, its growth even in salt deficient media enhance its selective isolation and identification from other Vibrio species and contaminants. This would be simple and rapid protocol for the rapid diagnosis of cholera disease in resource limited laboratories. However, *Vibrio cholerae* in VBNC form cannot be isolated from conventional methods and hence, molecular methods stands to be rapid and sensitive methods for detection of *Vibrio cholerae* from clinical and environmental samples. It is necessary for all epidemiological laboratories to get equipped by molecular tools for regular surveillance of food and water samples. The availability of standard laboratory facilities, preventive and preparedness for curative measures are indeed required to avoid the consequences of cholera outbreak.

Abbreviations:
APW: Alkaline Peptone water
AST: Antimicrobial Susceptibility Test
CLED: Cystine lactose electrolyte deficient agar
CTAB: Cetyltrimethylammonium-bromide
DFA: Direct Fluorescent Antibody
DVC: Direct Viable Count
FISH: Fluorescent In-Situ Hybridization
IFA: Indirect Fluorescent Antibody
LB: Luria Bertani
MHA: Mueller Hinton Agar
NA: Nutrient Agar
NB: Nutrient Broth
PBS: Phosphate Buffer Saline
PCR: Polymerase Chain Reaction
PPL: Poly-L-Lysine
SDS: Sodium Dodecyl Sulfate
TCBS: Thiosulfate citrate bile salts sucrose agar
TCP: Toxin Co-regulated Pili
VBNC: Viable but non-culturable
WHO: World Health Organization

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