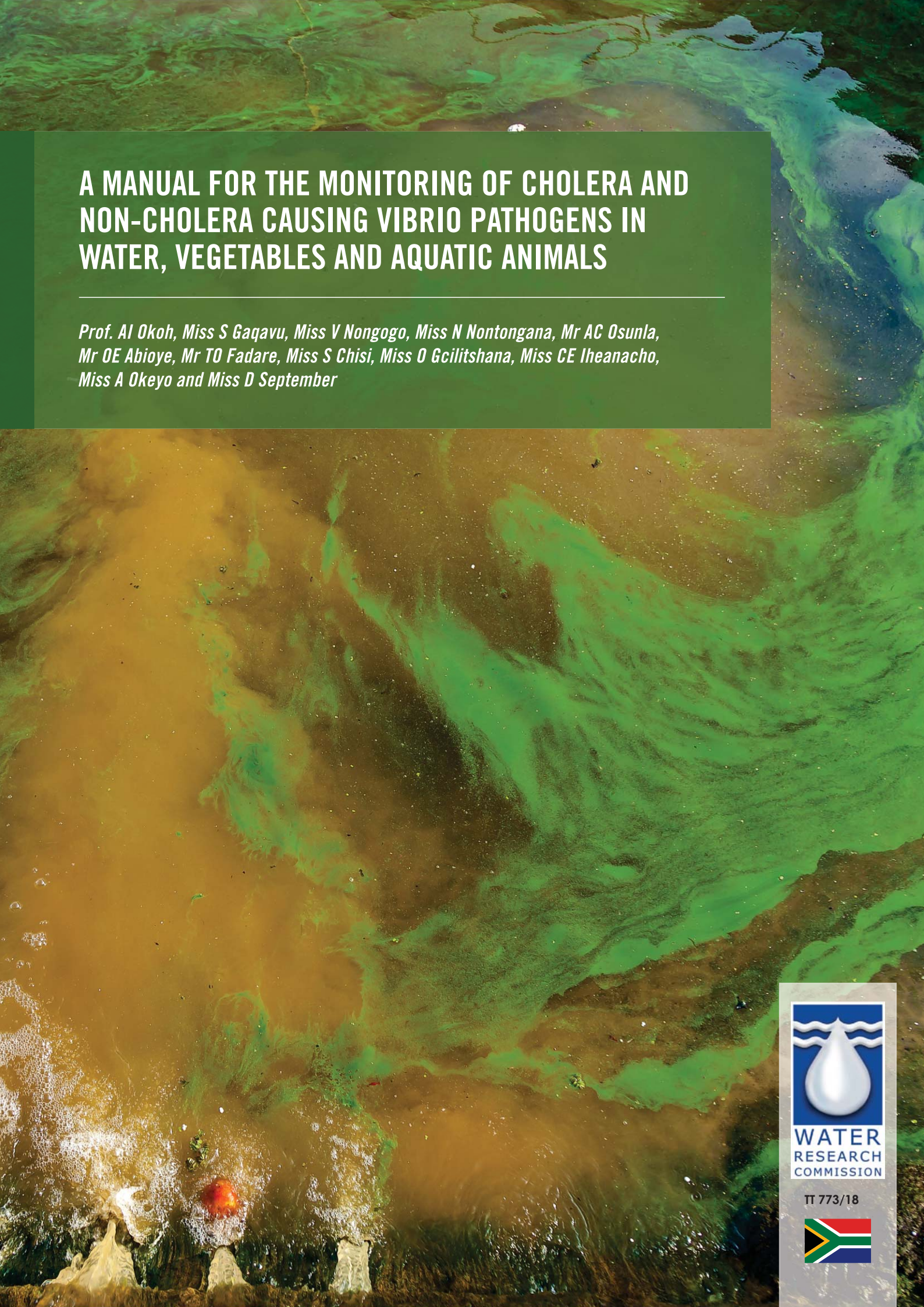

A MANUAL FOR THE MONITORING OF CHOLERA AND NON-CHOLERA CAUSING VIBRIO PATHOGENS IN WATER, VEGETABLES AND AQUATIC ANIMALS

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WATER RESEARCH COMMISSION

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A MANUAL FOR THE MONITORING OF CHOLERA AND NON-CHOLERA CAUSING *VIBRIO* PATHOGENS IN WATER, VEGETABLES AND AQUATIC ANIMALS

Report presented to the
WATER RESEARCH COMMISSION

by

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1 INTRODUCTION

The Eastern Cape Province is reportedly the most impoverished province in South Africa, with a low socio-economic status and high burden of disease. The province has the lowest proportion of people with access to potable water supplies and sanitation, a paucity of electricity, particularly in poorer communities, and a high level of poverty and infant deaths (BLACKSASH, 2010; Okoh *et al.*, 2015). Despite significant progress in water quality management, anomalies still persist (Mema, 2010). Some households in the Eastern Cape still reportedly use water from streams, rivers, boreholes, springs, dams, and so on. These water sources are also used for other activities such as irrigation and harvesting of fish and other aquatic animals. Thus, environmental quality (microbial and physicochemical) monitoring is eminently important.

Enteric pathogens such as some members of the *Vibrio* genus are known for many disease outbreaks worldwide (Griffith *et al.*, 2006), and, unfortunately, there is paucity of information on the epidemiology of *Vibrio* pathogens in Eastern Cape (Igbinosa and Okoh, 2009; Igbinosa and Okoh, 2010 and Okoh *et al.*, 2015). These bacteria are known to cause diarrhea, accounting for a substantial degree of morbidity and mortality in different age groups worldwide (Obi *et al.*, 2004; Okoh *et al*., 2015). The most comprehensively studied Vibrio pathogen, (notorious for its effect on the small intestine through the release of enterotoxins, causing infection in humans mostly through the ingestion of contaminated water and food) is *V. cholerae*. Non-cholera causing vibrios that notably infect humans, such as *V. parahaemolyticus*, cause acute gastroenteritis, diarrhoea and abdominal pain in individuals who eat contaminated seafood (usually raw or undercooked sea food such as oysters) and less commonly wound infections when exposed to sea water. *Vibrio vulnificus* is known to be extremely virulent and can cause 3 types of infections, namely, 1) acute gastroenteritis from eating raw or undercooked seafood 2) necrotizing wound infections when injured skin is exposed to contaminated water; and 3) invasive septicaemia when the bacteria invades the blood stream (80 times more likely in immunocompromised individuals) (Tantillo *et al*., 2004; Oliver, 2005; Anuradha, 2011). *V. fluvialis* causes cholera-like bloody diarrhoea, wound infection and primary septicaemia in immunocompromised individuals (Igbinosa and Okoh, 2010). *Vibrio alginolyticus* is also medically significant. It causes otitis media (ear infection) and also wound infection, particularly from practices such as seaweed wound dressing with unsterilized sea weeds (Reilly *et. al*., 2011). *Vibrio mimicus* is a Vibrio species that mimics *V. cholerae* in its infectivity. It is known to also cause gastroenteritis, which is transmitted from eating raw sea food (Hasan *et. al*., 2010).

1.1 History of cholera infections in South Africa

Documented cholera outbreaks in South Africa date back as far as the 1980s, when sporadic outbreaks occurred. However, in August 2000, South Africa experienced one of the worst cholera epidemics nationwide, which resulted in the deaths of 232 people and the infection of

up to 106 389 people. The epidemic affected KwaZulu-Natal, Gauteng, Mpumalanga and the Northern (currently Limpopo) Provinces. The 2000/2001 epidemic subsided with 3901 reported cases and 45 deaths in Mpumalanga, Eastern Cape and Kwazulu-Natal in 2003. In 2004, 1773 cases of cholera infections and 29 deaths were reported in Mpumalanga's Nkomazi region. In the same year, 738 people were diagnosed with cholera in the Eastern Cape, four of whom died. Two deaths were also reported in the North West Province, with 260 cases of infection (Le Roux, 2004; Health24, 2008; Olaniran *et al.*, 2011). In 2007, 80 diarrhoea-related child deaths occurred in the Eastern Cape, in the greater Barkly East area, Ukhahlamba District Municipality (Bateman, 2009). Although, no recent cholera outbreaks have been reported in the country, in 2009 cholera was confirmed to be endemic in South African water resources by a spokesperson from the South African Ministry of Health (Mail & Guardian, 2009; DWA, 2014).

Currently the guidelines that exist in South Africa (South African National Guidelines for Cholera control) only cover the monitoring of cholera outbreaks. With this document, we have tried to increase the spectrum of coverage to other vibrio pathogens which have also been implicated in high degrees of morbidity and mortality in human populations. This manual should therefore be used as a complementary document to the "The National Guidelines on Epidemic Preparedness and Response". Available online:

https://www.idealclinic.org.za/docs/Protocols/National%20Guidelines%20on%20Epidemic%20Preparedness%20and%20Response%20.pdf.

2 MONITORING AND SURVEILANCE OF *VIBRIO* SPECIES

General monitoring and surveillance should be conducted on environments of interest for cost effectiveness; for instance, areas where human cases of diarrhoea have been detected or are increasing (hotspots) and, neighbouring communities to boarder countries where incidents of Vibrio infections have been reported. Screening water and food-sources in these areas will lead public health officials to the sources of infection. Identification of hotspots may be done by mining the NHLS laboratory databases for *Vibrio*-positive cases and establishing potential sources i.e. water or food. How to tackle incidences of outbreaks is articulated below:

2.1 Tackling the incidence of an outbreak

2.1.1 Setting off an incident alarm

An outbreak alarm of acute diarrhoea is set off when there are unusually high numbers of cases reported in one week. A continuous report of more cases during a period of more than one week, particularly with common features such as clustering of cases in one area where people share the same water supply, may also set off an alarm for an outbreak. Lastly, an

abnormally high number of deaths caused by acute diarrhoea would raise a more significant alarm.

2.1.2 Outbreak investigation

It is essential to conduct a field investigation immediately after an alarm of a possible outbreak has been raised. The rapid response teams must collect stool samples from suspected cases depending on the number of cases and whether the reported cases were clustered in one area. When an outbreak is clinically verified, it is necessary to further investigate environmental factors that could aid the transmission and spread of the outbreak.

2.1.3 Coordinating the response

The committee for cholera control set up as indicted previously (Surveillance and monitoring of *Vibrio* pathogens) must be immediately activated. This committee will take full responsibility for coordinating responses with regards to combating and controlling the outbreak. The committee should oversee the official reporting of an outbreak of acute watery diarrhoea by the various health directorates to the Department of Health, and take the appropriate measures to contain it. During the first few days, the committee should reassess risks of the outbreak on public health and demand the mobilization of resources and emergency supplies, usually at national level. The committee should, during the outbreak, daily monitor the outbreak and follow up decisions taken on implementing containment-specific procedures.

2.1.4 Surveillance

It is necessary to report all suspected and laboratory confirmed cases of acute watery diarrhoea in all affected communities, including those made from selected reporting centres. It is important to use the Case Series Form prepared during the pre-epidemic phase to report all suspected cases in order to obtain laboratory confirmation on *Vibrio cholerae*. It is also necessary to activate the following procedures, considered fundamental to the surveillance of acute watery diarrhoea:

- Active case search in areas where there is laboratory confirmation of *Vibrio cholerae*.
- Using a unified case definition when reporting on all suspected cases.
- Daily reporting on cases and deaths (including zero cases).
- Preparing a case series report for all suspected cases that match the standard definition.

- Setting up a community-based mobile team in addition to surveillance centres to report on all suspected cases.
- Weekly analysis of data in case series reports to better understand the direction of the spread and the changing of the epidemiological pattern of the outbreak.
- Geographical mapping to show case distribution and to determine the areas where most cases are concentrated in order to optimize the targeting of affected areas with chlorination and promotion of sanitation.

2.1.5 Reporting

Reporting must be comprehensive once there is laboratory confirmation on the outbreak of acute watery diarrhoea caused by *Vibrio cholerae*. Data is collected daily from all surveillance units and sent to the Cholera Control Committee with a report on epidemiological situation and the general trend of the outbreak. While the basic purpose of the general reporting system is to deal with documented information in a clear and comprehensible manner, the main purpose of these reports is to:

- Calculate incidence rate and case fatality rate.
- Evaluate the spread and development of the outbreak.
- Plan extra supplies for treatment and containment procedures and for the appropriate changes of procedures.
- Assess control and containment procedures.

2.1.6 Case management

It is essential to apply proper unified management for all suspected cases in order to prevent fatalities and reduce the risk of infection. Therefore, the following procedures should be implemented:

- Distribution of case management and IPC guidelines and algorithms to assess the severity of dehydration to all health care providers in areas affected by the epidemic.
- Setting up isolation wards in hospitals.
- Providing the appropriate treatment to all suspected cases (patients suffering from severe dehydration in isolation wards) in accordance with the national treatment plan.

- Cleaning and disinfection of patients' beds and bed covers using disinfectants or through boiling, and the safe disposal of medical waste of patients (vomit and excretion /stools)
- Allocating separate lavatories to the patients in the hospital.
- Providing patients families with relevant information on prevention practices in their homes.
- Ensuring health care providers comply with personal hygiene procedures (washing hands with soap and water, cleaning and disinfection of food).
- Setting up temporary cholera treatment centres to improve access to treatment.
- Assessing estimated need for essential medicines and emergency supplies in order to manage cases pursuant to the current epidemiological situation, so as to determine the critical value of reserves and rapidly replace depleted stocks and prevent shortages.

2.1.7 Environmental control procedure

Environmental control interventions must include:

- Treatment of drinking water from environmental sources.
- Improving sanitation.
- Promoting personal hygiene at home.
- Ensuring the safety of food.
- Handling of contaminated material properly.

While the geographic distribution of suspected cases answers questions regarding which areas to target regarding the promotion of sanitation, it is necessary to adopt the following special procedures in order to contain the outbreak, by identifying environmental risk factors and stressing the following:

- Access to sufficient amounts safe drinking water/Boiling water when not treated.
- Improving access to sanitation.
- Chlorination of government water supplies used for drinking (surface and ground water) and making it free of contamination.
- Providing access to adequate amount of soaps and antiseptics at home.

- Promoting healthy behaviour especially hand washing with soap and water.
- Improving food safety procedures.
- Disinfecting contaminated objects.

3 *VIBRIO* PATHOGENS IN WASTEWATER, FRESH WATER AND FOOD

3.1 Wastewater and freshwater resources

Wastewater is composed of several components such as black water and grey water, which are primarily products of their sources which in turn determine its physicochemical and biological characteristics. Hence, the impact that a particular wastewater effluent will have on a surrounding receiving waterbody or environment is a direct result of its composition. In addition, freshwater resources serve as the main water resources in rural areas used for drinking, cooking, and irrigation in communities which have little or no access to safe drinking water. These freshwater resources easily become polluted as a result of population growth, land development along river banks, and urbanization. Continuous pollution has resulted in various water-associated disease epidemics in both developed and developing countries. This leads to poor health due to various water-related illnesses. This section of the report will focus on the monitoring of *Vibrio* pathogens in wastewater effluents.

3.2 Water Sampling

Water/wastewater sampling should be conducted according to the Quality of Domestic Water Supplies, Volume 2: Sampling Guide (Water Research Commission No: TT 117/99). Available online: http://www.wrc.org.za/Knowledge%20Hub%20Documents/Research%20Reports/TT-117-99.pdf.

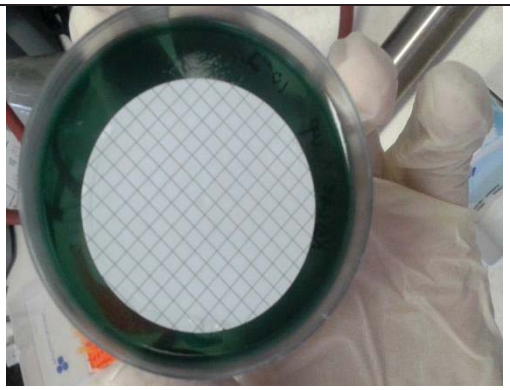
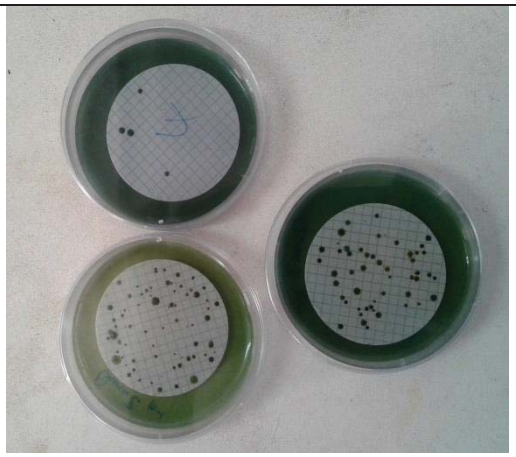
3.3 Laboratory Analysis

3.3.1 Enumeration of presumptive *Vibrio* pathogens

The cultivation and enumeration of presumptive *Vibrio* pathogens should be done following the techniques and principles described by APHA (American Public Health Association, American Water Works Association, & Water Environment Federation, 2005).

Table 1: Protocol for the enumeration of presumptive *Vibrio* pathogens.

Description	**Illustrations**
Membrane filtration technique: filter sample through nitrocellulose membrane filters (0.45 µm pore size, millipore), making relevant dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) using sterile saline solution (0.08% w/v). Samples should be filtered in triplicates (total volume of 100 ml for every filtration).	 **Figure 1:** 0.45 µm pore size millipore filter placed on top of filter cup base  **Figure 2:** Filtration apparatus with sample
Incubation/growth of presumptive *Vibrio* pathogens: place the filter on thiosulphate citrate bile salts sucrose (TCBS) agar plates (clearly labelled) and incubate aerobically at 37℃ for 24 hr. Counts obtained (distinct green and yellow colonies) should be expressed as CFU/100ml.	 **Figure 3:** Clearly labelled TCBS agar plate ready for use

	 **Figure 4:** Filter placed on TCBS plate after sample is filtered
Isolation of presumptive *Vibrio* colonies: isolate presumptive *Vibrio* colonies picking distinctive colonies on TCBS plates (5-10 per plate), purified and preserved in glycerol (20%) at -80°C for further analysis.	 **Figure 5:** Presumptive *Vibrio* colonies on TCBS agar.

3.4 Surveillance strategy for monitoring *Vibrio* pathogens in Seafood

The last few decades have seen numerous research reports from several parts of the globe on the detrimental role that *Vibrio*-contaminated seafood plays in human health and economic losses (Bonnin-Jusserand, *et al.,* 2015; Lafferty *et al.,* 2015). These findings suggest the need to monitor seafood in the environment for the presence of pathogenic *Vibrio* species. The *Vibrio* pathogens of concern are as articulated by food and drug administration (WHO and FAO 2002) and include *V. cholerae, V. parahaemolyticus, V. fluvialis, V. furnissii*, *V. hollisae, V. mimicus, V. metschnikovii, V. vulnificus, V. alginolyticus, V. carchariae, V. cincinnatiensis, V. damsel* and *V. motoeusi.* Of these, *V. cholerae, V. Parahaemaolyticus, V. fluvialis, V. mimicus*, *V. vulnificus, V. alginolyticus* and *V. furnissii* are most reported to cause human infections.

The World Health Organization (WHO) has documented that *Vibrio* infections may be contracted from aquatic animals, in particular from seafood. Seafood includes molluscs, crustaceans and fish (WHO & FAO, 2002). Therefore it is crucial to periodically monitor foods of aquatic origins, especially in infections hotspot areas, to predict a possible seafood related *Vibrio* outbreak.

3.5 Sampling and sample processing

3.5.1 What to sample?

The seafood types typically implicated in *Vibrio* outbreaks which need to be monitored are molluscs (e.g., oyster, mussel, and abalone), crustaceans (e.g., crabs, prawn, and lobster) and fish. Aquatic animals that are peculiar to a geographical location and of importance in the aquatic environment's food chain are to be included among the samples to be monitored. Samples should include both processed and raw (unprocessed) seafood.

3.5.2 How to sample?

Aquatic animals slated for sampling should be collected as described in the Vibriology section of the microbiological methods and analytical manual (Andrews and Hammack, 2003).

3.5.3 Where to sample?

Sampling should be taken from natural aquatic environments, seafood markets, retails shops and aquaculture farms.

3.6 Sample processing

3.6.1 Pre-treatment of samples

The aquatic animal samples should be aseptically handled. Samples should be collected in sterile sampling bags or stomacher bags and transferred to the laboratory on ice within six hours of collection. Samples must not be allowed to have direct contact with the ice since this could affect the integrity of the sample. The following should also be noted when preparing samples especially molluscs and crustaceans.

- Carefully dislodge contaminants (e.g., sand) using sterile distilled water.
- If necessary, scrub the back of mollusc shell to remove algae and shell debris under running tap water and allow to drain. Thereafter, aseptically open the bivalve. **Do not dip bivalves in water** because they might open up and the internal content needed for microbial analysis may be contaminated in the process (Mannas *et al.,* 2014).

- A minimum of 10 live individual shellfish which contain at least 50 g of flesh and intravascular fluid should be harvested for use in the analysis (CEFAS 2015).
- Relevant anatomical sites of fish (gill, flesh, intestine and possibly fins) should be separately analysed when dealing with fish samples.

3.7 Determination of presumptive *Vibrio* counts

Estimation of presumptive *Vibrio* bacteria counts should follow after classical microbiological methods and analytical manual described by the US Food and Drug and Administration (Andrews and Hammack, 2003). The general description of the procedure is given in Figure 6.

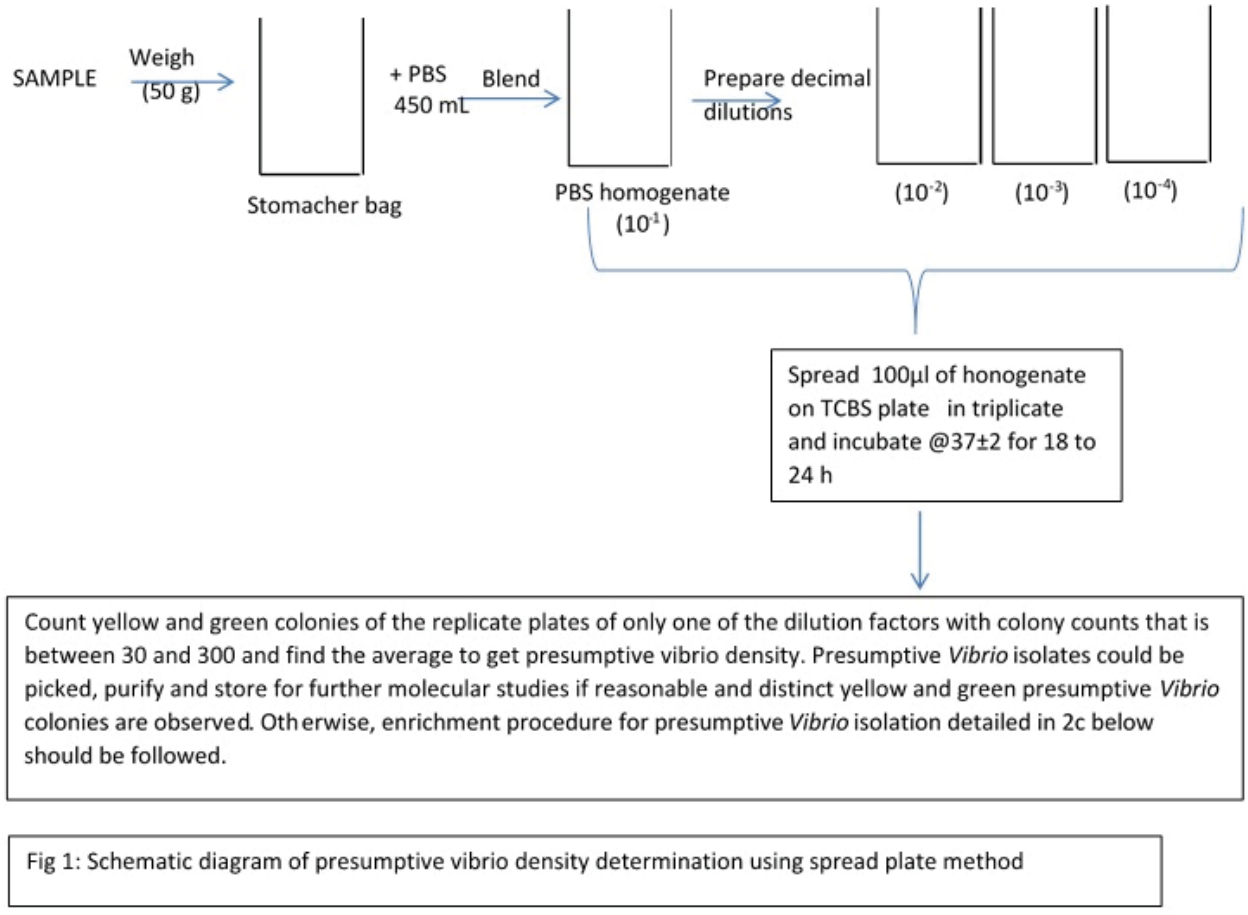


Figure 6: Flow-chart of presumptive *Vibrio* counts determination using spread plate method.

3.8 Total *Vibrio* density determination using Most Probable Number (MPN)-PCR method

The MPN-PCR method should be employed when total *Vibrio* density is required. This method can also be applied if the total density of a specific *Vibrio* species is required. The procedure is as described in Figure 7. The description here uses 3 MPN tubes by 6 dilutions.

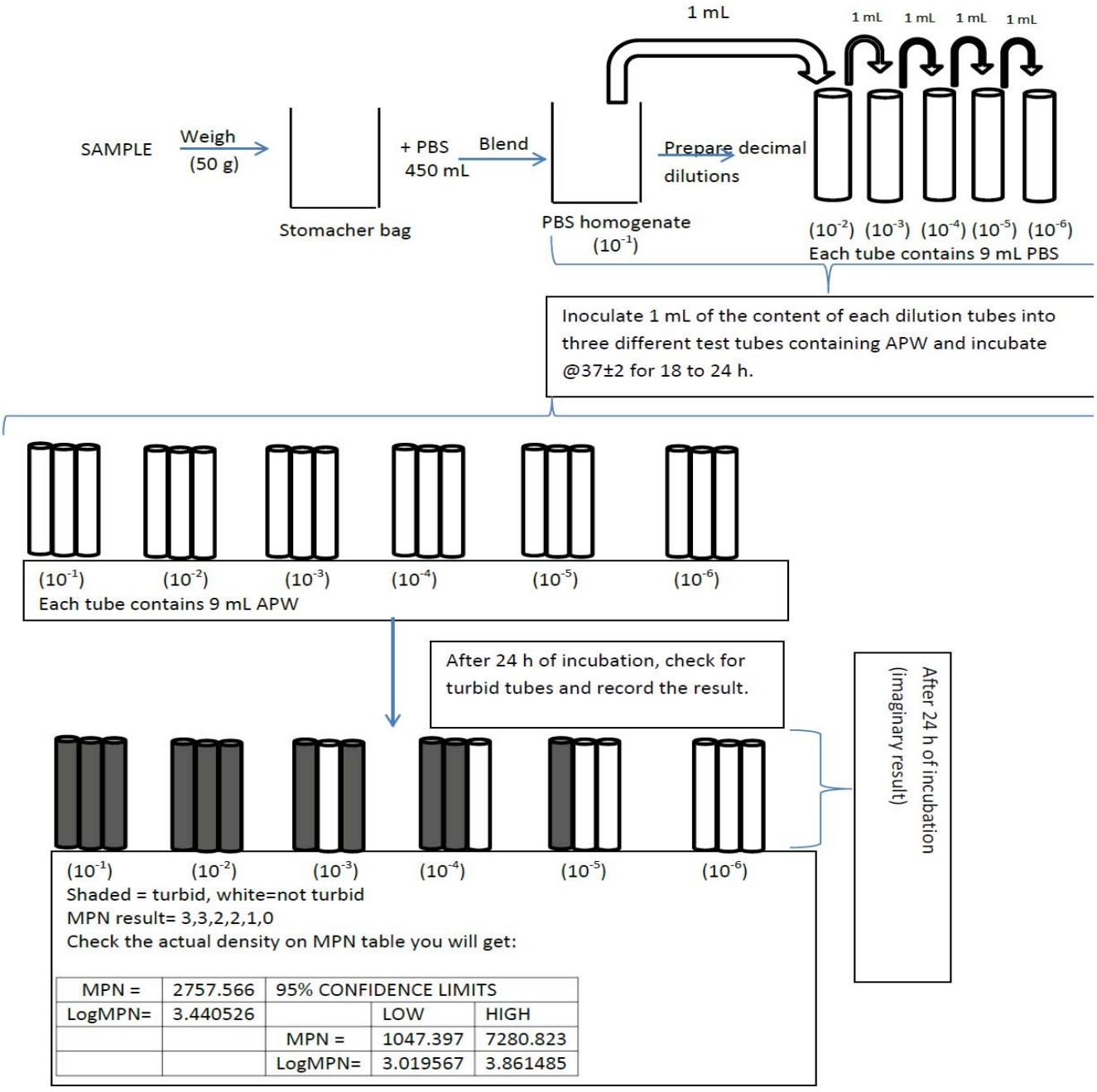


Figure 7: Schematic diagram of *Vibrio* density determination using MPN-PCR approach.

Given that the presence of other organisms besides *Vibrio* could make the APW turbid, the result obtained here is taken as presumptive *Vibrio* density in MPN/g. To obtain the actual *Vibrio* density, DNA is extracted from the turbid tubes and used as the template in PCR for *Vibrio* genus detection using specific primers listed in Table 3. The turbid tubes that are positive for *Vibrio* spp. will then be used for the determination of total *Vibrio* density. If one assumes that the turbid tubes which are positive for the *Vibrio* genus in the imaginary example given above are 3 of 10^{-1}, 2 of 10^{-2}, and 1 of 10^{-3}; the MPN result becomes 3,2,1,0,0,0, while the corresponding total *Vibrio* density in MPN/g is calculated using the MPN excel spreadsheet developed by the FDA. The output will be as shown in the example in Table 2. The excel spreadsheet can be downloaded from the FDA site at: https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm.

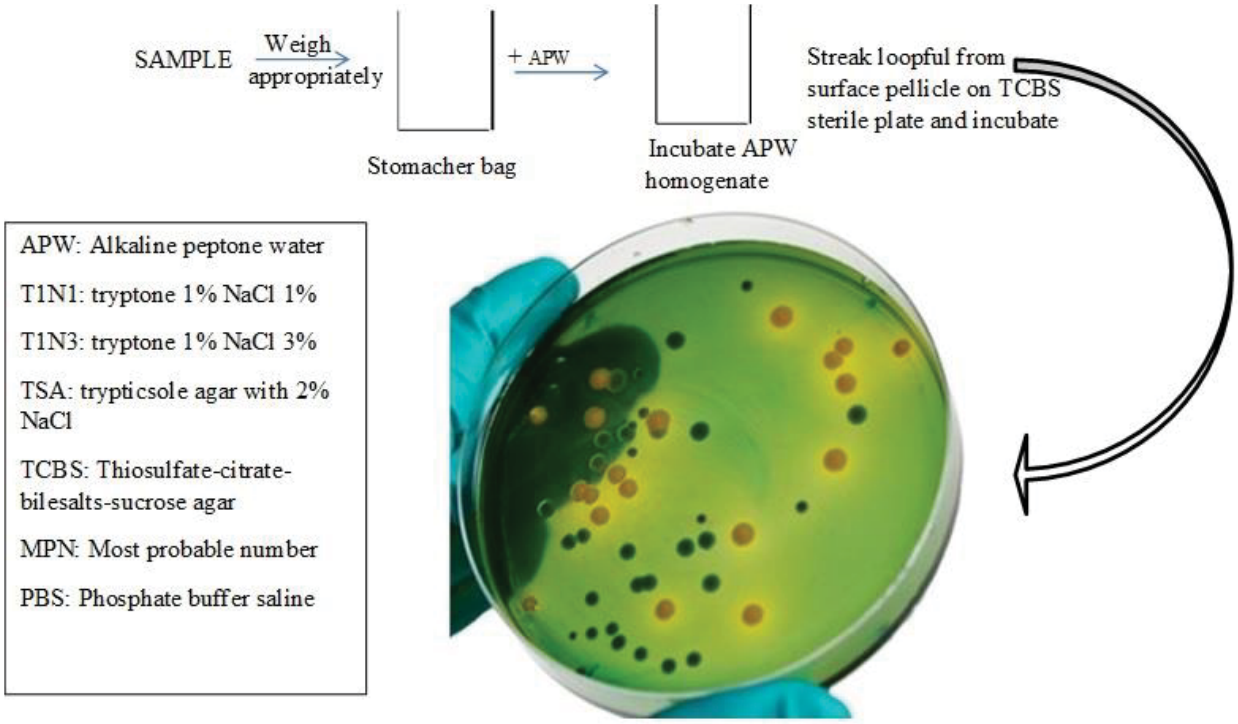
Table 2: An excerpt from Bacteriological Analytical Method (BAM) MPN Excel spreadsheet showing total *Vibrio* density of *Vibrio* positive MPN tubes.

Concentration		**95% confidence limit**		
MPN =	146.6262		Low	High
LogMPN=	2.166212	MPN =	44.28062	486.3961
		LogMPN=	1.645917	2.686506

To determine the density of a particular *Vibrio* species, DNA templates (from the turbid tubes above) which are positive for *Vibrio* genus must be subjected to PCR using specie specific primers. In conclusion the actual density of the species of interest must be determined from the MPN table.

3.9 Enrichment of samples for the isolation of presumptive *Vibrio* spp.

In cases where the density of *Vibrio* in a sample is too low to be detected by the direct plating described above, these samples must be enriched with alkaline peptone water (APW) and processed in accordance with the recommendation of Bacteriological Analytical Manual (BAM). This procedure is summarised in the schematic diagram below:



1. Streak to ensure distinct colonies **2.** Carefully pick 7 to 10 distinct green and yellow colonies and carefully purify them on sterile T1N1, T1N3 or TSA-2% agar **3.** Extract DNA from the purified isolates **4.** Run PCR protocol for confirmation using genus and species specific primers. **5.** Prepare a glycerol stock of confirmed isolates for reference and further study purposes.

Note:

1. 25 g of the sample should be blended with 225 ml of APW. 2475 ml of APW should be used in the case of raw oyster and when very high *Vibrio* contamination is suspected.
2. Incubation of APW homogenate should be done for 6 to 8 h and 24 h (most especially in the case of processed samples) at 35 ± 2°C. Incubation should be done for 18 to 21 h in the case of raw oyster at 42 ± 2°C.
3. The dominant pathogenic *Vibrio* population in the incubated homogenate differs at each incubation regime as stated in point 2 above, therefore TCBS sterile plates should be streaked at the expiration of each incubation regime to optimise the isolation of the pathogenic *Vibrio* spp. present in the sample under Vibriology.

3.10 Detection of pathogenic *Vibrio* in vegetables

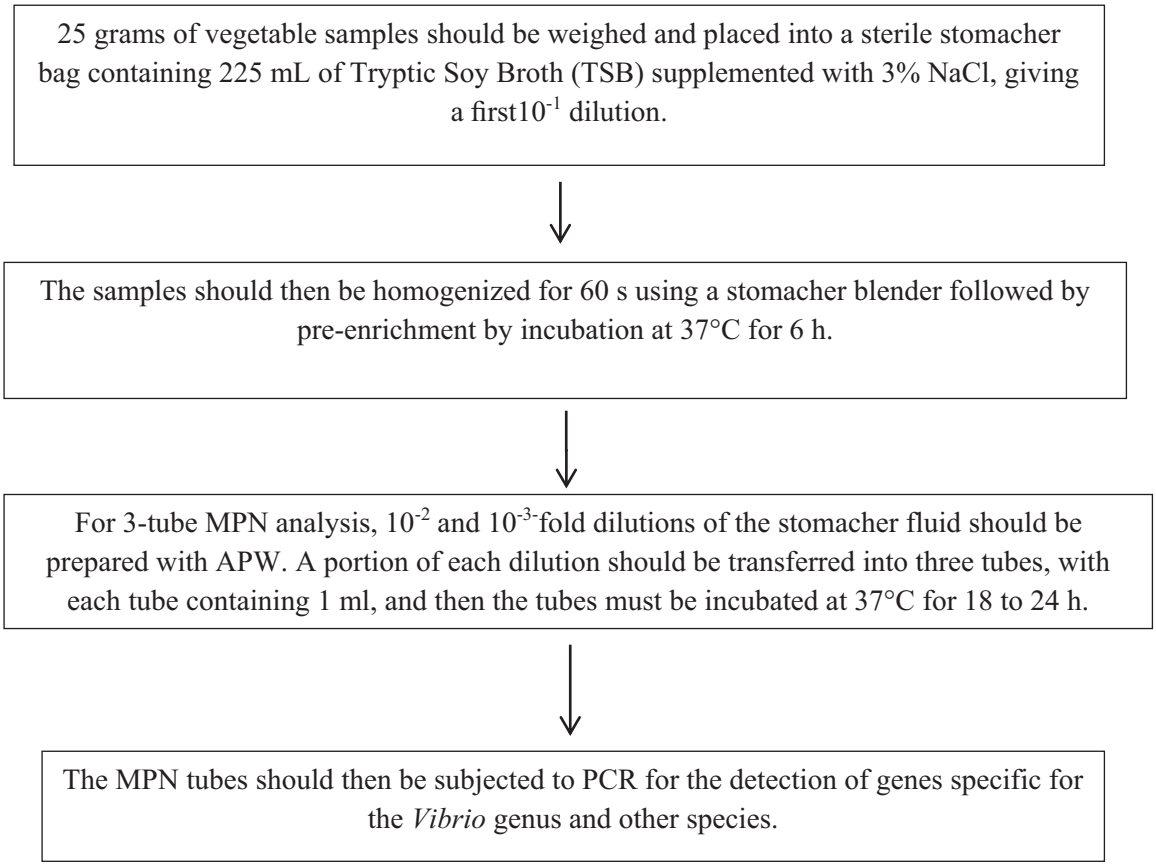
3.10.1 Isolation of *Vibrio cholerae* and other Vibrio pathogens from vegetables

Freshly harvested vegetables are frequently cultured as sentinel specimens for surveillance purposes to identify and monitor the Vibrio pathogens and to determine the risk of transmission in the population. For the isolation of Vibrio species, homogenates from the

maceration of 25 grams of vegetables in sterile stomacher bag containing 225 mL of Alkaline Peptone Water (APW) should be incubated at 37℃ under aerobic conditions for 18 h. After 18 h of incubation and enrichment, a loopful of enriched mixture should be streaked onto the surface of the selective media, Thiosulphate Citrate Bile salts Sucrose (TCBS**)** agar, and incubated at 37℃ for 18 h. Green and yellow colonies with typical Vibrio species morphology should then be isolated, purified on nutrient agar and preserved in 20% glycerol at -80°C for further analysis.

3.10.2 Enumeration and isolation of *Vibrio* species in vegetable samples

The most probable number (MPN) enumeration method should be based on the bacteriological analytical manual standard method (Andrews and Hammack, 2003). The flow of the procedure is presented in the chart below. The density of *Vibrio* species in vegetables will be expressed as MPN/g.



3.11 Molecular analysis for the characterization of *Vibrio* species

DNA extraction

1. Presumptive *Vibrio* colonies to be used for DNA extraction should be freshly (18-24 h old) grown on nutrient agar plates at 37℃.
2. The single colony on nutrient agar should be picked from plates and suspended in 200 µl of sterile distilled water in a sterile microfuge tube and lysed by boiling at 100℃ for 10 minutes.
3. After boiling, the mixture is centrifuged at 11,000×g for 2 min to precipitate cell debris.
4. The cell lysates (5 µl) obtained is used as template DNA in PCR assay.
5. Amplification of the relevant *Vibrio* species gene(s) should be done using species-specific primers listed in Table 3.

Table 3: list of primers used for molecular identification.

Target species	**Primers**	**Sequences (5 ' 3')**	**Target gene**	**Amplicon size (bp)**	**Reference**
Vibrio genus	V16S-700F V16S-1325R	(F)CGG TGA AAT GCG TAG AGA T (R)TTA CTA GCG ATT CCG AGT TC	16S rDNA	663bp	Kwok *et al.,* 2002
V. cholerae	V.compW F V.compW R	(F)CACCAAGAAGGTGACTTTATTGTG (R) GAACTTATAACCACCCGCG	ompW	302	Dutta *et al.,* 2013
V. parahae-molyticus	Vp.toxR R Vp.toxR F	(F)GTC TTC TGA CGC AAT CGT TG (R)ATA CGA GTG GTT GCT GTC ATG	toxR	897	Tarr *et al.,* 2007
V. vulnificus	Vv. hsp-326F Vv. hsp-697R	(F)GTC TTA AAG CGG TTG CTG C (R)CGC TTC AAG TGC TGG TAG AAG	hsp60	410	Wong *et al.,* 2002
V. fluvialis	Vf- toxR F Vf- toxR R	(F)GAC CAG GGC TTT GAG GTG GAC (R)AGGATACGG CACTTGAGTAAGACTC	toxR	217	Osori and Klose, 2000
V. mimicus	VM-F VM-Rmm	(F)CAGGTTTGYTGCACGGCGAAGA (R)YCTTGAAGAAGCGGTTCGTGCA	dnaJ	177	Nhung *et al.,* 2007
V. alginoly-ticus	Vg gyrB F Vg gyrB R	(F)GAGAACCCGACAGAAGCGAAG (R)CCTAGTGCGGTGATCAGTGTTG	gyrB	337	Zhou *et al.,* 2007

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