Simple, Rapid and on Spot Dye-based Sensor for the Detection of Vibrio Load in Shrimp Culture Farms

Sweta Binod Kumar

Central Salt and Marine Chemicals Research Institute CSIR

Ambika Hemant Shinde

Central Salt and Marine Chemicals Research Institute CSIR

Maheshawari Jagadishbhai Behere

Central Salt and Marine Chemicals Research Institute CSIR

Dhruvi Italiya

Central Salt and Marine Chemicals Research Institute CSIR

Soumya Haldar (✉ shaldar@csmcri.org)

Central Salt and Marine Chemicals Research Institute <https://orcid.org/0000-0002-8776-5238>

Research Article

Keywords: Kit, Water, Vibrio, Aquaculture, Probiotics, Detection

Posted Date: March 6th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-265744/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

For the detection of *Vibrio* bacteria, a kit involving two-step method was developed. In the first step a specific media was added in the water sample which selectively promote the growth of vibrios and inhibit the growth of other bacteria. The second step involved addition of dye-based sensor (already developed in our previous work) in the sample which detect the active *Vibrio* and changed the colour of the sample to red/pink. The vibrio detection kit was optimized on five different species of *Vibrio* (*V. cholerae* and *V. parahaemolyticus*, *V. campbellii*, *V. harveyi* & *V. proteolyticus*) and two negative control bacteria (*Escherichia coli* and *Bacillus subtilis*). The kit was further evaluated on aquaculture pond water and probiotics used in aquaculture farms. It successfully estimated *Vibrio* concentration of all the five strains and in aquaculture ponds. The negative control bacteria and probiotics were not sensed by the kit. Hence, the kit developed here is perfect for the detection of *Vibrio*, especially in aquaculture farms.

1. Introduction

The members of *Vibrio* genus are asporogenous Gram-negative rods that may have a single, rigid curve or are straight rods. *Vibrio* are motile and possess single polar flagellum. They are glucose fermenters and do not produce gas. Most of them produce oxidase and catalase (Kaysner and DePaola 2004). Among them, *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* are well recognized human pathogens (Bonnin-Jusserand et al. 2019). *V. cholerae* causes cholera and it is indirectly transmitted through contaminated water supplies. Apart from drinking water, vibrios are also the major microbiota of the ecosystems like marine water, estuarine ecosystem and aquaculture farm. Many of them are potent pathogens for the animals reared in aquaculture (Chatterjee et al. 2012 and Haldar et al. 2011). The *Vibrio* spp. causes vibriosis, which is a most predominant diseases among organisms bred in aquaculture and other fishes. It is also accountable for mortality of cultured aquaculture organisms globally (Lavilla-Pitogo et al. 1998 and Chen et al. 2000). The species of *Vibrio* which are generally related with shrimp diseases are *V. parahaemolyticus*, *V. anguillarum*, *V. harveyi*, *V. alginolyticus*, *V. splendidus* and *V. vulnificus* (Chatterjee et al. 2012 and Haldar et al. 2010). Therefore, considering the epidemiological significance of the *Vibrio* spp. both in public health and aquaculture, there is an earnest need to develop a rapid and portable kit to detect them in the water samples in simpler way to prevent any disease outbreak which leads to spread of disease and huge economic loss respectively.

Our group has developed a multiplex PCR based method for accurate identification of some of the important *Vibrio* spp., but this method requires initial screening in selective media, subsequent DNA extraction and amplification through PCR (Haldar et al. 2010 and Neogi et al. 2010). However, this method needs sophisticated laboratory facility which is generally not available in the shrimp farms. Similarly, many techniques and methods have been established for the detection of *Vibrio* spp. for example, typing methods, molecular based detection methods, culture-based identification, antibody-based assays etc. (Ramamurthy et al. 2020, Bisha et al. 2012, Kaysner and DePaola 2004). Although, many of these methods are rapid but also very expensive and need skilled persons to operate them. The traditional microbiological methods which are used commonly to detect them are very tedious and time-consuming. Also, onsite testing without microbiological laboratory and skilled manpower is not possible for any of these methods.

Recently, our group has developed a dye-based sensor which senses the total bacteria in the water just by dipping 1 cm² coated membrane in 1 mL of water sample (Binod et al. 2018). However, when the kit was introduced in the aquaculture settings; on the basis of feedback from the end user it was strongly felt that along

with the total bacterial count, specific kit was also needed which can specifically detect the selected *Vibrio* load of the pond water. Hence, with the help of this dye-based sensor we have made an attempt to develop a kit for easy, rapid and affordable detection of *Vibrio* spp. onsite. This kit involves two-steps in which the first step selects *Vibrio* and inhibits other bacterial species in the given sample by adding the designed medium. The second step will then detect *Vibrio* in the sample by adding dye-based kit by changing the colour of the sample into pink/red.

Therefore, the main objectives of this study were (1) development of vibrio detection kit based on the dye-based sensor, (2) optimization of the prepared kit on different standard *Vibrio* species taking *E. coli* and *B. subtilis* as negative control and (3) evaluation of the kit in aquaculture pond water.

2. Materials And Methods

2.1 Materials

Polyvinylidene fluoride (MW = 573 KDa, Solef, Solvay, France), polyester fabric (Filtration Sciences Corporation, USA), N, N dimethyl formamide (Qualigen, India), D-(+)-Glucose anhydrous (Hi media, India), Graphene oxide (Sigma-Aldrich) and 2, 3, 5-Triphenyltetrazolium chloride ($\geq 99.0\%$ SIGMA, Life Science) were used to prepare the asymmetric membranes. Reverse osmosis treated water was used for the membrane preparation.

Nutrient Agar, Nutrient Broth, Luria-Bertani Broth and TCBS Agar (Hi media, India) were used for all microbiology studies. *Escherichia coli* (NCIM2065), *Bacillus subtilis* (NCIM2920), *Vibrio cholerae* (N16961), *Vibrio parahaemolyticus* (IDH02640), *Vibrio campbellii*, *Vibrio harveyi*, *Vibrio proteolyticus* (Isolated from seawater and identified in our laboratory) were used for testing and evaluating the vibrio kit. Also, commercial probiotic containing *Paracoccus pantotrophus* (PondDtox, Novozymes) was used to test the vibrio kit.

Casein enzymic hydrolysate, Yeast extract, Proteose peptone, Sucrose, Sodium thiosulphate, Sodium citrate, Sodium deoxycholate, Sodium chloride, Oxgall, Sodium lauryl sulphate, Bile salts and Potassium tellurite (HiMedia, India) were used to prepare *Vibrio* selective medium.

2.2 Preparation of dye - based sensor for the detection of bacteria

The dye-based sensor was prepared as described in our previous paper (Binod et al. 2018). In brief, PVDF membrane of 7% W/W concentration was prepared using dimethyl formamide as solvent. 5% glucose (pH 8.6) was dissolved in the non-solvent medium (water). The membrane was then cut into 1 x 1 cm² pieces and autoclaved. The membrane pieces were then dip-coated in the prepared solution of filter sterilized tetrazolium dye (5 mg/mL), sterile graphene oxide (25µg/mL) and sterile nutrient broth inside laminar air flow for 24 h. Membrane pieces were then taken out from the solution and allowed to dry inside the laminar.

2.3 Preparation of selective growth media for *Vibrio* species

Selective media of different compositions were prepared which promoted the growth of different *Vibrio* species and inhibited the growth of other bacterial species. Total six types of such media compositions were prepared (Table S1 to S5), out of which only one medium was selected for further experiments (Table 1) as the other five media were showing inconsistent results (Table S6 to S14).

2.4 Application of selective growth medium for the detection of different *Vibrio* species

For the evaluation of selective growth medium, initially two *Vibrio* species were selected. *V. cholerae* and *V. parahaemolyticus* were used as positive control and *E. coli* and *B. subtilis* were used as negative control. Subsequently, three more *Vibrio* strains commonly reported from shrimp culture water (*V. campbellii*, *V. harveyi* and *V. proteolyticus*) were used to validate the efficiency of the prepared medium. Various concentration of the prepared medium (100, 200, 300, 400, 500 & 1000 µL) was standardized on different cell densities (10^2 - 10^8 CFU/mL) at 5, 15 and 30 min for all the selected bacteria. Sterile water was used as the diluent for the preparation of bacterial solution. All the selected positive and negative control bacteria were grown in nutrient broth for 12 h and washed subsequently with physiological saline (0.9 wt% NaCl) 3-4 times (3500 rpm for 15 min) to remove any traces of the nutrient from the bacterial cells. The cells were then resuspended in the diluent. The washed cells were then diluted for different concentrations and spread on nutrient agar plates simultaneously to find out the bacterial concentration.

The dye-based sensor of size 1 cm² was then dipped into the bacterial solution containing the medium and kept at 37°C. The colour change in the water was then monitored periodically.

2.5 Assessment of vibrio detection kit in the water sample spiked with probiotics

To check whether the kit is detecting probiotics in the water sample; a range of dilutions of *Vibrio* culture was mixed with a specific cell concentration of probiotics and tested with the vibrio detection kit. In detail, the probiotic was grown in the sterile pond water and was diluted subsequently so that the bacterial concentration reaches to 10^4 CFU/mL approximately. The selected *Vibrio* strain was grown in the LB media and its different dilutions were prepared in sterile pond water. The diluted probiotic (100 µL) of 10^4 CFU/mL cell concentration was then added to all the different dilutions of *Vibrio*. All the mixtures were spread on Nutrient Agar plates and TCBS plates to determine the total bacterial load and *Vibrio* concentration respectively. As comparison standards, another set was prepared containing different dilutions of probiotic and *Vibrio* separately. The dilutions of probiotics and *Vibrio* were spread on Nutrient Agar plates and TCBS plates respectively to determine the total bacterial load.

Each of the prepared mixtures and separate dilutions were then inoculated in the vibrio detection kit containing 500 µL of the selective growth medium, which were incubated for 15 min and the dye-based sensor of size 1 cm² was then dipped into the kits and kept at 37°C. The colour change in the water was then monitored periodically. All the experiments were done in duplicates.

2.6 Evaluation of vibrio detection kit in two different aquaculture farms

Applicability of the vibrio detection kit was tested on two different aquaculture farms which have unrelated environmental and water parameters and located distantly from each other. Water samples from aquaculture farms were collected from Bhavnagar, Gujarat (India) and Bhimavaram, Andhra Pradesh (India) (Fig 1). Both the ponds are practicing intensive type of culture (100/m²) with regular use of different probiotics and chemicals to improve the productivity.

For sample collection, polypropylene bottles (for physicochemical parameters) and sterile screw cap vials (for microbiology) were obtained from the local market. Physicochemical parameters (Salinity, Total Dissolved Solids and pH) was measured for all the collected aquaculture pond water samples with manual refractometer (ATAGO), digital TDS meter (Eutech) and digital pH meter (Eutech) respectively.

For the total *Vibrio* count, the water samples were spread on TCBS agar plate.

One milliliter of all the water samples was collected in sterile transparent container (e.g. 1.5/2 mL vials) containing 500 µL of the selective growth medium. It was incubated for 15 min and the dye-based sensor of size 1 cm² was then dipped into the water samples and kept at 37°C (the samples from Bhimavaram were kept at room temperature, 25-28°C approximately). The colour change in the water was then monitored periodically. All the experiments were done in triplicates.

3. Results And Discussion

3.1 Dye-based bacterial detection kit

In our previous study the principle and properties of dye-based bacterial detection kit has already been explained (Binod et al. 2018). In brief, the membrane used as the kit was prepared by wet phase separation technique. The replacement of solvent by nonsolvent in the formation process of asymmetric membrane results in the creation of macro voids which gets filled by glucose particles present in the water (non-solvent) during the diffusional exchange. Additives like glucose; tetrazolium and graphene oxide were impregnated onto the membrane surface so that they could leach in surrounding medium and subsequently get utilized by bacteria present in the water.

3.2 Detection of different *Vibrio* strains with the aid of dye-based sensor and prepared media

The principle behind the vibrio detection kit is that the specific medium added in the water sample allows the growth of only *Vibrio* spp. and inhibits the growth of other unspecific bacteria. Subsequently, the dye-based sensor detects the active *Vibrio* and change the colour of the sample to red/pink.

The prepared medium contains proteose peptone and yeast extract which provides nitrogenous compounds, vitamin B complex and other essential growth nutrients. Bile salt derivative i.e. oxgall and sodium citrate inhibit gram-positive bacteria and coliforms (Howard 1994). A good source of Sulphur is also present in the form of Sodium thiosulphate. For the metabolism of vibrios, sucrose is added as a fermentable carbohydrate.

Initially, different concentrations of the selected medium were used for a range of cell densities of positive and negative control bacteria for the optimization of the medium concentration used in the vibrio detection kit. The selected medium quantity from 100 µL to 400 µL showed the colour change of the negative control bacteria for all the different bacterial concentration (Table 2). Therefore, the medium concentration ranging from 100-400 µL was not sensitive for positive control bacteria and hence, cannot be used for vibrio detection kit. Again, the 1000 µL quantity was giving inconsistent results for varying bacterial concentration. For higher bacterial concentration, the colour change was slow and for less bacterial load the colour change was faster when compared with the results of all the other medium concentration (Table 2). Finally, the 500 µL concentration of medium was selected which was showing delayed or no colour change in the negative control bacteria and had consistent results with respect to the different bacterial concentrations.

For the optimization of medium concentration (explained in the above paragraph), the incubation time of 15 min was selected because short duration of incubation could not inhibit the growth of nonspecific bacteria in the medium effectively and too long incubation might give inconsistent result as the vibrio can grow in the medium exponentially affecting the time of colour change. Therefore, to confirm the optimum incubation time for the selected medium, three different incubation time was selected i.e. 5 min, 15 min and 30 min.

In case of *Vibrio cholerae* and *Vibrio parahaemolyticus*, faster rates of color development were observed when the incubation time was increased from 5 minutes to 15 minutes. However, when the incubation time was increased further to 30 minutes, it did not significantly increase or alter the rate of color development in corresponding dilutions of bacterial load (Table 3).

In case of *Escherichia coli*, lesser span of incubation resulted in faster color development in higher bacterial counts and obtained an irregular pattern for lower bacterial load. This was expected due to reduced time for effective selectivity of the medium. Thus, higher, incubation time of 15 min observed significantly slower rates of color development. Further, 30 min incubation span observed similar durations of color development for all the bacterial counts. In case of *Bacillus subtilis*, no color development was observed at any bacterial load irrespective of incubation span. This may be by virtue of effective selectivity of the medium (Table 3).

The incubation span, may therefore, be restricted to 15 minutes as it optimally enhanced color development rates in case of *Vibrio spp.* and, prolonged the same in case *E. coli* and *Bacillus sp.* so as to facilitate a threshold limit.

In conclusion, the vibrio detection kit was prepared with 500 µL medium concentration with incubation time of 15 min. To validate the efficiency of the kit further experiments were done with other positive control bacteria e.g. *V. proteolyticus*, *V. harveyi* and *V. campbellii*.

The Table 4 clearly shows that the vibrio detection kit is very sensitive for *Vibrio spp.* and it is showing delayed/no colour change for negative control bacteria. Hence, the kit can be used effectively for the detection of *Vibrio* in any water source. Fig 2 illustrates the colour change by the kit; in which positive control bacteria changed the colour of water and the negative control bacteria did not show any colour change. All the experiments were performed for three times and time taken to change the colour was same. Therefore mean of time taken to change the colour was not mentioned.

Finally, after all the standardization the average time intervals were obtained which correlated to the approximate cell densities of *Vibrio* which is shown in the Table 5. Hence, a rough estimate of *Vibrio* in the water samples can be determined by using these time periods.

3.3 Testing vibrio detection kit in mixed culture of *Vibrio* and probiotics

Probiotics are commonly used in the aquaculture farms for various purposes like enhancing the innate immunity of shrimps, achieve better growth and specially to mitigate the proportion of harmful *Vibrio spp.* (Martínez Cruz et al. 2012). So, it is necessary to check whether the presence of probiotic bacteria of non-vibrio origin in the pond is affecting the sensitivity of kit. Hence, one *Vibrio* strain was selected and mixed with the commercially available probiotic to assess the vibrio detection kit.

The total bacterial count and *Vibrio* count of mixed culture (*Vibrio* and probiotics) shows that the probiotics and *Vibrio* both are coexisting in the mixture. Because in the higher dilutions of total bacterial count there is an increase in bacterial cell concentration whereas low bacterial load was observed in the higher dilutions of *Vibrio* count (Table 6). Now to test whether the probiotic present in the mixture was detected by the kit; the timings of colour change of mixed culture dilutions was compared with the timings of the colour change of only *Vibrio* culture dilutions. Table 6 shows that the *Vibrio* cell density (for all dilutions) in both the mixture as well as in the *Vibrio* culture alone was same and the time period of colour change corresponding to these cell concentrations were also comparable. Hence, there was no effect of probiotic on the timing of colour change by the kit. The complete experiment was repeated three times and similar trend was observed.

To further confirm the results, probiotic of different concentrations was prepared and checked with the vibrio detection kit. No colour change was observed in any of the dilutions of probiotics (table 7). So, the kit did not detect commercial probiotics present in the pond water. Thus, the kit is reliable when used for *Vibrio* contamination detection in aquaculture pond water.

3.4 Application of vibrio detection kit in two different aquaculture farms

It is really required to evaluate any kit in the field to confirm that it is properly working in diverse and fluctuating environmental conditions. The dye-based bacterial detection kit was already tested in wide variety of environmental samples and it perfectly worked in all types of water having different physicochemical properties (Binod et al. 2018). The vibrio testing kit could detect *Vibrio* in the ponds of Bhavnagar aquaculture farm of having quite high salinity. When compared to the standard chart prepared for *Vibrio* detection concentration (Table 4 and 5), the kit perfectly estimated the *Vibrio* load in the different ponds i.e. for $10^2 - 10^3$ CFU/mL *Vibrio* concentration and the time of colour change was 5:30-7:50 h approximately (Table 8). Again, the different ponds were having unlike characteristics. For example, the water of Pond 1 had too much hardness and dead fishes were also present in the water for which flocculants (HydroFloc™) and zeolite was added into the pond to improve its water quality. In Pond 2 also HydroFloc and disinfectant were added. Probiotics was supplemented in the Pond 3 and Pond 4. In spite of these many additions to the pond, the kit could correctly estimate the *Vibrio* concentration in the various ponds.

Further, at Bhimavaram a wide range of pond water was tested for the presence of *Vibrio* with the aid of kit e. g. Mixed pond water used for algae culture (Pond 1), effluent treatment pond water (Pond 2), culture pond water (Pond 3), grow out pond water (Pond 4 & 5), brine water reservoir (Pond 6) and fresh water reservoir (Pond 7). Each water had different physicochemical properties. Compared to the Bhavnagar aquaculture farm, the water of Bhimavaram pond water was having lower salinity and TDS. But here also the *Vibrio* load was $10^2 - 10^3$ CFU/mL for which the kit took 10:00 – 12:00 h for colour change (Table 9). The delay in colour change was may be due to the incubation temperature (Section 2.6) and/or less salinity of the water. The control sample and the water from freshwater reservoir did not have any *Vibrio* count on TCBS plates and the kit also did not change its colour which contained those samples (Table 9). This surely indicates that the kit did not give false positive results. Nonetheless, it was confirmed from this result that the kit can even work at room temperature and it can be directly used onsite. Further, the kit can be standardized according to the salinity and temperature of particular field where the kit is to be used and can be distributed directly to the farmers working at the farm. So, when the colour of the kit changes its colour in very less time compared to the normal time of colour change; the farmers can immediately raise alarm about the increase in *Vibrio* load in pond water.

Hence, there is no influence of different additives and probiotics on the working mechanism of the vibrio detection kit. Moreover, it can work effectively on the field and can evade the problem of transporting samples to the laboratory which are many times very far from the farm and affect the final result.

4. Conclusion

For the detection of *Vibrio*, a unique kit was developed having very simple operating mechanism. It is affordable, simple and does not need any skilled personnel for its operation. The kit requires only 500 µL of the selective medium to inhibit unspecific bacteria within 15 min. Even low concentration (10^2 CFU/mL) of the *Vibrio* can be detected within 6-7 h. Other specific bacterial detection kit can also be prepared on the basis of this kit. This will be

very useful for the initial detection of harmful bacteria especially *Vibrio* spp. from water and can act as an alarm to warn about the presence of particular pathogen in water source. It can be operated onsite and visual analysis is enough for result interpretation. Hence, it is perfect for inexperienced people like aquaculture farmers.

Declarations

ACKNOWLEDGMENTS

Authors sincerely acknowledge analytical division of CSIR-CSMCRI for providing all the instrument facilities. SBK acknowledges Department of Biotechnology, Govt. of India and AHS acknowledges Council of Scientific Industrial Research, India for their Senior Research Fellowship and financial support respectively. The manuscript has been assigned CSIR-CSMCRI – 2/2020 registration number.

Funding

The work was performed with internal fund of the institute. Sources of fellowships received by SBK and AHM has been mentioned in the acknowledgement part.

Conflict of Interest/Competing interest:

The authors declare that they have no conflict of interest.

Ethics approval

The manuscript has not been submitted to any other journal and the work is original and not have been published elsewhere in any form or language (partially or in full). Results has been presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation. Plagiarism has been checked throughout the manuscript.

Consent to participate

All the coauthors have given their consent to participate as coauthors in the manuscript.

Consent for publication

No third party data has been included without proper citation. Therefore, this is not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Code availability

Not Applicable

Authors' contributions

SBK performed the experiments and interpreted the data. AHS and BMJ performed the experiment and help in writing manuscript. DI performed the part of experiment. SH has conceptualized the work and prepared the

References

1. Binod S, Hemant A, Mehta R, Bhattacharya A (2018) Simple , one-step dye-based kit for bacterial contamination detection in a range of water sources. *Sensors Actuators B Chem* 276:121–127. <https://doi.org/10.1016/j.snb.2018.08.077>
2. Bisha B, Simonson J, Janes M, et al (2012) A review of the current status of cultural and rapid detection of *Vibrio parahaemolyticus*. *Int J Food Sci Technol* 47:885–899. <https://doi.org/10.1111/j.1365-2621.2012.02950.x>
3. Bonnin-Jusserand M, Copin S, Le Bris C, et al (2019) *Vibrio* species involved in seafood-borne outbreaks (*Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*): Review of microbiological versus recent molecular detection methods in seafood products. *Crit Rev Food Sci Nutr* 59:597–610. <https://doi.org/10.1080/10408398.2017.1384715>
4. Chen FR, Liu PC, Lee KK (2000) Lethal attribute of serine protease secreted by *Vibrio alginolyticus* strains in kuruma prawn *Penaeus japonicus*. *Zeitschrift fur Naturforsch - Sect C J Biosci* 55:94–99. <https://doi.org/10.1515/znc-2000-1-218>
5. Haldar S, Chatterjee S, Sugimoto N, et al (2011) Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an *Artemia* model. *Microbiology* 157:179–188
6. Haldar S, Neogi SB, Kogure K, et al (2010) Development of a haemolysin gene-based multiplex PCR for simultaneous detection of *Vibrio campbellii*, *Vibrio harveyi* and *Vibrio parahaemolyticus*. *Lett Appl Microbiol* 50:146–152
7. Howard BJ (1994) *Clinical and pathogenic microbiology*. Mosby
8. Kaysner CA, DePaola AJ (2004) *BAM: Vibrio*
9. Lavilla-Pitogo CR, Leaño EM, Paner MG (1998) Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. *Aquaculture* 164:337–349. [https://doi.org/10.1016/S0044-8486\(98\)00198-7](https://doi.org/10.1016/S0044-8486(98)00198-7)
10. Martínez Cruz P, Ibáñez AL, Monroy Hermosillo OA, Ramírez Saad HC (2012) Use of Probiotics in Aquaculture. *ISRN Microbiol* 2012:1–13. <https://doi.org/10.5402/2012/916845>
11. Neogi SB, Chowdhury N, Asakura M, et al (2010) A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Lett Appl Microbiol* 51:293–300
12. Ramamurthy T, Das B, Chakraborty S, et al (2020) Diagnostic techniques for rapid detection of *Vibrio cholerae* O1/O139. *Vaccine* 38:A73–A82. <https://doi.org/10.1016/j.vaccine.2019.07.099>
13. Chatterjee S, Haldar S (2012) *Vibrio* Related Diseases in Aquaculture and Development of Rapid and Accurate Identification Methods. *J Mar Sci Res Dev S* 1(1): 1-7. <https://doi.org/10.4172/2155-9910.s1-002>

Tables

Table 1. Composition of selective growth medium for *Vibrio* spp.

Ingredients	g/50mL
Proteose peptone	1.7
Yeast extract	0.9
Sodium thiosulphate	1.7
Sodium citrate	1.7
Oxgall	1.4
Sucrose	3.4
Sodium chloride	1.7
pH at 25 °C	8.6 ± 0.2

Table 2. Effect of selected medium concentration on the colour change of the vibrio detection kit for various cell densities of positive and negative control bacteria

Bacteria	Media conc. (µL)	Time taken (in hours) for color development by corresponding bacterial load (CFU/mL)									
		10 ²	10 ³					10 ⁴	10 ⁵	10 ⁶	10 ⁷
<i>Vibrio cholerae</i>	100	14:30	10:30					6:45	3:30	2:00	00:35
	200	14:30	11:00					6:45	3:30	2:00	00:35
	300	15:00	11:40					9:00	4:00	2:15	00:40
	400	15:00	12:00					9:00	4:15	2:15	1:00
	500	15:30	12:30					10:00	5:00	2:30	00:50
	1000	11:10	11:00					10:00	8:00	6:30	6:10
<i>Vibrio parahaemolyticus</i>	100	14:00	10:00	10 h	10 h 45 min	10 h 45 min	11 h	6:00	3:00	1:30	00:10
	200	14:00	10:00	10 h	10 h 45 min	10 h 45 min	11 h	6:15	3:00	1:30	00:15
	300	16:00	10:45					9:30	3:30	1:30	00:15
	400	16:00	10:45					10:15	3:30	2:00	00:30
	500	16:30	11:00					10:15	3:30	2:00	00:45
	1000	8:00	6:10					6:00	4:00	3:00	1:45
<i>Escherichia coli</i>	100	15:30	12:30					8:00	5:00	2:00	00:35
	200	15:30	12:30					8:15	6:45	2:30	00:35
	300	16:35	14:00					10:00	7:15	5:00	3:30
	400	17:00	14:00					10:00	10:00	6:15	5:30
	500	-	-					-	24:00	24:00	16:00
	1000	-	-					-	-	-	-
<i>Bacillus subtilis</i>	100	18:00	14:00					9:00	8:30	8:00	6:00
	200	18:00	14:00					9:00	8:30	8:15	6:00
	300	18:40	15:15					11:00	11:00	10:00	8:00
	400	20:00	16:00					12:00	12:00	10:00	10:00
	500	-	-					-	-	-	-
	1000	-	-					-	-	-	-

(-): no color development

Table 3. Effect of incubation time on the colour change of the vibrio detection kit for various cell densities of positive and negative control bacteria

Bacteria	Incubation Time	Time taken (in hours) for color development by corresponding bacterial load (CFU/mL)					
		10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷
<i>Vibrio cholerae</i>	5 mins	8:58	7:44	6:32	5:08	3:04	0:26
	15 mins	7:23	6:39	5:51	4:50	2:55	0:17
	30 mins	7:50	6:17	5:23	4:27	2:38	0:31
<i>Vibrio parahaemolyticus</i>	5 mins	6:51	6:11	5:46	4:21	3:06	1:30
	15 mins	6:36	5:46	4:50	3:46	2:56	0:45
	30 mins	6:50	5:40	5:05	3:35	2:40	0:50
<i>Escherichia coli</i>	5 mins	NA	-	24:20	-	-	5:08
	15 mins	NA	24:20	21:40	20:40	19:40	17:10
	30 mins	NA	23:55	-	23:55	23:25	19:55
<i>Bacillus subtilis</i>	5 mins	-	-	-	-	-	-
	15 mins	-	-	-	-	-	-
	30 mins	-	-	-	-	-	-

NA: data not available, (-): no color development

Table 4. Concentration detection of different positive and negative control bacteria by vibrio detection kit

Bacteria	Time taken (in hours) for color development by corresponding bacterial load (CFU/mL)					
	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷
<i>V. cholerae</i>	8:30	8:00	6:00	5:00	3:15	00:45
<i>V. parahaemolyticus</i>	6:00	5:15	4:30	3:15	2:00	00:45
<i>V. proteolyticus</i>	6:45	5:30	4:45	3:45	2:30	1:30
<i>V. harveyi</i>	6:00	5:15	4:00	3:15	1:45	00:45
<i>V. campbellii</i>	5:00	4:30	3:00	2:30	1:30	00:45
<i>E. coli</i>	-	24:00	22:00	21:30	20:45	16:30
<i>B. subtilis</i>	-	-	-	-	-	-

(-): no color development

Table 5. Detection of *Vibrio* spp. concentration by vibrio detection kit

Bacterial load (CFU/mL)	Time taken (in hours) for color development by the corresponding bacteria		
	<i>Vibrio</i> spp.	<i>E. coli</i>	<i>B. subtilis</i>
10 ²	6:00 - 7:00	beyond 1 day	-
10 ³	5:00 - 6:00	beyond 1 day	-
10 ⁴	4:00 - 5:00	upto 22:00	-
10 ⁵	3:00 - 4:00	upto 21:30	-
10 ⁶	1:30 - 3:00	upto 20:45	-
10 ⁷	0:15 - 1:30	upto 16:30	-

(-): no color development

Table 6. Comparison of colour change after spiking probiotics in different dilutions of *Vibrio* with colour change timings of only *Vibrio* dilutions

Bacterial dilutions	<i>Vibrio</i> + probiotic *		<i>Vibrio</i>	<i>Vibrio</i> + probiotic*	<i>Vibrio</i>
	Total Bacterial Count (Nutrient Agar) in CFU/mL	Total <i>Vibrio</i> Count (TCBS) in CFU/mL	Total <i>Vibrio</i> Count (TCBS) in CFU/mL	Colour change (h)	Colour change (h)
Neat	9.8 X 10 ⁶	9.5 X10 ⁶	9.0 X 10 ⁶	2:00	1:50
10 ⁻¹	1.8 X10 ⁶	1.5 X10 ⁶	1.0 X10 ⁵	3:30	3:25
10 ⁻²	1.5 X10 ⁵	3.7 X10 ⁴	1.0 X10 ⁴	5:10	4:55
10 ⁻³	1.2 X10 ⁵	3.9 X 10 ³	1.9 X10 ³	6:40	6:25
10 ⁻⁴	1.5 X 10 ⁴	4.4 X 10 ²	2.2 X 10 ²	8:10	8:05
10 ⁻⁵	1.8 X10 ⁴	3.0 X 10 ¹	9.0 X10 ¹	9:10	10:05
10 ⁻⁶	1.8 X10 ⁴	≤ 10 ⁰	≤ 10 ⁰	12:40	11:05

*900 µL of *Vibrio* dilutions + 100 µL of 10⁴ CFU/mL conc. Of probiotic

Table 7. Colour change detection of different dilutions of probiotic by vibrio detection kit

Bacterial dilutions	Total Probiotic Count (Nutrient Agar) in CFU/mL	Colour change (h)
Neat	4.1 X10 ⁶	-
10 ⁻¹	1.0 X10 ⁵	-
10 ⁻²	1.3 X10 ⁴	-
10 ⁻³	2.6 X 10 ³	-
10 ⁻⁴	4.1 X 10 ²	-
10 ⁻⁵	1.0 X 10 ¹	-
10 ⁻⁶	≤ 10 ⁰	-

(-): no color development

Table 8. *Vibrio* concentration detection in Bhavnagar aquaculture pond by vibrio detection kit

Water Sample	pH	Salinity (‰)	TDS (Total Dissolved Solids) (ppt)	Vibrio Count (CFU/mL)	Color Development (h)
Pond 1	7.62	46	32.7	1.3×10^3	5:30
Pond 2	7.4	59	32.3	1.3×10^3	7:50
Pond 3	7.6	50	33.0	8.2×10^2	7:50
Pond 4	7.4	45	30.8	2.9×10^2	7:35
Reserve	7.95	25	36.2	7.7×10^2	5:30

Table 9. *Vibrio* concentration detection in Bhimavaram aquaculture farm by vibrio detection kit

Water Sample	pH	Salinity (‰)	TDS (Total Dissolved Solids) (ppt)	Vibrio Count (CFU/mL)	Color Development (h)
Pond 1	8.8	24.3	25.5	2×10^3	10:00
Pond 2	7.7	3.15	3.41	1.3×10^3	10:00
Pond 3	7.7	25.4	24.5	4.4×10^2	10:30
Pond 4	8.3	5.09	5.23	6.0×10^2	12:00
Pond 5	7.9	4.61	4.82	4.5×10^2	12:00
Pond 6	7.9	12.2	17.5	3.2×10^2	10:30
Pond 7	8.3	0.51		Uncountable colourless colonies	-
*Control	7.2	0.56		Uncountable colourless colonies	-

*Provided by the farm owner

(-): no color development

Figures

Figure 1

Sampling sites from aquaculture farms located in India. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Digital image of vibrio detection kit; tested for different positive and negative control bacteria.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryESPR.docx](#)