Surface Immuno-Functionalisation for the Capture and Detection of *Vibrio* Species in the Marine Environment: A New Management Tool for Industrial Facilities

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Abstract

Bacteria from the genus *Vibrio* are a common and environmentally important group of bacteria within coastal environments and include species pathogenic to aquaculture organisms. Their distribution and abundance are linked to specific environmental parameters, including temperature, salinity and nutrient enrichment. Accurate and efficient detection of *Vibrios* in environmental samples provides a potential important indicator of overall ecosystem health while also allowing rapid management responses for species pathogenic to humans or species implicated in disease of economically important aquacultured fish and invertebrates. In this study, we developed a surface immuno-functionalisation protocol, based on an avidin-biotin type covalent binding strategy, allowing specific sandwich-type detection of bacteria from the *Vibrio* genus. The assay was optimized on 12 diverse *Vibrio* strains, including species that have implications for aquaculture industries, reaching detection limits between 7 × 10^3 to 3 × 10^5 cells mL^-1_. Current techniques for the detection of total *Vibrios* rely on laborious or inefficient analyses resulting in delayed management decisions. This work represents a novel approach for a rapid, accurate, sensitive and robust tool for quantifying *Vibrios* directly in industrial systems and in the environment, thereby facilitating rapid management responses.

Introduction

*Vibrios* are a Gram-negative bacterial genus found in both tropical and temperate marine environments [1–3]. In recent years there has been growing interest in the dynamics of *Vibrio* populations, because many strains are pathogenic to humans and marine animals and represent a significant threat to the aquaculture industry and human health [4,5]. A global estimate of disease losses to aquaculture by the World Bank in 1997 was approximately US$3 billion per annum with *Vibrios* playing a significant role [6]. There is evidence that *Vibrio*-associated diseases are increasing globally, including seafood-linked human poisonings [7], human wound infections through contact with contaminated waters [8,9] along with diseases reported in corals, molluscs and fish [10–13]. These increases in *Vibrio* distribution and virulence have been linked to climate change [14] and other environmental perturbations associated with human activities [15–17]. Given the emerging threat of marine diseases and their potential to detrimentally impact the aquaculture industries, there is a growing need for establishing rapid, on-site detection techniques for pathogenic marine bacterial groups, including the *Vibrios*.

Current techniques for detecting *Vibrios* in the environment are focused on the detection of specific strains, such as *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, known to affect economically important aquaculture species as well as human health. Approaches used to examine these bacteria rely on time-consuming procedures, including culturing or quantitative molecular biological approaches (e.g. quantitative PCR), often resulting in management decisions being made days after the collection of samples [18–20]. Tools incorporating biosensor technology which allow real-time quantitative assessment of *Vibrio* populations in environmental samples offer considerable advantages over well-established methods, including low analysis cost, relatively short time-to-result, high potential for miniaturisation, and the possibility of performing the measurements *in situ* without technical expertise. Biosensing devices also allow for online monitoring of water systems enabling the development of near real-time ecosystem and aquaculture species health and disease surveillance platforms.
Previous efforts to make *Vibrio* biosensors have generally focused on the detection of human pathogenic strains [21,22]. This study develops and optimises a robust functionalisation protocol allowing the specific capture of total *Vibrios* in seawater samples using selected anti-*Vibrio* antibodies as the recognition elements. We describe the optimisation of a sandwich-type assay using the avidin-biotin affinity as the strategy for the immobilisation of the capture antibodies, and horse-radish peroxidase (HRP) as the label for the detection antibody. We show the assay to be robust with real samples obtained from mulloway fish larvae (*Argyrosomus japonicas*) rearing tanks and that the technique allows for rapid identification of *Vibrio* strains previously implicated as pathogens within aquaculture settings. This work represents a major step towards the development of a biosensor for the detection of *Vibrios* in aquaculture and natural settings and the management of aquaculture facilities.

**Materials and Methods**

**Ethics statement**

This study was carried out in strict accordance with the recommendations in “A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research” [23]. The protocol was approved by the Animal Care and Ethics Committee of the NSW Department of Primary Industries (Fisheries) (Permit Number: 93/1). Larvae were reared under optimal conditions as described in [24] and all efforts were made to minimize suffering.

No specific permission was required for this location and none of the field studies conducted for this study involved endangered or protected species.

**Bacterial strains and growth conditions**

Bacterial strains used in this study are listed in Table 1. At least seven out of twelve *Vibrio* species included in this work have been described as potential aquaculture pathogens [28]. The different strains were grown at 26°C in marine broth 2216 medium (Difco) overnight. For the surface immuno-functionalisation protocol, fresh cultures were aliquoted into 1.5 ml eppendorf tubes, and centrifuged for 10 minutes at 14,000 g. The resultant supernatants were washed, centrifuged for 10 minutes at 14,000 g and reconstituted in marine agar (2216 medium; Difco). Prior to experiments, frozen samples using selected anti-*Vibrio* antibodies as the recognition elements. We describe the optimisation of a sandwich-type assay using the avidin-biotin affinity as the strategy for the immobilisation of the capture antibodies, and horse-radish peroxidase (HRP) as the label for the detection antibody. We show the assay to be robust with real samples obtained from mulloway fish larvae (*Argyrosomus japonicas*) rearing tanks and that the technique allows for rapid identification of *Vibrio* strains previously implicated as pathogens within aquaculture settings. This work represents a major step towards the development of a biosensor for the detection of *Vibrios* in aquaculture and natural settings and the management of aquaculture facilities.

**Affinity test and colorimetric detection of *Vibrio* strains using targeted polyclonal antibodies**

Horseradish peroxidase (HRP) labelled anti-*Vibrio* rabbit polyclonal antibody (HRP-αVib Pab) (Kirkegaard & Perry Laboratories Inc; Washington, D.C., US) was assessed for affinity against a range of *Vibrio* bacterial strains (Table 1) and checked for cross reaction with another non-*Vibrio* ubiquitous bacterium found in marine systems, *Serratia marcescens* [30]. The stability of the all the antibodies used in this study was given by the manufacturer and is 3 months at 4 degrees Celsius and 1 year at −20 degrees Celsius. No loss of activity was observed during the 12 month experimental period. Increasing serial concentrations of target *Vibrio* strains (from 1 to 10⁶ cells mL⁻¹), *S. marcescens* (negative control) and the antibody manufacturer’s positive control cell extracts were adsorbed onto the multiwell plate (Nunc maxisorb, Sigma-Aldrich) surfaces. The wells were then washed (3 washes with 400 μl of PBS (10 mM pH 7.4) containing 0.05% v/v Tween (PBST; Sigma-Aldrich)) and their surfaces blocked through addition of 200 μl bovine serum albumin (BSA 2% (v/v); Sigma-Aldrich) for 2 hours at 37°C. After another washing procedure, 100 μl of a 1/500 HRP-αVib Pab solution was added to the wells (standard dilution recommended by the antibody supplier KPL, Inc.) for 1 hour at room temperature. The final washing step involved 3 washes with 400 μl of PBST, followed by 2 washes with 400 μl PBS. One hundred μLs of 3,3’,5,5’-Tetramethylbenzidine (TMB; Sigma-Aldrich) HRP substrate was then introduced to each plate well and left to develop for 10 min at room temperature. The reaction was stopped with 50 μL of stop solution (Sigma-Aldrich), and colour development was recorded at 450 nm using a plate reader (Fluostar optima, BMG labtech Pty. Ltd.).

**Surface immuno-functionalisation and capture antibody immobilisation**

To obtain a surface that would specifically and efficiently capture *Vibrio* cells, we developed and optimised a protocol allowing for maximum coverage and optimum orientation of the antibodies (yielding maximum binding capacity). Any remaining uncovered surface was blocked with BSA to avoid any non-specific adsorption of cells to the surface (see Figure 1). Unless otherwise stated, all washing procedures consisted of two consecutive washes with PBST, followed by two washes with 400 μl of PBS. Briefly, 100 μl of neutravidin (20 μg mL⁻¹ in PBS) was added to the Nunc maxisorb plate wells for 1 hour at 37°C followed by a washing step. These concentration and incubation conditions were optimised in a previous study [31]. Next, 100 μl of biotin labelled anti-*Vibrio* rabbit polyclonal antibody (Bt-αVib Pab; KPL Inc; Washington, D.C., US) (20 μg mL⁻¹ in PBS) was directly added to the well surface of the 96 well plates for 1 hour at 37°C. The wells were washed, blocked with 200 μl 2% BSA (w/v) in PBS solution for 2 hours at 37°C, and washed again. The negative controls consisted of wells functionalised with neutravidin and BSA but no antibody.

**Bacterial detection on an immuno-functionalised surface**

Once the surface had been functionalised, the optimum conditions for bacterial cell capture were assessed by testing the appropriate contact times and the optimum amount of detection antibody (HRP-αVib Pab). The wells functionalised with Bt-αVib Pab or BSA only (negative controls for unspecific adsorption), were incubated with serial inoculations of between 1 to 1×10⁹ cells mL⁻¹ bacteria, washed and incubated with 100 μl of HRP-αVib Pab at room temperature (22±1°C) mimicking field conditions. For each condition tested, 3 replicates were carried out and the average and standard deviation of the absorbance values calculated. The assay limit of detection (LOD) was calculated as the mean of 10 blanks (assay carried out in the absence of bacteria) plus 3 times the standard deviation and expressed in cells mL⁻¹. IC50 is the concentration in cells mL⁻¹ generating 50% of the maximum assay absorbance (A) signal and was calculated as follows: (Amax – (Amax - Amin))/2. The assay sensitivity was
calculated as the slope of the linear component of the curve and is expressed in absorbance units per unit of bacteria concentration (AU mL cell$^{-1}$).

Detection of *Vibrios* within mulloway fish larvae rearing tanks

A field test of the assay was conducted in the context of a mulloway rearing trial, where kaolin clay (Premium Clay, Boral Cements Blue Circle, Australia; 5 mg/L) and algal paste (Instant Algae, Reed Mariculture, USA; 3.5 mL/1000 L) were being evaluated as media for ‘greenwater’ in the rearing of mulloway (*Argyrosomus japonicus*) larvae. Fertilized eggs were sourced from captive mulloway broodstock at the Port Stephens Fisheries Institute (NSW, Australia) and hatched in 450 L incubators (salinity 32; 22$^\circ$C). After hatching, the larvae were randomly stocked (4 larvae L$^{-1}$) into six 2000 L experiment tanks filled with disinfected seawater (ozone 0.5–1.0 ppm; salinity 3232 ppt). The tanks had conical bottoms and were fitted with an upwelling manifold positioned at the base of the tank. Water flowed out of the tank through a 500 mm mesh-covered standpipe into a 200 L sump, where the water was returned to the tank via the manifold at 24 L min$^{-1}$. In addition, 100% of the tank volume was exchanged daily with disinfected seawater. Artificial light was provided overhead from fluorescent lamps at 225–400 Lux with a photoperiod of 12 h light (0900 h to 2100 h) and 12 h dark [24]

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**Table 1.** Bacterial strains used in this study.

| Species               | Strain   | Source location |
|-----------------------|----------|-----------------|
| *Vibrio rotiferianus* | DAT722   | UTS$^1$ [25]    |
| *Vibrio alginolyticus*| 12G1     | UTS [26]        |
| *Vibrio harveyi*      | ATCC14126| ATCC$^2$        |
| *Vibrio natriegens*   | C5       | UTS             |
| *Vibrio campbellii*   | C7       | UTS             |
| *Vibrio parahaemolyticus* | C8 | UTS             |
| *Vibrio cholerae* (non 01/0139) | S10 | UTS [27]        |
| *Vibrio corallilysticus* | BAA-450 | ATCC            |
| *Vibrio shiloi*       | BAA-91   | ATCC            |
| *Vibrio splendidus*   | 33125    | ATCC            |
| *Vibrio ordalii*      | 33509    | ATCC            |
| *Vibrio tubiashii*    | 19109    | ATCC            |
| *Serratia marcescens* | BAA-632  | ATCC            |

$^1$University of Technology, Sydney Culture Collection.

$^2$American Type Culture Collection.

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**Figure 1.** Sandwich-type format carried out in ELISA plate wells for the capture and detection of *Vibrio* cells. The well surface is coated with neutravidin and blocked with bovine serum albumine. The biotinylated anti-*Vibrio* polyclonal antibody is added and bacterial cells are then captured and detected using an horse radish peroxidase linked anti-*Vibrio* polyclonal antibody that can be detected colorimetrically using a spectrophotometer at 450 nm.

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until 6 days post hatch, after which time photoperiod was increased to 18L:6D. Water temperature was 22.0 ± 1°C.

Algae and clay treatments were added to the tanks every day after the daily water change. Fish larvae were fed rotifers (Brachionus plicatilis) enriched with Sparkle and Spresso (INVE Aquaculture, Belgium) according to manufacturer’s instructions at a target density of 10 rotifiers mL$^{-1}$ from 2 days post hatch (dph). From 12 dph, larvae were fed Artemia enriched with Algamac 3030 (Aquafoma Biomarine, CA, USA) according to manufacturer’s instructions. Surface skimmers were used during all experiments to remove oil debris from the water surface. Dissolved oxygen, water temperature, salinity and pH were measured daily using a multi-parameter water quality probe (Horiba U-10, Horiba Ltd., Japan). The trial was terminated at 26 dph and the larval survival rates were determined at harvesting.

At 14 dph, 50 mL sterile Falcon tubes were used to collect water samples from each of the mulloway larvae rearing tanks (n = 3 samples per tank). Conservative estimates of Vibrios and marine bacteria were made using the TCBS culturing method and compared to estimates made using the ELISA detection test. 100 μL of fresh tank samples were added in triplicate to functionalised 96 well plates. A V. parahaemolyticus pellet (positive control) was resuspended in filtered seawater from the tanks water supply and used within the same plate in order to obtain a standard detection curve. The detection of captured Vibrio cells was then carried out using the developed ELISA method.

Results and Discussion

Antibody affinity to target Vibrios

Twelve temperate and tropical Vibrio strains were used to assess the specificity of the polyclonal antibodies used in this study. There was substantial variation in absorbance signal amplitudes which ranged between 0.08 and 0.45 (see Figure 2), demonstrating differences in the affinity of the antibody for the different Vibrio strains. However, the limit of detection (LOD) remained similar for all strains tested, ranging from 1 × 10$^4$ to 5 × 10$^5$ cells mL$^{-1}$ (Table 2). V. parahaemolyticus and V. tubiashii showed a lower LOD than all other strains (1 × 10$^3$ cells mL$^{-1}$ and V. parahaemolyticus was chosen as the target for subsequent development and optimisation of the immuno-functionalised surface experiments due to its relevance for human health issues. No cross-reactivity or cross-binding was observed for the non-target control strains due to its relevance for human health issues. No cross-reactivity or cross-binding was observed for the non-target control

Optimal conditions for Vibrio cell capture and detection

To reduce costs for a future Vibrio biosensor and limit non-specific adsorption, dilutions of 1/500 to 1/10000 were conducted to determine the lowest effective concentration for detection. Dilutions of 1/2500 and higher affected the assay sensitivity, not allowing the detection to occur (Figure S3a). The LODs obtained from 1/1000 and 1/500 dilutions were the lowest, showing similar values of 5 × 10$^3$ cells mL$^{-1}$ (Figure S3a). However, the non-specific binding of the antibody increased significantly when the 1/100 dilution was used. To limit the non-specific signal, the 1/1000 dilution was considered optimal.

In an attempt to reduce assay time and develop a simplified protocol for the development of a potential Vibrio biosensor, the HRP-αVib Pab and the target cells were incubated together for 1 hour at 37°C prior to capture them onto the functionalised surface, thus removing a washing step. However, the efficiency of bacterial capture and detection was dramatically reduced (Figure S3b). This loss in the capture and detection efficiency is likely due to a steric constraint due to excessive cell coverage by HRP-αVib Pab which does not allow further capture onto the functionalised surface, thus this step is not recommended in the final protocol.

Optimised incubation times for both the capture (binding of cells onto functionalised well surfaces) and detection (binding of HRP labelled antibody onto cells) steps were determined to be 30 minutes (see Figure S4). No improvement of the LOD was observed with incubation times longer than 30 min with the HRP-αVib Pab (Figure S4c), which is favourable for developing an assay due to the short time-to-result. Incubation periods shorter than 30 min considerably affected both the capture and detection efficiency.

An affinity test using optimised conditions demonstrated an even distribution of the detection curve for the different Vibrio strains and confirmed that the negative control did not bind with the antibody (Figure 3). The results for the Vibrio strains and the positive control were more similar compared to the initial experiment when the well plates were not functionalised (Figure 2) and thus functionalisation of the well plate surface was critical to the assay development. Potentially, the high proportion of cell debris contained in the lyophilised positive control resulted in an increased amount of antigen binding onto the bare well surfaces in the initial experiment compared to the specific capture provided by the functionalised surface. Additionally, the amplitude of the signals obtained for the Vibrio strains tested were higher due to the enhanced capture capacity of the surface for entire cells.
The LOD, IC50 and sensitivity values obtained when using the functionalisation strategy considerably improved for each strain tested (Table 2). The LOD obtained for all twelve strains tested in this study ranged between $7 \times 10^3$ to $3 \times 10^4$ cells mL$^{-1}$ which represents an improvement of over one order of magnitude compared to the method involving direct adsorption presented in the preliminary experiment (Table 2).

Detection of Vibrios in seawater

To assess whether the physical and chemical properties of seawater had an antagonistic effect on the capture and detection of Vibrio species, the absorbance signals obtained after direct adsorption of bacterial strains to the well surface for one hour followed by detection using a 1/500 dilution of horseradish peroxidase anti-Vibrio antibody (HRP-aVib Pab) incubated for one hour onto the surface were measured. The absorbance signals were obtained for each strain tested and compared to the negative control.

### Table 2. LOD, IC50 and sensitivity values obtained for each Vibrio strain with or without functionalising the plate surface.

| STRAIN             | LOD (cells mL$^{-1}$) | IC50 (cells mL$^{-1}$) | Sensitivity (AU/mL cell$^{-1}$) | LOD (cells mL$^{-1}$) | IC50 (cells mL$^{-1}$) | Sensitivity (AU/mL cell$^{-1}$) |
|--------------------|-----------------------|------------------------|---------------------------------|-----------------------|------------------------|---------------------------------|
| Vibrio rotiferianus| $5 \times 10^5$       | $8 \times 10^5$        | 0.039                           | $3 \times 10^4$       | $5 \times 10^5$        | 0.064                           |
| Vibrio alginolyticus| $5 \times 10^5$       | $8 \times 10^5$        | 0.049                           | $9 \times 10^4$       | $4 \times 10^5$        | 0.067                           |
| Vibrio harveyi     | $5 \times 10^5$       | $8 \times 10^5$        | 0.078                           | $8 \times 10^4$       | $2 \times 10^5$        | 0.079                           |
| Vibrio natriegens  | $3 \times 10^5$       | $10^6$                 | 0.051                           | $3 \times 10^4$       | $10^5$                 | 0.071                           |
| Vibrio campbellii  | $3 \times 10^5$       | $3 \times 10^6$        | 0.052                           | $9 \times 10^3$       | $5 \times 10^5$        | 0.087                           |
| Vibrio parahaemolyticus| $10^5$               | $2 \times 10^5$        | 0.121                           | $7 \times 10^4$       | $2 \times 10^5$        | 0.129                           |
| Vibrio cholerae S10| $5 \times 10^5$       | $8 \times 10^5$        | 0.024                           | $8 \times 10^4$       | $2 \times 10^5$        | 0.087                           |
| Vibrio corallilyticus| $3 \times 10^5$       | $3 \times 10^6$        | 0.103                           | $8 \times 10^4$       | $10^5$                 | 0.128                           |
| Vibrio shiloi      | $5 \times 10^5$       | $2 \times 10^7$        | 0.047                           | $8 \times 10^4$       | $5 \times 10^5$        | 0.070                           |
| Vibrio splendidus  | $3 \times 10^5$       | $3 \times 10^4$        | 0.050                           | $9 \times 10^4$       | $4 \times 10^5$        | 0.069                           |
| Vibrio ordali      | $5 \times 10^5$       | $7 \times 10^4$        | 0.046                           | $9 \times 10^3$       | $4 \times 10^5$        | 0.085                           |
| Vibrio tubiashii   | $10^5$                | $5 \times 10^3$        | 0.116                           | $7 \times 10^3$       | $4 \times 10^4$        | 0.122                           |

[Figure 2. Antibody affinity for different Vibrio strains. Absorbance signals obtained after direct adsorption of bacterial strains to the well surface for one hour followed by detection using a 1/500 dilution of horseradish peroxidase anti-Vibrio antibody (HRP-aVib Pab) incubated for one hour onto the surface. doi:10.1371/journal.pone.0108387.g002]
the bacterial cells, the sensitivity of the assay was tested in an environmentally realistic sample matrix. We inoculated PBS, 0.2 μm filtered and unfiltered seawater collected from Iron Cove, Sydney, with *V. parahaemolyticus* and *S. marcescens* separately. Similar results were obtained for all sample types (Figure 4) with no visible negative effect of the seawater matrix on the test efficiency and no increase of the non-specific signal. When inoculating filtered seawater or PBS with increasing concentrations of *V. parahaemolyticus*, the LOD remained constant for both media at $7 \times 10^3$ cells mL$^{-1}$ which shows the applicability of the assay developed to environmental samples.

Interestingly, when using unfiltered seawater to prepare our serial dilution of *V. parahaemolyticus* cells, the results showed a slightly lower LOD at $6 \times 10^3$ cells mL$^{-1}$. An enumeration of total *Vibrio* using TCBS agar plate culture revealed a conservative *Vibrio* estimate of $5 \times 10^2$ cells mL$^{-1}$ in the collected sample. This would explain this increase in the detection signal of the assay as we are also detecting the Vibrios already present in the sampled water.

**Detection of Vibrios in mulloway fish rearing tanks**

A standard curve obtained with the ELISA method using cultured *V. parahaemolyticus* showed a linear relationship between Absorbance and cell abundance between $1 \times 10^4$ and $1 \times 10^9$ cells ml$^{-1}$ with an $R^2$ of 0.9904 ($p<0.05$; Figure 5). The equation fitting the values obtained for this region of the standard curve was used to calculate the *Vibrio* concentrations within mulloway tank samples when samples showed absorbance values above the limit of detection. The comparative estimates of total *Vibrio* abundance in the tank samples using both culturing or ELISA detection methods are shown in Table 3. When within the limit of detection, the counts obtained from the ELISA method showed good correspondence with those obtained using the TCBS plate culture method, but those for the ELISA were slightly higher, most likely because this technique enables the detection of total *Vibrio* cells and not only cultivable cells, and because the culture methods only estimate cells able to grow on the medium. These results confirm that the ELISA method can be used as a reliable warning system when *Vibrio* bacteria concentrations reach values above $10^4$ cells ml$^{-1}$ in aquaculture tanks, providing results within 70 min after sampling.

**Conclusions**

This study developed a robust surface functionalisation strategy for the capture and colorimetric detection of *Vibrio* cells, and represents an important step for the monitoring of emerging *Vibrio*-related diseases in industrial facilities such as aquaculture systems and the marine environment. The results confirm the efficiency of the functionalisation method by anchoring biotinylated PAb to a neutravidin coated surface and that non-specific adsorption of cells was avoided with the BSA blocking step. No significant cross reactivity was found against the control bacterium tested using this commercial antibody. The assay enables the
detection of *Vibrio* cells in seawater samples within 70 minutes, with the LOD ranging between $7 \times 10^3$ to $3 \times 10^4$ cells mL$^{-1}$ without the need for a prior enrichment step. While these LOD are considerably higher than those obtained using other methods such as quantitative PCR which can allow detection of single cells in a sample, they are acceptable for environmental detection and monitoring in aquaculture systems as $1 \times 10^4$ *Vibrio* cells mL$^{-1}$ is standardly present in these environments [2,4,36] with the infective dose ranging between $10^4$ and $10^8$ cells mL$^{-1}$ [37]. Therefore, this assay has a significant potential for the rapid detection of *Vibrio* outbreaks in marine systems providing a cost effective, short time-to-result test.

Work is underway to develop the ELISA assay further, and an electrochemical detection format has been developed using this functionalization strategy to immobilise the antibodies onto a gold screen-printed electrode. The secondary antibodies were then

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**Figure 4. Effect of the capture medium.** Signals obtained for increasing *Vibrio parahaemolyticus* (dashed line) or *S. marcescens* (solid line) cell concentrations when carrying out the capture step either in PBS (■), filtered (▲) or unfiltered (●) natural seawater. doi:10.1371/journal.pone.0108387.g004

**Figure 5. Linear part of the ELISA standard curve.** Absorbance values obtained for increasing *V. parahaemolyticus* cell concentrations ranging from $1 \times 10^6$ to $1 \times 10^8$ cells ml$^{-1}$. doi:10.1371/journal.pone.0108387.g005
detected using amperometric detection of the HRP label [38]. Such a tool contributes significantly to further reducing the LOD obtained in this study, allowing a future miniaturisation and automation of a Vibrio online monitoring system. Another development of this method would involve using antibodies which are specific to particular Vibrio species of interest for the aquaculture industry in order to detect specific emerging pathogenic Vibrios in rearing facilities. This strategy has recently been used for the screening of V. Harveyi in shrimps [39]. These developments will allow us to detect Vibrios rapidly and thus help improve aquaculture yields and provide intervention opportunities if outbreaks occur.

Supporting Information

Figure S1 Antibody affinity for different Vibrio strains. 
Absorbance signals obtained after direct adsorption of the commercial positive control cells to the well surface for one hour followed by detection using a 1/500 dilution of horseradish peroxidase anti-Vibrio antibody (HRP-aVib Pab) incubated for one hour to allow binding onto the surface. (TIF)

Figure S2 Biotinylated anti-Vibrio antibody (Bt-aVib Pab) concentration optimization. Signals obtained after wells pre-coated with 20 μg mL⁻¹ neutravidin (solid lines) and functionalised with increasing concentrations of Bt-aVib Pab were exposed to different concentrations of V. parahaemolyticus. Wells in which a 80 μg mL⁻¹ Bt-aVib Pab solution was added when no neutravidin was present were also exposed to V. parahaemolyticus (dashed line). Bt-aVib Pab concentrations tested were (●) 0, (▲) 10, (■) 20, (□) 40 and (▲) 60 μg mL⁻¹. A 1/500 dilution of horseradish peroxidase anti-Vibrio antibody (HRP-aVib Pab) was used for the detection of the captured cells. (TIF)

Figure S3 Horseradish peroxidase anti-Vibrio antibody (HRP-aVib Pab) concentration optimization. Signals obtained after wells pre-coated with 20 μg mL⁻¹ neutravidin and functionalised with 20 μg mL⁻¹ biotinylated anti-Vibrio antibody (Bt-aVib Pab) were exposed to different concentrations of V. parahaemolyticus and detected using increasing HRP-aVib Pab dilutions: (■) 1/500, (□) 1/1000, (●) 1/2500, (○) 1/5000 and (▲) 1/10000. (A) The cells and the HRP-aVib Pab were incubated successively onto the functionalised surface or (B) both the cells and the HRP-aVib Pab were incubated together, before being placed in contact with the surface. (TIF)

Figure S4 Capture and detection time optimisation. 
Absorbance signals obtained after exposure of wells pre-coated with 20 μg mL⁻¹ neutravidin and functionalised with 20 μg mL⁻¹ biotinylated anti-Vibrio antibody (Bt-aVib Pab) to different concentrations of V. parahaemolyticus using increasing contact time with the cells (A) 5, (B) 10, (C) 30 and (D) 60 mins) and the 1/1000 horseradish peroxidase anti-Vibrio antibody (HRP-aVib Pab): (■) 5 mins, (○) 30 mins and (●) 60 mins. (TIF)

Author Contributions

Conceived and designed the experiments: OFL. Performed the experiments: OFL. Analyzed the data: OFL. Contributed reagents/materials/analysis tools: OFL ML JRS DGB SSF MD. Wrote the paper: OFL ML JRS DGB SSF MD.

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