Duplex electrochemical biosensor for the detection of the *tdh* and *trh* virulence genes of *Vibrio parahaemolyticus* in oysters

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Abstract

Vibrio parahaemolyticus is a bacterium present in estuarine environments. Since the first outbreak in Japan in 1950, it has been a dominant cause of foodborne infections throughout the world. *Vibrio parahaemolyticus* strains can accumulate in shellfish and cause gastroenteritis. The thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) encoded by *tdh* and *trh* genes, respectively, are considered major virulence factors in *V. parahaemolyticus*. Conventional methods used for *V. parahaemolyticus* monitoring in seafood, based on microbiological counts, are time consuming and laborious, and they cannot identify virulence genes. Therefore, a duplex electrochemical biosensor for the detection of the *tdh* and *trh* genes of *V. parahaemolyticus* in oysters was developed. Oyster homogenates were prepared, enriched for bacterial content, and DNA was extracted. Then, a duplex PCR with tailed primers was performed to amplify both genes. Afterwards, sandwich hybridisation assays were carried out on magnetised working electrodes of an array. The electrochemical biosensor was able to detect the *tdh* and *trh* genes from *V. parahaemolyticus* strains, and no cross-contaminations were observed between electrodes. The limit of detection for both genes was 10 pg/µL and no cross-reactivity was observed when using other *Vibrio* species and non-*Vibrio* pathogens. The biosensor was able to detect as low as 1 CFU of *V. parahaemolyticus* in oyster homogenate. Screening of oysters from an infectivity experiment and comparison with other techniques proved the proper performance of the biosensor and its applicability to the analysis of natural samples, with added advantages of specificity, duplexing capability, portability and provided virulence information.

Keywords: electrochemical biosensor, sandwich hybridisation assay, *V. parahaemolyticus*, *tdh*, *trh*, oyster.

1. Introduction

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium, present in estuarine environments (Nelapati, Nelapati, & Chinnam, 2012). Since the first outbreak in Japan in 1950, with 272 total cases including 20 deaths (Fujino et al., 1953), *V. parahaemolyticus* has been a dominant cause of foodborne infections throughout the world (Letchumanan, Chan, & Lee, 2014; Su & Liu, 2007). In the United States, *V. parahaemolyticus* is estimated to cause 45,000 illnesses per year (CDCP, 2019). In China, 322 gastroenteritis outbreaks involving 9,041 illnesses and 3,948 hospitalizations were reported from 2003 to 2008 (Wu, Wen, Ma, Ma, & Chen, 2014), and 1,488 infections were identified in the southern coastal region of China from 2007 to 2012 (Li et al., 2014). Several sporadic but noteworthy outbreaks have also been reported over the last 20 years in European countries, such as Norway, France and the United Kingdom (WHO, 2021). The high prevalence of *V. parahaemolyticus* in seafood presents a great threat to human health.

The thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) encoded by the *tdh* and *trh* genes, respectively (Zhang & Austin, 2005), are considered major virulence factors in *V. parahaemolyticus* (Ceccarelli, Hasan, Huq, & Colwell, 2013) and are closely related to its pathogenicity. Virulent *V. parahaemolyticus* strains can accumulate in shellfish and cause gastroenteritis when it is raw, undercooked or mishandled (Drake, DePaola, & Jaykus, 2007). Conventional methods used for *V. parahaemolyticus* monitoring in seafood, based on microbiological counts, are time consuming and laborious (Bonnin-Jusserand et al., 2019). Additionally, they cannot identify virulence genes. Therefore, simple, rapid and specific techniques are pursued (Wu et al., 2019). To address this necessity, several biosensors have been developed to monitor foodborne pathogens, including *V. parahaemolyticus*. Some of them are based on antibodies (Seo et al., 2010; Sha et al., 2016; Wang et al., 2019; Zhou et al., 2020), aptamers (Ahn et al., 2018; Hu, Shen, Tan, Yuan, & Gan, 2021; Hu et al., 2022; Jiang, Sun, Guo, & Weng, 2021; Wang et al., 2020; Wei et al., 2021), or even both biomolecules together (Teng et al., 2017). However, although such biosensors might be specific, they do not detect virulence genes either, because the biorecognition molecule used is not targeting genetic material. Biosensors based on molecular techniques are thus crucial. In 2012, an electrochemical biosensor for the *tlh* gene of *V. parahaemolyticus* was developed using glassy carbon electrodes modified with graphene oxide and poly-L-lysine as a transducer, and methylene blue as an electrochemical indicator to monitor the hybridisation event (Sun et al., 2012). In 2015, the same group used electroreduced graphene oxide, titanium dioxide nanowires and chitosan-modified carbon ionic liquid electrodes for the same purpose (Wang et al., 2015). Although both biosensors detected synthetic complementary DNA and discriminated it from non-complementary and mismatched sequences, they were applied to only one oyster sample. More recently, Nordin et al. (Nordin, Yusof, Abdullah, Radu, & Hushiarian, 2017)developed another electrochemical biosensor for the detection of the *tlh* gene of *V. parahaemolyticus*. They used screen-printed carbon electrodes modified with polylactide-stabilised gold nanoparticles, and also methylene blue as a redox indicator. The biosensor was able to discriminate between complementary, non-complementary and mismatched oligonucleotides, showed no cross-reactivity against other foodborne pathogens, and was able to identify *V. parahaemolyticus* in fresh cockles. However, validation was again limited to a couple of samples.

Despite the great threat to human health and the urgent need for accurate, rapid and sensitive tools for the screening of pathogenic *V. parahaemolyticus* in seafood, biosensors targeting

virulence genes, fully validated, are still lacking. Therefore, in this work, a duplex electrochemical biosensor for the detection of the *tdh* and *trh* genes of *V. parahaemolyticus* in oysters was conceived (Fig. 1). To this purpose, oyster homogenates were prepared, enriched for bacterial content, and DNA was extracted. Then, a duplex PCR was performed to simultaneously amplify the *tdh* and *trh* genes of *V. parahaemolyticus*. The PCR primers were modified with oligonucleotide tails to provide double-stranded DNA (dsDNA) amplicons flanked by single-stranded DNA (ssDNA). The PCR solution containing both *tdh* and *trh* amplicons was split and subsequently incubated with magnetic beads (MBs) coated with specific capture probes. The tails for the forward primers of each amplicon were different and allowed hybridisation with the corresponding *tdh* and *trh* capture probes on the MBs, avoiding the need for DNA denaturisation before the application to the biosensor. The tails for the reverse primers of both amplicons were the same and allowed hybridisation with a horseradish peroxidase (HRP)-labelled reporter probe, common to *tdh* and *trh*. Each oligocomplex was immobilised on one of the magnetised working electrodes of an array and, after addition of enzyme substrate and mediator that produce an insoluble precipitate, the reduction current intensity was measured with amperometry. After optimisation of several experimental parameters, the performance of the biosensor was characterised. Finally, its applicability to the analysis of oyster samples, both spiked and infected with *V. parahaemolyticus*, was evaluated and compared to other techniques.

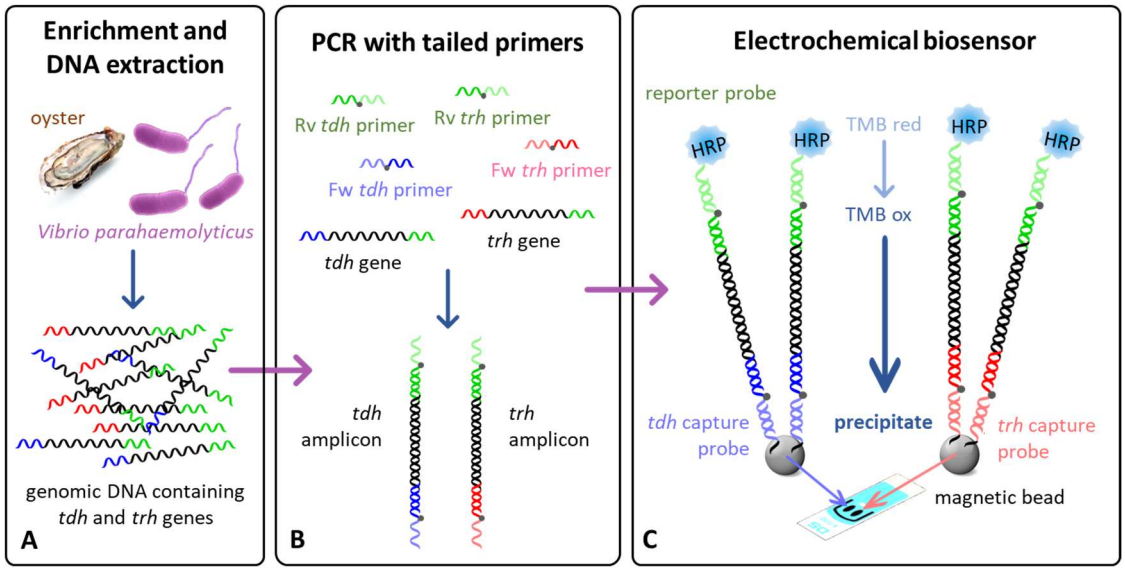

Figure 1. Schematic representation of the strategy: A) Enrichment of bacteria from infected oysters and genomic DNA extraction; B) Duplex PCR with tailed primers for the amplification of *tdh* and *trh* virulence genes of *V. parahaemolyticus*; and C) Electrochemical biosensor with oligocomplexes immobilised on magnetised working electrodes of an array.

2. Materials and methods

2.1. Samples

Strains of *V. parahaemolyticus* CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-), LO8 (*tdh*-, *trh*+) and LO10 (*tdh*-, *trh*-) were used for the development of the duplex PCR coupled to the sandwich hybridisation assay and biosensor. In the specificity study, strains of *V. parahaemolyticus* CAIM 1773 (*tdh*+, *trh*+), as well as *Vibrio aestuarianus* CECT 625, *Vibrio alginolyticus, Vibrio anguillarum* CECT 7199*, Vibrio harveyi* CECT 525*, Vibrio splendidus* CECT 528*, Aeromonas salmonicida* CECT 5231*, Pseudomonas reinekei* IRTA 18-8 and *Staphylococcus*

equorum IRTA 18-6 were used. CAIM strains were obtained from the Collection of Aquatic Important Microorganisms (http://www.ciad.mx/caim/CAIM.html). CECT strains were obtained from the Spanish Type Culture Collection (https://www.cect.org). LO strains were isolated from oyster samples from Alfacs Bay (Ebro Delta, NW Mediterranean) in 2019 (Roque et al., 2009). IRTA strains were isolated from *Argyrosomus regius* in 2018. *Vibrio alginolyticus* strain was kindly provided by HIPRA.

Depurated oyster samples were collected from a depuration plant in Alfacs Bay (Ebro Delta, Spain). Oysters were scrubbed, shucked, and homogenised with a stomacher (Lab Blender 400). Spiked oyster homogenates were obtained by adding 1 mL of *V. parahaemolyticus* CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-), LO8 (*tdh*-, *trh*+) and LO10 (*tdh*-, *trh*-) strains at different colony-forming unit (CFU) dilutions to 1 mL of oyster homogenates.

Some other depurated oysters, also from Alfacs Bay, were immersed in aquaria containing 4.5 L of UV-treated seawater (5 oysters/aquarium) to be infected experimentally. To this purpose, 4.5-mL inocula of CAIM 1772 (*tdh*+, *trh*+), LO8 (*tdh*-, *trh*+) or LO10 (*tdh*-, *trh*-) strains at 10^9 CFU/mL were added to each aquarium. Thus, the final concentration of *V. parahaemolyticus* in each aquarium was 10^6 CFU/mL. Additionally, 0.1-mL inocula of CAIM 1772 (*tdh*+, *trh*+) strain at 10^9 CFU/mL was injected to the oysters and these were immersed in another aquarium. A control aquarium with no inoculum was also included in the experiment. The experiment was performed at room temperature in a static aerated system. Phytoplankton was added to the aquaria for the purpose of feeding and to stimulate bacteria uptake by the oysters. After 4 h, oysters were sampled and homogenised.

For the enrichment, 2-mL spiked or 1-mL infected oyster homogenates were incubated with sterile alkaline peptone water (final volume of 10 mL) for different time periods (3 h, 6 h and overnight) at 37 °C on an orbital shaker (Roque et al., 2009).

2.2. DNA extraction

Total DNA was extracted using the Qiagen DNeasy Blood and Tissue kit, according to the manufacturer's protocol. Briefly, 150 µL of enriched oyster homogenate were mixed with 180 µL of buffer ATL and 20 µL of proteinase K. After 3 h at 56 °C, 200 µL of buffer AL and 200 µL of ethanol were added, and DNA extraction was carried out using spin columns. Elution was performed with 200 µL of AE buffer. DNA purity and quantity were assessed by measuring the A260/A280 ratio in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). All samples prepared for use in experiments needed to have values between 1.8 and 2.0 to be considered further. Extracted DNA was stored at 4 °C until analysis.

2.3. Tailed primer design and duplex PCR

The *tdh* and *trh* primers used in this work were based on those described by Ward and Bej (Ward & Bej, 2006) and modified with oligonucleotide tails to result in dsDNA amplicons flanked by ssDNA to be detected through complementary capture and reporter probes in a sandwich hybridisation format. Tailed primers and probes were synthesised by Biomers (Ulm, Germany). Tailed primers and probes were analysed *in silico* using the Basic Local Alignment Tool (BLAST) on the National Center for Biotechnology Information website to confirm that the primer sequences were an exclusive match for the appropriate *Vibrio* annotated sequences within the data base. Primer specificity and potential presence of primer-dimers was checked using Multiple Primer Analyser software (Thermo Fisher Scientific, Madrid, Spain) and by electrophoresis of the duplex PCR products in 2% *w/v* agarose gel.

DNA was amplified using the Invitrogen Taq DNA kit (Thermo Fisher Scientific, Madrid, Spain). Each 50-µL PCR mixture contained: 2.5 µL of 2 µM *tdh* forward primer (0.1 µM in the mixture),

2.5 µL of 2 µM *tdh* reverse primer (0.1 µM in the mixture), 1.25 µL of 2 µM *trh* forward primer (0.05 µM in the mixture), 1.25 µL of 2 µM *trh* reverse primer (0.05 µM in the mixture), 2 µL of 50 mM magnesium chloride, 3 µL of 10 mM dNTPs, 5 µL of PCR buffer 10x, 30.3 µL of DNA-free molecular biology grade water, 0.2 µL of 5 U/µL of Taq polymerase and 2 µL of genomic DNA. DNA-free molecular biology grade water was used as a negative control (no template control, NTC). Different primer concentrations were also tested in the optimisation with sandwich hybridisation assay (0.2 µM and 0.2 µM, 0.4 µM and 0.1 µM, and 0.4 µM and 0.2 µM *tdh* and *trh* primers, respectively, in the mixture). The reaction took place in a Nexus Gradient Thermal Cycler (Eppendorf Ibérica, Madrid, Spain). The following test parameters were selected: initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C (54.0 °C – 64.2 °C in the annealing temperature optimisation) for 30 s and elongation at 72 °C for 30 s, with termination by a final extension of 5 min at 72 °C. The PCR products of the oyster samples from the infectivity experiment were visualised by electrophoresis in 2% *w/v* agarose gel.

2.4. Sandwich hybridisation assay and biosensor

Tween-20, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, TMB enhanced liquid substrate, 6-mercapto-1-hexanol, skimmed milk powder, potassium phosphate dibasic, potassium phosphate monobasic and sodium chloride were obtained from Sigma-Aldrich (Tres Cantos, Spain).

Magnetic oligocomplexes were prepared as follows: 1) 10 µL of maleimide-activated MBs (PureCube maleimide-activated MagBeads, Cube Biotech, Monheim, Germany) were transferred to a tube; 2) 100 µL of 500 nM thiolated capture probe in PBS (100 mM phosphate, 150 mM NaCl, pH 7.4) were added; 3) 100 µL of 100 µM 6-mercapto-1-hexanol in PBS were added to block any non-functionalised maleimide groups; 4) 100 µL of 5% *w/v* skimmed milk in PBS were added to avoid non-specific adsorption and, finally, capture probe-MB conjugates were resuspended in 10 µL of PBS-Tween. When the amounts of MBs varied, volumes were adjusted proportionally. Once the conjugates were prepared: 5) 4.5 µL of conjugate were added to a new tube and placed on a magnetic stand to remove the supernatant; 6) 5 µL of PCR product and 40 µL of PBS (also 20 and 2 µL of PCR product and 25 and 43 µL of PBS, respectively, in the optimisation) were incubated; 7) 50 µL of 10 nM HRP-labelled reporter probe in PBS-Tween were added; and 8) magnetic oligocomplexes were resuspended in 45 µL of PBS-Tween. All steps were performed with agitation for 30 min at room temperature, except for the capture probe conjugation step, which was incubated at 4 °C overnight. After each step, magnetic oligocomplexes were rinsed three times with PBS-Tween, by placing the tube on the magnetic separation stand and removing the PBS-Tween. In the sandwich hybridisation assay, used for the optimisation of the experimental parameters, the protocol continued as follows: 9) 20 µL of magnetic oligocomplex were placed into a new tube and the supernatant was removed; 10) 125 µL of TMB liquid substrate were added and incubated for 10 min; 11) tubes were placed on a magnetic separation stand and 100 µL were collected to measure the absorbance at λ = 620 nm. Absorbance measurements were performed with a microplate reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA) and data were collected and evaluated with Gene 5 software.

In the sandwich hybridisation biosensor, steps from 1 to 7 were the same. Then: 8) magnetic oligocomplexes were resuspended in 4.5 µL of PBS-Tween (instead of 45 µL); 9) 2 µL of the *tdh* magnetic oligocomplex were immobilised on one of the working electrodes of the screen-printed carbon dual electrode array (DRP-X1110 from Dropsens, Oviedo, Spain) with a

homemade magnetic support on the reverse side, and 2 µL of the *trh* magnetic oligocomplex were immobilised on the other one (since oligocomplexes were resuspended in 10-fold lower volumes, the amount on the electrodes was the same as in the tubes); 10) supernatants were removed and 80 µL of TMB enhanced liquid substrate were added and incubated for 2 min; 11) a potential of -0.2 V vs. Ag/AgCl was applied for 5 s at each working electrode and current intensities were measured at 0.5 s. Amperometric measurements were performed with an AUTOLAB PGSTAT128N potentiostat from Metrohm Autolab (Utrecht, The Netherlands) and data were collected and evaluated with Nova 2.1.4 software.

2.5. Quantitative Polymerase Chain Reaction (qPCR)

qPCR amplifications were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Alcobendas, Spain). Single reactions for *tdh* and *trh* were performed. Each 20-µL qPCR mixture contained: 10 µL of 2x Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad, Alcobendas, Spain), 0.5 µL of 20 µM *tdh* or *trh* forward primer, 0.5 µL of 20 µM *tdh* or *trh* reverse primer, 7 µL of DNA-free molecular biology grade water and 2 µL of genomic DNA. DNA-free molecular biology grade water was used as a negative control (no template control, NTC). All reagents were prepared in a master mix except for the DNA. The following test parameters were selected: initial denaturation at 95 °C for 5 min, 39 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 20 s and elongation at 72 °C for 30 s. Fluorescence data were collected at the end of each cycle. A final melt curve step was included to confirm specific amplification.

3. Results and discussion

3.1. Tailed primer design and duplex PCR

Tailed primers were based on those described by Ward and Bej (Ward & Bej, 2006). The primers for *tdh* were 44 bp in length and amplified the 229 bp region between nucleotides 170 and 438 of this gene. The primers for *trh* were 41 bp (forward primer) and 40 bp (reverse primer) in length and amplified the 207 bp region between nucleotides 82 and 287 of this gene. GC content was between 39-47%. Tailed primers and probes sequences are detailed in Table 1.

Table 1. Tailed primers and probes for *tdh* and *trh*. Tails are underlined.

Name	Sequence (5'-3')
Forward *tdh* primer with tail	cat cgc acg aat ata ata ca5 gta rag gtc tct gac ttt tgg ac
Reverse *tdh* primer with tail	tgt aaa acg acg gcc agt 5ct aca gaa tya tag gaa tgt tga ag
Forward *trh* primer with tail	gtt ttc cca gtc acg ac5 cca tcm ata cct ttt cct tct cc
Reverse *trh* primer with tail	tgt aaa acg acg gcc agt 5ac ygt cat ata ggc gct taa c
tdh capture probe	tgt att ata ttc gtg cga tgt ttt ttt ttt ttt tt-SH
trh capture probe	gtc gtg act ggg aaa act ttt ttt ttt ttt tt-SH
Reporter probe	HRP-act ggc cgt cgt ttt aca

Gel electrophoresis of the amplicons obtained when running the PCR at different primer annealing temperatures (54.0 °C – 64.2 °C) is shown in Fig. S1. Brightest bands were observed between 54.8 °C and 56.1 °C and, therefore, 55 °C was chosen as the optimal primer annealing temperature. Gel electrophoresis of the PCR products also demonstrated that the designed

tdh and *trh* tailed primers were specific for *tdh* and *trh* genes, respectively, showing specific bands at the estimated molecular weight and no primer-dimer formation (Fig. S2).

3.2. Sandwich hybridisation assay

Several experimental parameters were optimised with the colorimetric sandwich hybridisation assay, which was conducted like the electrochemical sandwich hybridisation biosensor, but measuring the absorbance from the oligocomplexes in suspension instead of the reduction current intensity from the oligocomplexes immobilised on the magnetised working electrodes.

First, the amount of amplicon used in the hybridisation was optimised. Trials were performed with 5 µL, 10 µL and 20 µL of amplicon, using 1 ng/µL of genomic DNA from CAIM 1772 (*tdh*+, *trh*+) strain in the duplex PCR and *tdh* capture probe-MB conjugate in the sandwich hybridisation assay. Absorbance values increased from 2 µL (0.625± 0.001) to 5 µL (1.030 ± 0.123) of amplicon, and then remained constant when using 20 µL (1.100 ± 0.086), indicating that the capture probe-MB conjugate was already totally coated with amplicons when using 5 µL of amplicon (saturation). Consequently, this amount of amplicon was used in the following experiments.

Then, the primer concentrations and ratio were optimised. Trials were performed with 0.2 µM and 0.2 µM, 0.4 µM and 0.1 µM, 0.4 µM and 0.2 µM, and 0.1 µM and 0.05 µM *tdh* and *trh* primer concentrations, respectively. In these experiments, apart from CAIM 1772 (*tdh*+, *trh*+) strain, LO8 (*tdh*-, *trh*+) and CAIM 1400 (*tdh*+, *trh*-) strains were also tested, using both *tdh* and *trh* capture probe-MB conjugates. Results using a concentration of 0.2 µM *tdh* and *trh* primers show that in the *tdh* system, the *tdh*+ CAIM 1400 strain was detected and the *tdh*- LO8 strain and the NTC were not, as expected (Fig. S3). However, the *tdh*+ CAIM 1772 strain (which is also *trh*+) was not detected. In the *trh* system, high absorbance values were measured elsewhere, and no differences between specific and non-specific responses were observed.

In order to get specific amplification for the CAIM 1772 (*tdh*+, *trh*+) strain in the *tdh* system and to reduce the non-specific amplification in the *trh* system, the *tdh* primer concentration was increased 2-fold (from 0.2 to 0.4 µM) and the *trh* primer concentration was decreased by half (from 0.2 to 0.1 µM). With this change, both *tdh*+ CAIM 1772 and CAIM 1400 strains were successfully detected in the *tdh* system (Fig. S4). However, the non-specific absorbance values from NTC and the *tdh*- LO8 strain increased. In the *trh* system, non-specific absorbance values decreased and the *trh+* LO8 strain could be successfully detected (although it was less evident than in the previous experiment). However, no amplification was achieved with the *trh*+ CAIM 1772 strain. These results suggest that a 0.1 µM *trh* primer concentration may be too low and cannot compete with the 0.4 µM *tdh* primer concentration. Therefore, the primer concentration ratio seems to be crucial.

In order to get specific amplification for the CAIM 1772 (*tdh*+, *trh*+) strain in both systems, the *trh* primer concentration was increased again 2-fold (from 0.1 to 0.2 µM) but maintaining the *tdh* primer concentration at 0.4 µM. With this change, an appropriate response was achieved from the *trh*+ CAIM 1772 strain in the *trh* system, and the response from the *trh+* LO8 strain increased again (Fig. S5). However, the non-specific absorbance value from NTC also increased. Regarding the *tdh* system, the results were better than in the previous experiment, since the non-specific absorbance values with the *tdh*- LO8 strain and NTC decreased. Although the *tdh* primer concentration was maintained from the previous experiment to this one, it seems that the higher *trh* primer concentration acts as an inhibitor of the *tdh* non-specific adsorption. Even though the non-specific response from NTC in the *trh* system was a problem to be solved,

it was reasonable to conclude that a ratio of 2:1 *tdh*:*trh* primers concentration is needed in the duplex PCR to achieve the proper amplification of both genes when present together in a sample.

To avoid possible non-specific bindings of the primers to the capture probes, between them (primer-dimer) and/or to genomic DNA (since both genes are related and very similar), the last optimisation step was to decrease both primers concentrations 4 times (from 0.4 µM and 0.2 µM to 0.1 µM and 0.05 µM *tdh* and *trh* primer concentration, respectively), maintaining the *tdh*:*trh* primer ratio. In both systems, the specific absorbance values slightly decreased, but were higher than the *tdh*-, *trh*- and NTC systems (Fig. S6). In fact, the non-specific absorbance value from NTC in the *trh* system decreased considerably (it was lower than in the *tdh* system). Thus, appropriate specific/non-specific absorbance value ratios were achieved. Consequently, 0.1 µM and 0.05 µM *tdh* and *trh* primer concentrations, respectively, were used in the development of the biosensor.

3.3. Sandwich hybridisation biosensor

The experimental conditions optimised in the colorimetric assay were used to develop the electrochemical biosensor. The difference between these techniques is the detection method. Instead of using the magnetic oligocomplexes in suspension, they are immobilised on the working electrodes of the dual array (each capture probe-MB on a different electrode), and the TMB liquid substrate is replaced with TMB enhanced liquid substrate. In the presence of HRP-labelled reporter probe, the enzyme reacts with this reagent and an insoluble precipitate is produced. This insoluble precipitate concentrates on the working electrodes, enhancing the electrochemical response. Apart from signal enhancement, this reagent allows physical separation of the resulting product, avoiding cross-contamination between electrodes of an array, which is much more difficult to overcome when using soluble TMB (Gaiani et al., 2022).

Results obtained with the electrochemical biosensor showed the same trend as those obtained with the colorimetric assay, indicating that the detection technique is not affecting the performance of the system (Fig. 2). In the *tdh* system, current intensities of 4,700 nA were reached with the *tdh*+ CAIM 1772 and CAIM 1400 strains, while 2,100 nA were obtained with the *tdh*- LO8 strain and NTC. In the *trh* system, current intensities were of around 3,700 nA with the *trh*+ CAIM 1772 and LO8 strains, 850 nA with the *trh*- CAIM 1400 strain and 1,500 nA with NTC. Despite the background currents, discrimination between positive and negative responses was achieved for both systems. Additionally, no cross-contaminations were observed between the two working electrodes of the dual array when using genomic DNA of CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh*+) strains, since each electrode provided a significant current intensity for the positive gene and a background current intensity for the negative gene. The physical separation of the working electrodes and the use of TMB enhanced liquid substrate allows duplex electrochemical detection using drop configuration (electrode in a horizontal position) instead of batch mode (electrode in a vertical position) (Piguillem et al., 2020). Therefore, small-volume samples can be used.

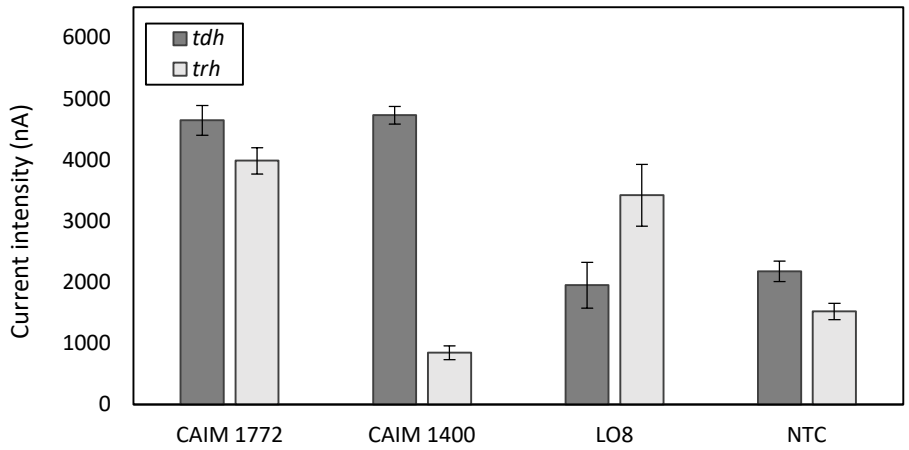

Figure 2. Current intensity values obtained in the duplex PCR coupled to the sandwich hybridisation biosensor on electrodes with *tdh* and *trh* capture probe-MB conjugates. Duplex PCR is performed using 0.1 µM *tdh* and 0.05 µM *trh* primer concentrations and 1 ng/µL of genomic DNA from CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh+*). DNA-free molecular biology grade water was used as a negative control (no template control, NTC).

Next step was the construction of the calibration curves for *tdh* and *trh* genes, which were performed using dilutions of genomic DNA from CAIM 1772 (*tdh*+, *trh*+) (Fig. 3). Curves were fitted to a sigmoidal logistic four-parameter equation using SigmaPlot 12.0 software (R^2 = 0.999 for *tdh* and R^2 = 0.998 for *trh*; P < 0.05). Relative standard deviations (RSDs) were between 0.4 and 20.0% (n = 3). Limits of detection (LODs), calculated as the mean of the corresponding NTC values plus 3 times their standard deviation, were 10 pg/µL for both *tdh* and *trh* genes. Calibration curves with CAIM 1400 (*tdh*+, *trh*-), LO8 (*tdh*-, *trh*+) and LO10 (*tdh*-, *trh*-) are shown in Fig. S7. As expected, no significant responses were obtained for the *trh* gene with CAIM 1400, the *tdh* gene with LO8 and both genes with LO10, regardless of the genomic DNA dilution. There are no other biosensors targeting the *tdh* and *trh* genes, and those developed for the *tlh* gene (Nordin et al., 2017; Sun et al., 2012; Wang et al., 2015) do not use genomic DNA, but synthetic oligonucleotides to construct the calibration curves, achieving LODs in the range of 10^{-12} to 10^{-13} M. Our LODs (in genomic DNA concentration units) are difficult to compare to molar concentrations. Nevertheless, they can be compared to those obtained by qPCR. In the work by Ward and Bej (Ward & Bej, 2006), the LODs were 200 pg for both genes. In our work, the LODs are 10-fold lower (20 pg). However, our working range is lower. Whereas they could detect genomic DNA amounts as high as 2 µg, our sigmoidal calibration curve indicates that our system starts to be saturated at 4 ng (2 ng/µL). This saturation is not probably due to the PCR step, but to the sandwich hybridisation assay on the MBs. If necessary, the working range could be enlarged by increasing the amount of MBs. However, in that case a compromise would have to be reached, since this change would increase the cost of the assay. In any case, the calibration curves obtained under our optimised conditions show good sigmoidal fittings.

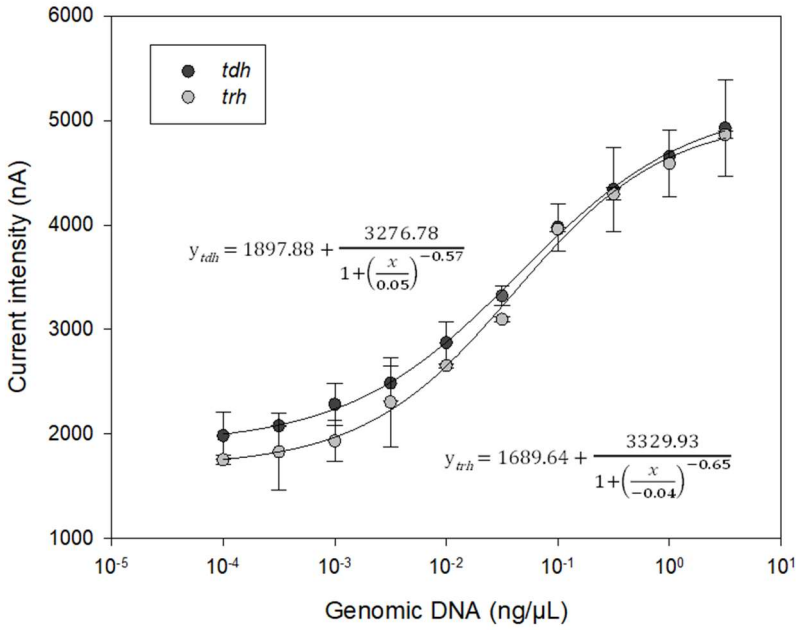

Figure 3. Calibration curves for the *tdh* and *trh* genes obtained with the electrochemical biosensor. Duplex PCR is performed using 0.1 µM *tdh* and 0.05 µM *trh* primer concentrations and genomic DNA from CAIM 1772 (*tdh*+, *trh*+) at different dilutions.

To evaluate the specificity of the biosensor, cross-reactivity experiments were performed using 1 ng/µL of genomic DNA (concentration near the saturation of the system in Fig. 3) from other *Vibrio* species (*V. alginolyticus, V. anguillarum, V. splendidus, V. harveyi* and *V. aestuarianus*) and non-*Vibrio* pathogens *(S. equorum, P. reinekei* and *A. salmonicida*) potentially present in shellfish samples, and results were compared to those obtained with *V. parahaemolyticus* (two more strains were included in this experiment, CAIM 1773 (*tdh*+, *trh*+) and LO10 (*tdh*-, *trh*-)) (Fig. 4). Specific detection (current intensity values above the corresponding threshold) was achieved only in the presence of the target genes with the corresponding capture probes. The non-targeted *Vibrio* species and non-*Vibrio* pathogens did not show any response above the LOD. These results demonstrate the excellent specificity of the system when confronted with other pathogens that could be present in seafood samples.

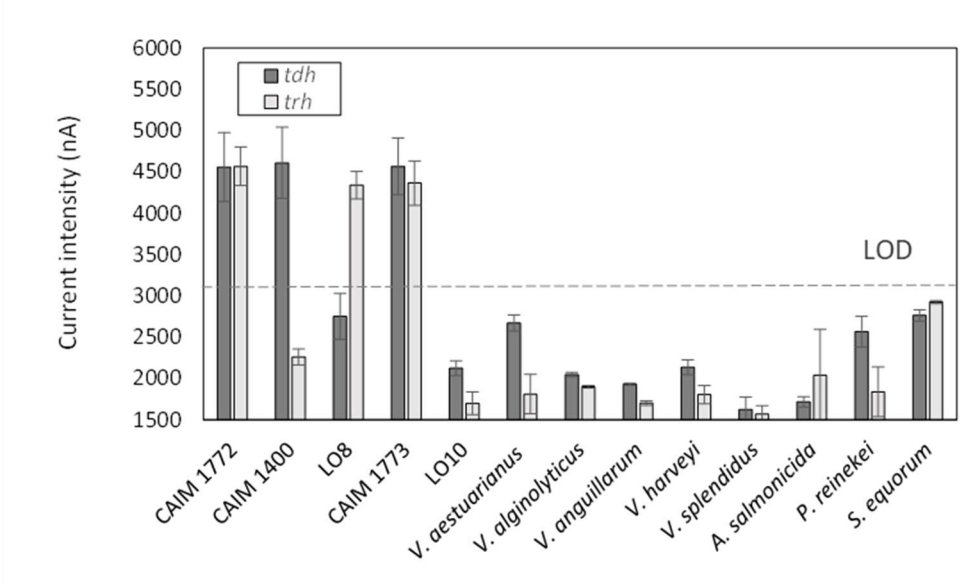

Figure 4. Specificity of the electrochemical biosensor. Duplex PCR is performed using 0.1 µM *tdh* and 0.05 µM *trh* primer concentrations and 1 ng/µL of genomic DNA from *V. parahaemolyticus* CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-), LO8 (*tdh*-, *trh*+), CAIM 1773 (*tdh*+, *trh*+), LO10 (*tdh*-, *trh*-), *V. aestuarianus*, *V. alginolyticus, V. anguillarum, V. harveyi*, *V. splendidus*, *A. salmonicida*, *P. reinekei* and *S. equorum*.

Repeatability and reproducibility of the biosensor were evaluated using 1 ng/µL of genomic DNA from CAIM 1772 (*tdh*+, *trh*+) and performing multiple measurements on the same day (intraday precision) and different days (interday precision), respectively. RSD for the measurements performed on the same day using the same capture probe-MB conjugate pools and PCR amplicons was 5.7% for *tdh* and 6.9% for *trh* (n = 3). RSD for the measurements performed on different days using different capture probe-MB conjugate pools and different PCR amplicons was 11% for *tdh* and 15% for *trh* (n = 5). These values are below the expected ones according to Horwitch (11% for the repeatability and 16% for the reproducibility when the analyte concentration is 1 ppm) (AOAC, 1993). Therefore, they show the appropriate reliability of the whole procedure (i.e., duplex PCR, sandwich hybridisation assay on magnetic beads and electrochemical detection) for both *tdh* and *trh* genes.

Regarding the storage stability of this biosensor, previous results have demonstrated that the capture probe-MB conjugates were stable at least 17 days at 4 and -20 °C, with a predicted stability of at least 3 months at -20 °C (Toldrà, Alcaraz, Diogène, O'Sullivan, & Campàs, 2019). This long-term stability makes possible to reduce the assay time, as large amounts of conjugates can be prepared many days in advance and stored until use.

3.4. Validation of the biosensor with oyster samples

To evaluate the applicability of the biosensor to the analysis of natural samples, *V. parahaemolyticus* CAIM 1772 (*tdh*+, *trh*+) CFU dilutions were spiked to oyster homogenate and enriched for different time periods (3 h, 6 h and overnight). This enrichment process had already been tested with different shellfish samples (oysters, mussels and clams) from the bays of the Ebro Delta containing different *V. parahaemolyticus* strains (Roque et al., 2009). Then, genomic DNA was extracted and analysed with the biosensor. Results with 3-h enrichment showed that the LOD for both genes was 10^2 CFU/mL (Fig. 5A). When using 6-h enrichment, it was possible to detect down to 1 CFU/mL and no differences were observed from 10 to 10^3 CFU/mL (Fig. 5B). The enrichment overnight showed a saturation of the system, even at 1 CFU/mL (Fig. 5C). This concentration was also detected with the *tdh* and *trh* systems when

spiking CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh*+), respectively, and using overnight enrichment. No responses were obtained with the *trh* and *tdh* systems when spiking CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh*+), respectively, neither LO10 (*tdh*-, *trh*-) (Fig. S8). Although enrichment was necessary to reach such low LODs (with no enrichment, LODs were 10^7 CFU/mL), these results indicate that the biosensor performed successfully in the analysis of oyster samples spiked with a *V. parahaemolyticus* strain. Since 1 mL of *V. parahaemolyticus* strain was used for the spiking, we can affirm that the biosensor is able to detect as low as 1 CFU. Ward and Bej (Ward & Bej, 2006) also had to enrich the oyster tissue homogenate to be able to detect 1 CFU with qPCR (they used overnight enrichment to ensure proper bacterial growth). Like in our work, no differences were observed among bacterial dilutions, likely due to saturation. Although enrichment leads to non-quantitative detection of this pathogen, it assures the detection of 1 CFU. Some of the biosensors based on antibodies also reported the need of an enrichment step to be able to detect 1 CFU (Seo et al., 2010; Zhou et al., 2020). It is important to mention that some immunosensors and aptasensors report LODs between 1 and 33 CFU without enrichment (Hu et al., 2021; Hu et al., 2022; Jiang et al., 2021; Sha et al., 2016; Teng et al., 2017; Wang et al., 2019; Wang et al., 2020; Wei et al., 2021). However, as previously mentioned, the biosensors based on antibodies and/or aptamers do not detect DNA and therefore cannot identify virulence genes, which may be present even in the absence of their expression.

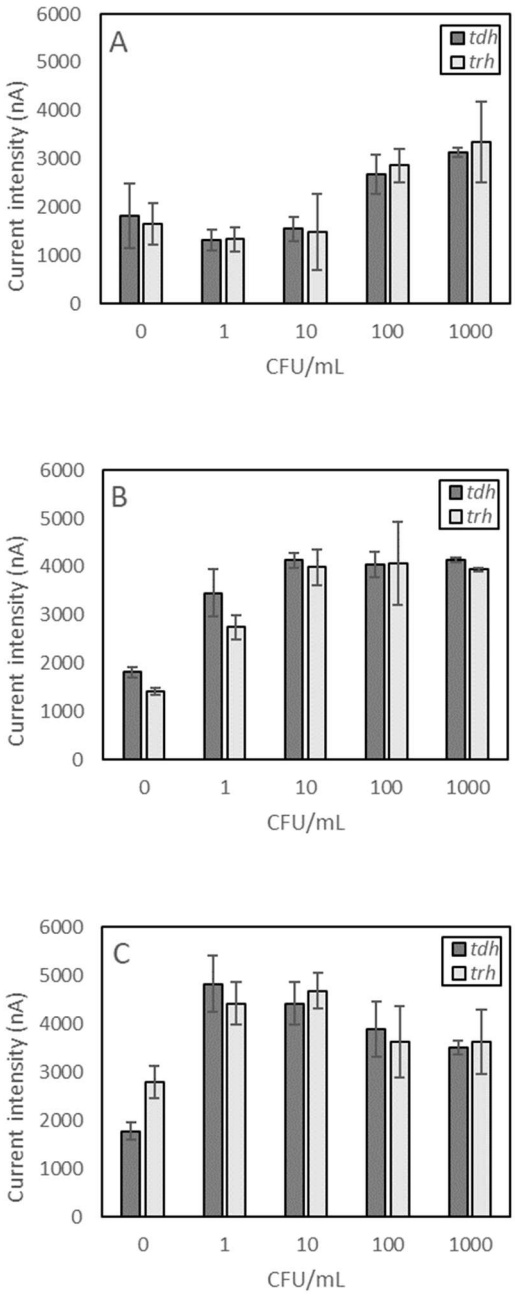

Figure 5. Current intensity values obtained in the duplex PCR coupled to the sandwich hybridisation biosensor on electrodes with *tdh* and *trh* capture probe-MB conjugates. Duplex PCR is performed using 0.1 µM *tdh* and 0.05 µM *trh* primer concentrations and dilutions of CAIM 1772 (*tdh*+, *trh*+) in oyster homogenate enriched for 3 h (A), 6 h (B) and overnight (C).

The biosensor was then applied to the screening of oysters obtained from an infectivity experiment, and results were compared with those obtained with the colorimetric assay, agarose gel and qPCR. The aim of this experiment was to detect as low as 1 CFU, which can be achieved with both 6-h and overnight enrichments. For practical reasons, oyster homogenates were enriched overnight. Since overnight enrichment does not permit discrimination between 1 CFU or more, results were provided as “positive” or “negative” (the threshold being the LOD, i.e. 1 CFU, common in all techniques). As it can be observed in Table 2, the correlation

between all techniques was excellent. Oysters from the control aquarium (no inoculum) were negative for both genes. Regarding the aquarium where CAIM 1772 (*tdh*+, *trh*+) was inoculated, 3 out of 5 oysters were positive and 2 were negative for both genes. Regarding the aquarium where LO8 (*tdh*-, *trh*+) was inoculated, all oysters were negative for *tdh* (as expected) and 4 out of 5 were positive for *trh*. Regarding the aquarium where LO10 (*tdh*-, *trh*-) was inoculated, all samples were negative (as expected). Regarding oysters to which CAIM 1772 (*tdh*+, *trh*+) was injected, 4 out of 5 oysters were positive and 1 was negative for both genes. The fact that not all oysters from the same aquaria with CAIM 1772 and LO8 provided the same results may be due to differences in the genetic background of individual oysters and their inherent resistance to *V. parahaemolyticus,* or different uptake/depuration kinetics. These results demonstrate that the biosensor is working properly.

Table 2. Results for the screening of *tdh* and *trh* in oyster samples obtained from an infectivity experiment with the electrochemical biosensor, the colorimetric assay, agarose gel and qPCR. LOD = 1 CFU. +: response above the LOD, -: response below the LOD.

	Electrochemical biosensor		Colorimetric assay		Agarose gel		qPCR	
	tdh	*trh*	*tdh*	*trh*	*tdh*	*trh*	*tdh*	*trh*
Control								
C1	-	-	-	-	-	-	-	-
C2	-	-	-	-	-	-	-	-
C3	-	-	-	-	-	-	-	-
C4	-	-	-	-	-	-	-	-
C5	-	-	-	-	-	-	-	-
CAIM 1772								
S1	-	-	-	-	-	-	-	-
S2	+	+	+	+	+	+	+	+
S3	+	+	+	+	+	+	+	+
S4	-	-	-	-	-	-	-	-
S5	+	+	+	+	+	+	+	+
LO8								
S6	-	+	-	+	-	+	-	+
S7	-	-	-	-	-	-	-	-
S8	-	+	-	+	-	+	-	+
S9	-	+	-	+	-	+	-	+
S10	-	+	-	+	-	+	-	+
LO10								
S11	-	-	-	-	-	-	-	-
S12	-	-	-	-	-	-	-	-
S13	-	-	-	-	-	-	-	-
S14	-	-	-	-	-	-	-	-
S15	-	-	-	-	-	-	-	-
CAIM 1772 (injection)								
S16	+	+	+	+	+	+	+	+
S17	+	+	+	+	+	+	+	+
S18	+	+	+	+	+	+	+	+
S19	+	+	+	+	+	+	+	+
S20	-	-	-	-	-	-	-	-

The International Commission on Microbiological Specifications for Foods recommends a limit of acceptability of 10^2-10^3 CFU/g for *V. parahaemolyticus* in seafood (ICMSF, 1986), the FDA guideline value being 10-fold higher (FDA, 2022). However, not all approved methods for *Vibrio* enumeration detect pathogenic strains. On this subject, the Scientific Committee on Veterinary Measures relating to Public Health of the European Commission issued an opinion, in which it is indicated that a strict risk quantification of *V. parahaemolyticus* infection from the consumption of seafood would require quantitative data on the occurrence of *tdh*-positive and *trh*-positive *V. parahaemolyticus* in seafood (EC, 2001). This Committee concluded that more data on occurrence and consumption, consensus with regard to infectious dose and tests able to characterise virulent strains are required to determine a numerical estimate of risk. Indeed, the Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs states that there is a need for development of reliable methods for microbial hazards such as *V. parahaemolyticus* (EC, 2005). Our biosensor is contributing to alleviate this need. In our work, considering that 1 mL of oyster homogenate weighs at least 1 g, the ICMSF and FDA guideline values are attained, even with only a 3-h enrichment. In fact, using 6-h or overnight enrichment, the biosensor is able to detect even lower *V. parahaemolyticus* amounts, and what is even more important: pathogenic *V. parahaemolyticus*. Once the limits are agreed and clearly stated, the biosensor can be implemented in monitoring programs, by using sample dilutions (similarly to the most probable number (MPN) strategy) and carefully selecting the enrichment time to meet the appropriate requirements.

All techniques in Table 2 were able to detect as low as 1 CFU. However, as mentioned above, the LOD (in amount of genomic DNA) of our strategy is lower than that of qPCR (Ward & Bej, 2006), and certainly lower that in the visual agarose gel. The electrochemical biosensor stands out for this and other reasons. The performance of the electrochemical biosensor and the colorimetric assay is the same, but the biosensor has the added advantages of the duplex electrochemical readout and the easier portability of the equipment. Compared to the agarose gel, our strategy provides additional signal amplification because of the use of the HRP label (rather than only the DNA amplification). In this work, qPCR has been performed in single mode, targeting the *tdh* and *trh* genes independently, since SYBR Green does not allow duplexing. Additionally, compared to conventional microbiological count methods, the biosensor provides information about the potential virulence of the strains. Another important feature of our strategy is that enrichment has been included in the protocol and, although it makes the total assay time longer, it also favours the detection of viable bacteria rather than merely DNA from dead cells. Finally, compared to other electrochemical biosensors, our biosensor is targeting the *tdh* and *trh* virulence genes of *V. parahaemolyticus*, uses genomic DNA for the calibration curves, and its applicability to the analysis of oyster samples has been fully validated (Table 3).

Table 3. Comparison of the performance parameters of different electrochemical biosensors targeting *V. parahaemolyticus*.

Target	Biorecognition molecule	Transducer	Technique	LOD	Samples	Ref.
Bacterial cell	Antibody	Glassy carbon electrode, and graphene oxide nanosheets modified with silver nanoparticles and $Ru(bpy)_2^{2+}$ as a redox label	Anodic stripping voltammetry	33 CFU/mL	Seawater	Wang, 2019
Bacterial cell	Aptamer	Screen-printed carbon electrode and methylene blue as a redox label	Square wave voltammetry	4 CFU/mL	Shrimps Fish	Hu, 2021
Bacterial cell	Aptamer	Screen-printed gold electrode modified with methylene blue, and a nano metal-organic framework modified with an antimicrobial peptide and ferrocene as a redox label	Square wave voltammetry	4 CFU/mL	Aquaculture water	Hu, 2022
Bacterial cell	Aptamer	Carbon electrode modified with molybdenum disulphide nanosheets, and $[Fe(CN)_6]^{3-/4-}$ as a redox indicator	Differential pulse voltammetry	5.74 CFU/mL	Shrimps	Jiang, 2021
Bacterial cell	Aptamer	Screen-printed carbon electrode, and gold nanoparticles modified with phenylboronic acid and ferrocene as redox labels	Differential pulse voltammetry	3 CFU/mL	Prawns	Wang, 2020
Bacterial cell	Aptamer	Glassy carbon electrode and ferrocene as a redox label	Fast scan cyclic voltammetry	1 CFU/mL	-	Wei, 2021
Bacterial cell	Antibody Aptamer	Gold electrode, gold nanoparticles as labels, and methylene blue as a redox indicator	Differential pulse voltammetry	2 CFU/mL	Fish	Teng, 2017
tlh gene	DNA	Glassy carbon electrode modified with carboxyl-functionalised graphene oxide and electropolymerised poly-L-lysine, and methylene blue as a redox indicator	Differential pulse voltammetry	169 fM synthetic DNA	Oysters	Sun, 2012
tlh gene	DNA	Carbon ionic liquid electrode modified with electroreduced graphene oxide, titanium dioxide nanowires and chitosan, and methylene blue as a redox indicator	Differential pulse voltammetry	317 fM synthetic DNA	Oysters	Wang, 2015
tlh gene	DNA	Screen-printed carbon electrode modified with polylactide-stabilised gold nanoparticles, and methylene blue as a redox indicator	Differential pulse voltammetry	2.16 pM synthetic DNA	Cockles	Nordin, 2017
trh gene *tdh* gene	DNA	Screen-printed carbon electrode array and HRP combined with TMB as a redox label	Amperometry	10 pg/µL genomic DNA 1 CFU	Oysters	This work

4. Conclusions

A duplex electrochemical biosensor for the detection of the *tdh* and *trh* virulence genes of *V. parahaemolyticus* in oysters was developed. The use of tailed primers in the duplex PCR provided *tdh* and *trh* amplicons able to hybridise with the corresponding capture probes on the proximal end and a reporter probe on the distal end. The physical separation of the working electrodes allowed the independent immobilisation of the *tdh* and *trh* capture probe-magnetic bead conjugates on the same array. Together with the use of an enzyme substrate and mediator that produce an insoluble precipitate after reaction with the enzyme label, the electrochemical duplex detection was achieved. After optimisation of the experimental parameters, the electrochemical biosensor was able to detect the *tdh* and *trh* genes from *V. parahaemolyticus* strains, and no cross-contaminations were observed between electrodes. The LOD for both genes was 10 pg/µL and no cross-reactivity was observed when using other *Vibrio* species and non-*Vibrio* pathogens. The biosensor was able to detect as low as 1 CFU of *V. parahaemolyticus* in a matrix of oyster homogenate. Screening of oysters from an infectivity experiment and comparison with other techniques proved the proper performance of the biosensor and its applicability to the analysis of natural samples, with added advantages of specificity, duplexing capability, portability and provided virulence information.

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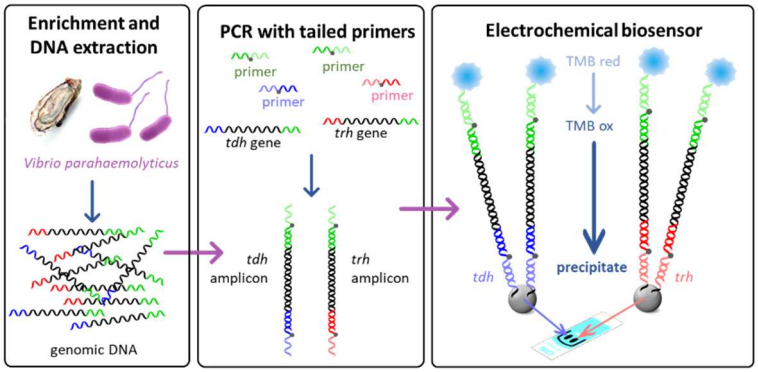
Graphical abstract



Supplementary material

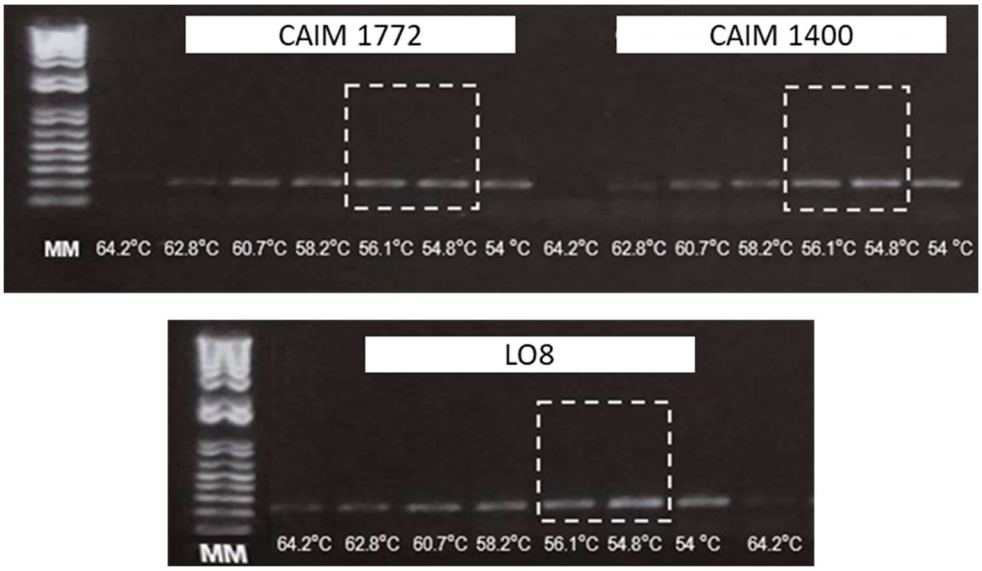


Figure S1. Gel electrophoresis of PCR products from CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh*+) strains when using different primer annealing temperatures.

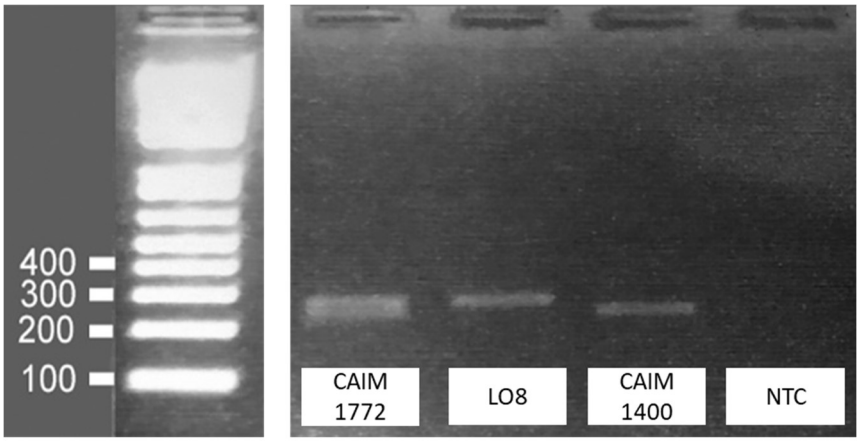


Figure S2. Gel electrophoresis of PCR products from CAIM 1772 (*tdh*+, *trh*+), LO8 (*tdh*-, *trh*+) and CAIM 1400 (*tdh*+, *trh*-) strains. DNA-free molecular biology grade water was used as a negative control (no template control, NTC).

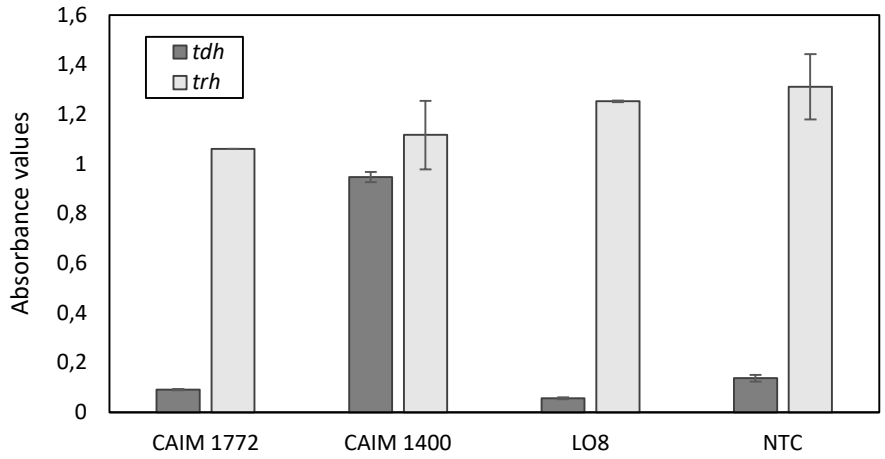


Figure S3. Absorbance values obtained in the duplex PCR coupled to the sandwich hybridization assay on *tdh* and *trh* capture probe-MB conjugates. Duplex PCR is performed using 0.2 µM *tdh* and 0.2 µM *trh* primer concentrations and 1 ng/µL of genomic DNA of CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh+*) strains. DNA-free molecular biology grade water was used as a negative control (no template control, NTC).

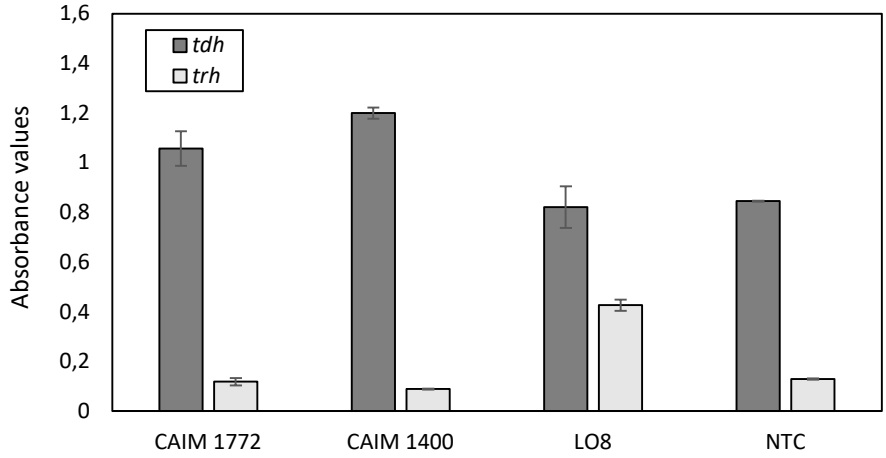


Figure S4. Absorbance values obtained in the duplex PCR coupled to the sandwich hybridization assay on *tdh* and *trh* capture probe-MB conjugates. Duplex PCR is performed using 0.4 µM *tdh* and 0.1 µM *trh* primer concentrations and 1 ng/µL of genomic DNA of CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh+*) strains. DNA-free molecular biology grade water was used as a negative control (no template control, NTC).

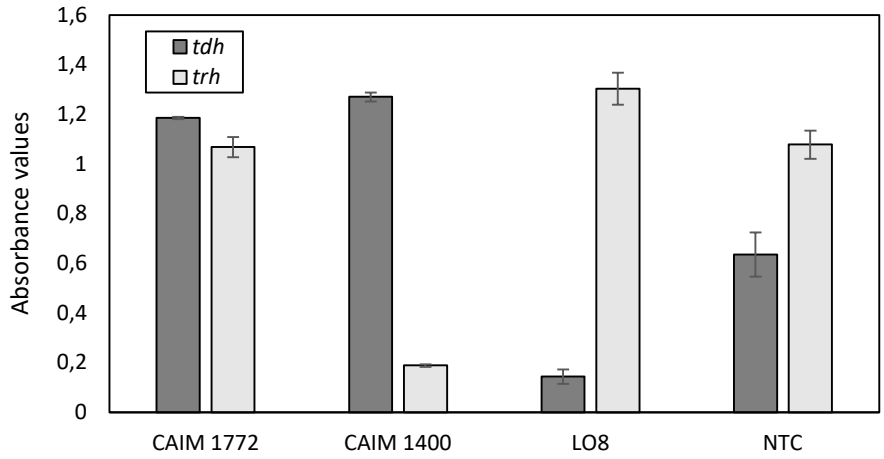


Figure S5. Absorbance values obtained in the duplex PCR coupled to the sandwich hybridization assay on *tdh* and *trh* capture probe-MB conjugates. Duplex PCR is performed using 0.4 µM *tdh* and 0.2 µM *trh* primer concentrations and 1 ng/µL of genomic DNA of CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh+*) strains. DNA-free molecular biology grade water was used as a negative control (no template control, NTC).

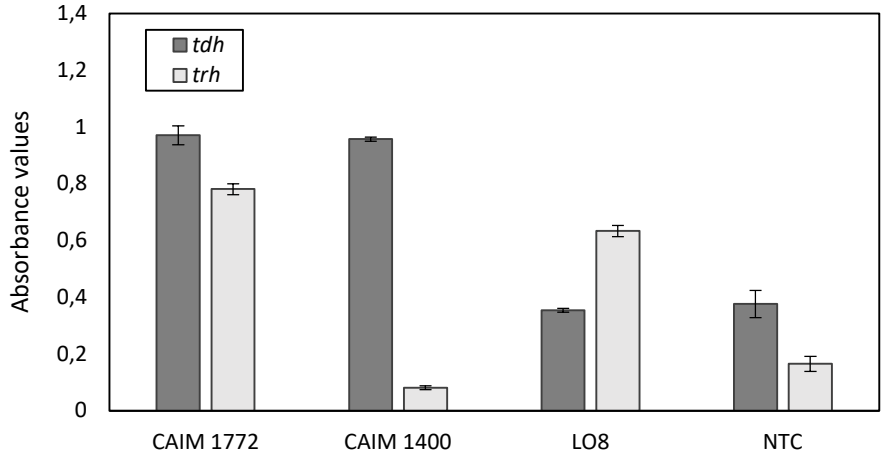


Figure S6. Absorbance values obtained in the duplex PCR coupled to the sandwich hybridization assay on *tdh* and *trh* capture probe-MB conjugates. Duplex PCR is performed using 0.1 µM *tdh* and 0.05 µM *trh* primer concentrations and 1 ng/µL of genomic DNA of CAIM 1772 (*tdh*+, *trh*+), CAIM 1400 (*tdh*+, *trh*-) and LO8 (*tdh*-, *trh+*) strains. DNA-free molecular biology grade water was used as a negative control (no template control, NTC).

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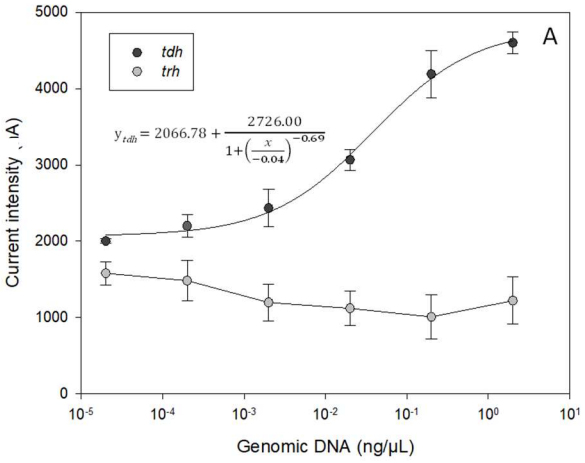

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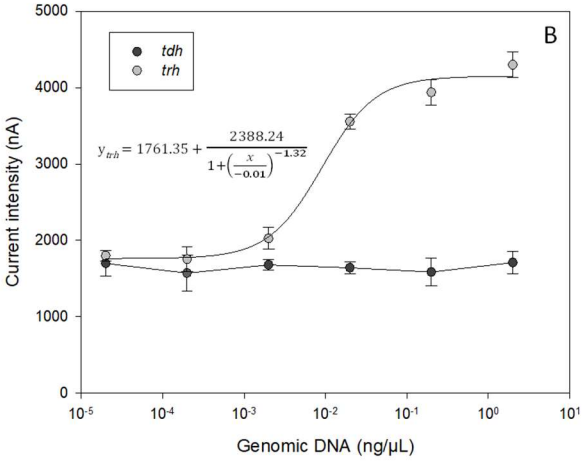

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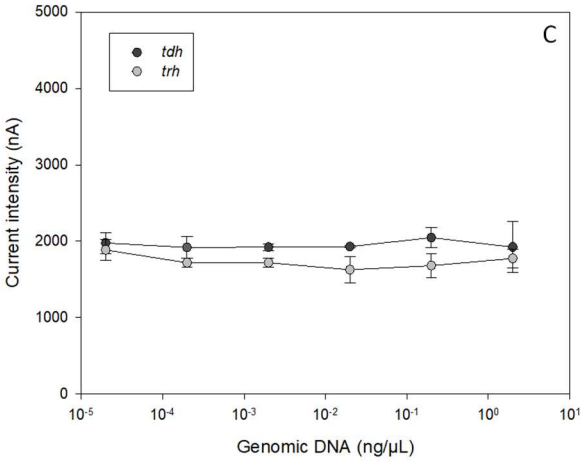

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206 **Figure S7.** Calibration curves for the *tdh* and *trh* genes obtained with the electrochemical
207 biosensor. Duplex PCR is performed using 0.1 µM *tdh* and 0.05 µM *trh* primer concentrations
208 and genomic DNA from CAIM 1400 (*tdh*+, *trh*-) (A), LO8 (*tdh*-, *trh*+) (B) and LO10 (*tdh*-, *trh*-) (C)
209 at different dilutions.

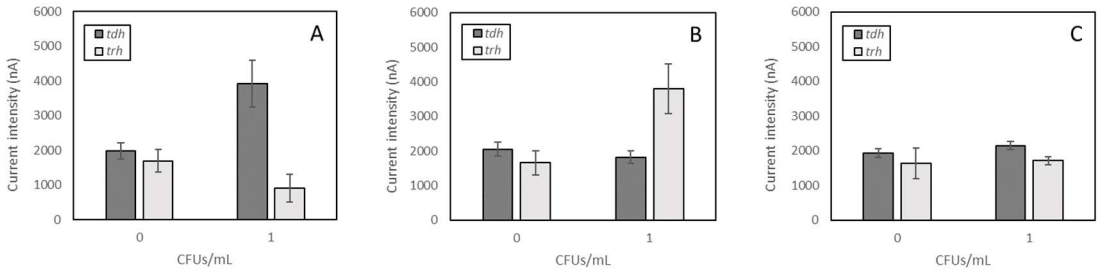

Figure S8. Current intensity values obtained in the duplex PCR coupled to the sandwich hybridisation biosensor on electrodes with *tdh* and *trh* capture probe-MB conjugates. Duplex PCR is performed using 0.1 µM *tdh* and 0.05 µM *trh* primer concentrations and 1 CFU of CAIM 1400 (*tdh*+, *trh*-) (A), LO8 (*tdh*-, *trh*+) (B) and LO10 (*tdh*-, *trh*-) (C) in oyster homogenate enriched overnight.